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Plasmalogen modulation attenuates atherosclerosis in ApoE- and ApoE/GPx1-deficient mice.
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TITLE: PLASMALOGEN MODULATION ATTENUATES ATHEROSCLEROSIS IN APOE- AND APOE/GPX1-DEFICIENT MICE.

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Abstract

Background and Aim: We previously reported a negative association of circulating plasmalogens (phospholipids with proposed atheroprotective properties) with coronary artery disease. Plasmalogen modulation was previously demonstrated in animals but its effect on atherosclerosis was unknown. We assessed the effect of plasmalogen enrichment on atherosclerosis of murine models with differing levels of oxidative stress.

Methods and Results: Six-week old ApoE- and ApoE/glutathione peroxidase-1 (GPx1)-deficient mice were fed a high-fat diet with/without 2% batyl alcohol (precursor to plasmalogen synthesis) for 12 weeks. Mass spectrometry analysis of lipids showed that batyl alcohol supplementation to ApoE- and ApoE/GPx1-deficient mice increased the total plasmalogen levels in both plasma and heart. Oxidation of plasmalogen in the treated mice was evident from increased level of plasmalogen oxidative by-product, sn-2 lysophospholipids. Atherosclerotic plaques in the aorta was reduced by 70% ($P = 5.69\text{E-}07$) and 69% ($P = 2.00\text{E-}04$) in treated ApoE- and ApoE/GPx1-deficient mice, respectively. A 40% reduction in plaque ($P = 7.74\text{E-}03$) was also seen in the aortic sinus of only the treated ApoE/GPx1-deficient mice. Only the treated ApoE/GPx1-deficient mice showed a decrease in VCAM-1 staining (-28%, $P = 2.43\text{E-}02$) in the aortic sinus and nitrotyrosine staining (-78%, $P = 5.11\text{E-}06$) in the aorta.

Conclusion: Plasmalogen enrichment via batyl alcohol supplementation attenuated atherosclerosis in ApoE- and ApoE/GPx1-deficient mice, with a greater effect in the latter group. Plasmalogen enrichment may represent a viable therapeutic strategy to prevent atherosclerosis and reduce cardiovascular disease risk, particularly under conditions of elevated oxidative stress and inflammation.

Word count: 245

Nonstandard Abbreviations and Acronyms

4-HNE	4-hydroxynonenal
ApoE^{-/-}	Apolipoprotein E-deficient mouse
ApoE^{-/-}GPx1^{-/-}	Apolipoprotein E/glutathione peroxidase-1- deficient mouse
BA	Batyl alcohol
CE	Cholesteryl ester
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PC	Phosphatidylcholine
PC(O)	Alkylphosphatidylcholine
PC(P)	Phosphatidylcholine plasmalogen/ alkenylphosphatidylcholine
PE(O)	Alkylphosphatidylethanolamine
PE(P)	Phosphatidylethanolamine plasmalogen/ alkenylphosphatidylethanolamine
ROS	Reactive oxygen species
SOD	Superoxide dismutase
VCAM-1	Vascular cellular adhesion molecule-1

Introduction

Oxidative stress is a contributing factor to the progression of atherosclerosis. Glutathione peroxidase-1 is an anti-oxidant enzyme which is expressed ubiquitously in mammalian cells; it detoxifies hydrogen peroxide, lipid hydroperoxide, and peroxynitrite ¹. Deficiency of glutathione peroxidase-1 in Apolipoprotein E-deficient mice (ApoE^{-/-}GPx1^{-/-}) was demonstrated to result in a significant increase in atherosclerosis after 24 weeks of high-fat (21% fat, 0.15% cholesterol) feeding as compared to mice which were deficient in Apolipoprotein E only (ApoE^{-/-}). This increase in atherosclerotic plaques was accompanied by an elevation in superoxide formation and protein nitration in the aorta ² as well as an increase in the expression of the pro-inflammatory markers, vascular cellular adhesion molecule-1 (VCAM-1) and receptor for advanced glycation products ³.

Plasmalogens (alkenylphosphatidylcholine, PC(P) and alkenylphosphatidylethanolamine, PE(P)) are subclasses of glycerophospholipids that are characterised by a *cis* vinyl ether bond linking an alkyl chain to the sn-1 position of the glycerol backbone. Plasmalogens are synthesised from the corresponding alkylphospholipids (alkylphosphatidylcholine, PC(O) and alkylphosphatidylethanolamine, PE(O)) by the action of a desaturase. Plasmalogens have been proposed to be atheroprotective, partly because of its anti-oxidant characteristics: (1) an enhanced electron density and low bond dissociation of the vinyl ether linkage which makes them more susceptible to reactive oxygen species (ROS) attack than allylic and alkyl linkages ⁴; (2) plasmalogen makes up one of the lipid components in cellular phospholipid bilayer which is a target of free-radical chemical reactions ⁴; and (3) the proposed slow propagation of the plasmalogen hemiacetal hydroperoxy radicals (ie. plasmalogen oxidative intermediate) ⁴. Indeed, the anti-oxidative role of plasmalogen was demonstrated in studies where supplementation of alkylglycerol (a precursor to plasmalogen synthesis) to plasmalogen deficient mutant Chinese Hamster Ovary cells and macrophage like cells, RAW.12 and RAW.108 were shown to improve resistance against ROS insults including

long-wavelength ultraviolet light and ROS generators^{5, 6}. In addition, plasmalogen was shown to have potential atheroprotective roles; Plasmalogen was demonstrated to be essential for intracellular cholesterol transport⁷ and in high-density lipoprotein (HDL)-mediated cholesterol efflux⁸, and recently, the inclusion of plasmalogen into reconstituted HDL improved the lipoprotein anti-apoptotic activity on endothelial cells⁹.

We have previously reported a negative association of circulating plasmalogens with both stable and unstable coronary artery disease; In parallel, we observed a positive association with the level of plasmalogen oxidative by-product, lysophosphatidylethanolamine (LPE)¹⁰. These findings suggested a depletion of plasmalogens in the patients due to oxidative degradation, implying a higher level of oxidative stress.

The modulation of plasmalogen concentration by oral administration of alkylglycerol has been demonstrated in humans and rodents¹¹, but the effect of plasmalogen modulation in atherosclerosis has not been previously investigated. Batyl alcohol (BA) is a naturally occurring alkylglycerol found in human plasma and tissues and is particularly abundant in human breast milk¹². Shark liver oil is a natural source of alkylglycerols that has been exploited as a nutraceutical for many years^{13, 14}. We hypothesised that modulation of plasmalogen concentration by BA would attenuate atherosclerosis progression. As a proof of concept that may show the atheroprotective mechanism of plasmalogen, here we assess the effect of plasmalogen enrichment in murine models of atherosclerosis with differing levels of oxidative stress; ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice.

Materials and Methods

Animal groups and diet study

Six-week old male C57/BL6 (Animal Resources Centre, WA, Australia), ApoE^{-/-} (Animal Resources Centre, WA, Australia), and ApoE^{-/-}GPx1^{-/-} (Alfred Medical Research and Education Precinct, VIC, Australia) mice, both on C57/BL6 background, were fed a high fat diet (22% fat, 0.15% cholesterol) (Specialty Feeds, WA, Australia), containing either 0% or 2% 1-O-octadecyl-rac-glycerol (batyl alcohol, Tokyo Chemical Industry, Astral Scientific, Australia) for 12 weeks (N=10/group). Power analysis was conducted prior to the animal experiment to ensure proper sampling. The animals were housed in standard conditions with unrestricted access to food and water at the Precinct Animal Centre of the Baker IDI Heart and Diabetes Institute. They were maintained on a 12 hr light and dark cycle in a pathogen free environment. Food intake was measured weekly by food pellet consumption. Body weights were determined weekly. After 12 weeks, animals were anaesthetised by Avertin (2,2,2-tribromoethanol) IP (0.3 mL of 2.5% solution per 20 g mouse; Sigma Chemical Co, USA) following food withdrawal for 3 hr, and organs were rapidly dissected and snap frozen. The experiment was approved and conducted in accordance to the principles devised by the Alfred Medical Research and Education Precinct Animal Ethic Committee under guidelines laid down by the National Health and Medical Research of Council of Australia (E/1345/2013/B).

Clinical measurements

Fasting blood glucose was measured by tail bleed using a glucometer (Accu-Chek, Roche Diagnostics, Australia) following 3 hr of food withdrawal and prior to the organ dissection. Blood was obtained via direct heart puncture during the organ dissection, and was collected into EDTA tubes. Plasma was separated from the blood via centrifugation at 1,485xg, room temperature for 10 min, and sucrose was added (final concentration of 0.6% (v/v)) as a cryoprotectant for lipoproteins^{15, 16} prior to storage at -80°C.

Plasma was thawed slowly on ice and then concentrations of total cholesterol and triglycerides were measured using commercial enzymatic kits on a COBAS Integra 400 Plus

blood chemistry analyser (Roche Diagnostics, Australia). Fasting plasma insulin was measured using an ELISA kit according to the manufacturer's instructions (ALPCO, USA).

Tissue homogenisation

Mice hearts were cut into two halves. The bottom half was snap frozen in liquid nitrogen and was subsequently homogenised in ice cold phosphate buffered saline (200 μ L, pH 7.6) containing 100 μ mol/L butylated hydroxytoluene using a Polytron electric homogeniser for 10 sec and then with a mini probe homogeniser for 15 sec at amplitude 23. The homogenate was stored at -80°C.

Lipoprotein fractionation

Lipoprotein fractionation was performed by density ultracentrifugation using a method adapted from Havel *et al.*¹⁷. Briefly, EDTA was added to the plasma (100 μ L) to a final concentration of 2 mmol/L and the density was adjusted to 1.019 g/mL in a final volume of 1.0 mL. The sample was centrifuged (435,680 xg, 16°C, 3 hr) in a TLA 120.2 rotor and Optima MAX-TL ultracentrifuge (Beckman Coulter, NSW, Australia). The top layer (400 μ L) of the sample, corresponding to the VLDL fraction was aspirated. The density of the remaining mixture was adjusted to 1.063 g/mL and overlaid with the same density solution to a final volume of 1.0 mL. The sample was centrifuged (435,680 xg, 16°C, 3 hr) and the top layer (400 μ L) corresponding to the LDL fraction was aspirated. The density of the remaining mixture was further adjusted to 1.21 g/mL and the volume made up to 1.0 mL. The samples were centrifuged (435,680 xg, 16°C, 16 hr) and the top layer (400 μ L) corresponding to HDL fraction was aspirated.

Lipid extraction

Prior to lipid extraction, samples were randomised to reduce bias. Lipids were extracted as previously described¹⁸. Briefly, plasma, lipoprotein or homogenised tissue was combined with internal standards (see Supplementary Table I) and the lipids were extracted using 20 volumes of chloroform:methanol (2:1). The extracted lipids were dried under a stream of nitrogen at 40°C and subsequently reconstituted in 1:1 mixture of water saturated butanol and methanol containing 5 mmol/L ammonium formate. Lipid extraction was performed on 10 μ L of heart homogenate (50 μ g protein) or plasma, 50 μ L aliquots of VLDL, LDL and HDL from C57/BL6 mice and HDL from the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice or 10 μ L aliquots of VLDL and LDL from the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice.

Liquid chromatography electrospray ionisation tandem mass spectrometry

Lipids were quantified using multiple reaction monitoring mode on a Agilent 1200 high pressure liquid chromatography system coupled to a Q/TRAP 4000 triple quadrupole mass spectrometer (AB SCIEX) using methodology described previously¹⁸ (see Supplementary Table II). Liquid chromatography separation was performed on a 2.1 x 100 mm C18 Poroshell column (Agilent, USA) at 300 μ L/min. The following gradient conditions were used: 10 % B to 100 % B over 13 min, 100 % B over 3 min, and a return 10 % B over 1 min, followed by 10 % B over 3 min. Solvents A and B consisted of water:tetrahydrofuran:methanol in the ratio of 60:20:20 and 5:75:20 respectively, both containing 10 mmol/L ammonium formate. The conditions for the tandem mass spectrometry of each lipid class are provided in Supplementary Table II. Different lipid species under the same lipid class were analysed the same way. The levels of individual lipid species were measured by taking a ratio of the area under the curve of lipid of interest to the area under the curve of internal standard of the corresponding lipid class. The ratio was then multiplied by the amount of internal standards added into the sample. Lipid classes were calculated from the sum of individual species within each class.

Lysophosphatidylcholine (LPC) were analysed by their fragmentation ions at m/z 184, whereas LPE were analysed by the neutral loss of 141 Da from the parent ion. The regioisomers of lysophospholipids such as sn-1 lysophospholipids where the fatty acid is at the sn-1 position of glycerol backbone were distinguished from the sn-2 isomers by their retention time on the chromatography; sn-2 isomers were eluted slightly earlier than sn-1 isomers^{19, 20}. The liquid chromatography method coupled with tandem mass spectrometry as described above allow separation and measurement of sn-1 and sn-2 isomers of lysophospholipids.

Quantification of atherosclerotic plaque

The entire aorta was cleaned of peripheral fat under a dissecting microscope (Olympus SZX9, Olympus Optical, Tokyo, Japan). The aorta was then stained with Sudan IV-Herxheimer's solution (BDH, Poole UK) and the *en face* technique was employed to assess the total and regional (arch, thoracic, and abdominal) plaque area as previously described²¹. Digitised photographs of the opened aortas were obtained using the dissecting microscope equipped with a digital camera (Axiocam colour camera; Carl Zeiss, North Ryde, NSW, Australia). Plaque area was quantified as the proportion of aortic intimal surface area occupied by red-stained plaque using Adobe Photoshop v.6.0.1 (Adobe Systems, Chatswood, NSW, Australia).

Frozen sections of aortic sinus were assessed as previously described^{2, 22}. Briefly, the sections (4 to 12 sections per mouse) were stained with Sudan IV-Herxheimer's solution and digitised photographs were taken using a light microscope (Olympus BX-50, Olympus Optical, Tokyo, Japan) equipped with the digital camera. The lesion area indicated by the red-stained plaque was quantified as lesion area (mm²) using Image-Pro Plus v.6.0 (Media Cybernetics, Rockville, USA). All assessments were made in a blinded manner. Necrotic core of the aortic lesions were analysed and quantified as the percentage (%) of area of lesion.

Immunohistochemistry

Immunohistochemical methods were employed as previously described²¹. Aortic sinus was frozen to allow for VCAM-1 staining (rat monoclonal, BD Pharmigen; 1:200) and F4/80 staining (rat monoclonal, Abcam; 1:75). Briefly, 4 µm frozen sections were fixed with cold acetone, air dried, and then quenched in 3% hydrogen peroxide in Tris buffered saline, pH 7.6 to prevent endogenous peroxidase activity. Sections were then blocked using 10% rabbit serum in Tris buffered saline, and incubated with primary antibodies overnight in a humidified chamber at 4°C. Sections were then incubated with biotinylated secondary antibodies anti-rat IgG (raised in rabbit, Vector Laboratories; 1:200) for 10 min at room temperature, followed by horseradish peroxidase-conjugated streptavidin (Vectastain Elite ABC Staining Kit, Vector Laboratories) for 30 min at room temperature. Signals were visualised with 3,3'-diamino-benzidine tetrahydrochloride/hydrogen peroxide (Sigma-Aldrich, USA). Subsequently, sections were counterstained in Mayer's haematoxylin, dehydrated, and coverslipped.

Aorta was paraffin-fixed to allow staining for nitrotyrosine (rabbit polyclonal, Millipore; 1:200) and 4-Hydroxynonenal (rabbit polyclonal, Millipore; 1:200). In brief, 5 µm of the aortic sections were de-waxed, hydrated, and quenched in 3% hydrogen peroxide in Tris buffered saline, pH 7.6. The sections were blocked using 10% horse serum in Tris buffered saline, and then incubated with primary antibodies overnight in a humidified chamber at 4°C. The sections were incubated with biotinylated secondary anti-rabbit IgG antibodies (raised in goat, Vector Laboratories; 1:500), followed by horseradish peroxidase-conjugated streptavidin as described above. The sections were subsequently processed in the same manner as the frozen sections as described above.

Data analysis and statistics

Mass spectrometric data were analysed using a Mann-Whitney U test, and were adjusted for multiple comparisons using the Benjamini Hochberg method, $P < 0.05$ was considered significant. Data were expressed as median (interquartile range). The lipidomic data were normalised to the relative total level of phosphatidylcholine in plasma and heart to allow a direct comparison of the relative lipid levels between the two sample types.

Data from the atherosclerotic plaque assessment and immunohistochemistry were analysed using Student t-test, comparing the control and BA-treated groups per individual genotype, $P < 0.05$ was considered significant. Data were expressed as mean $\pm$ SEM.

Results

Body weight and plasma lipid profile

In both the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} genotypes, mice fed a high fat diet without BA supplementation had a higher body weight in the final week of the study as compared to those that received BA supplementation (Table 1). In ApoE^{-/-} mice, there was 17.4% difference ($P < 0.001$) in the final weight between the two dietary groups, whereas in ApoE^{-/-}GPx1^{-/-} mice, there was a 10.7% difference ($P < 0.01$) (Table 1). All mice showed an increase in body weight across the study period (Supplementary Figure I). However, both ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice experienced slight weight loss after the first week on the BA supplementation, after which the weight increased weekly (Supplementary Figure I). Food intake significantly differed between the untreated and 2% BA-treated groups of all genotypes (control vs. BA-treated, g/day: 4.03 ± 0.14 vs. 3.20 ± 0.08 , $P < 0.001$ in C57/BL6; 5.06 ± 0.18 vs. 2.98 ± 0.09 , $P < 0.001$ in ApoE^{-/-}; 3.55 ± 0.22 vs. 3.13 ± 0.11 , $P = 0.08$ in ApoE^{-/-}GPx1^{-/-}).

There was no significant difference in the levels of plasma cholesterol in C57/BL6 and ApoE^{-/-} mice that were supplemented with BA as compared to their corresponding controls (ie. 0% BA groups). However, plasma cholesterol was reduced by 30.2% ($P < 0.01$) in BA-treated ApoE^{-/-}GPx1^{-/-} mice (Table 1). The sum of lipoprotein cholesteryl esters and free cholesterol as determined by mass spectrometry showed a similar trend with a larger decrease in cholesteryl ester (24.2%) relative to free cholesterol (10.8%) in the BA-treated ApoE^{-/-}GPx1^{-/-} mice although neither of these were significant (Table 1). Analysis of lipoprotein cholesterol, also by mass spectrometry, showed that in ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, the bulk of cholesterol was within the VLDL pool; however, the reduction of cholesterol in the treated ApoE^{-/-}GPx1^{-/-} mice was primarily within the LDL pool (42.6%, $p < 0.05$, Table 1). The BA-treated C57/BL6 and ApoE^{-/-} mice showed 28.9% ($P < 0.01$) and 19.1% ($P = 0.445$) higher levels of triglycerides compared to their corresponding control groups (Table 1). Whereas the treated ApoE^{-/-}GPx1^{-/-} mice showed a 24.9% ($P = 0.0904$) lower level (Table 1). The levels of fasting blood glucose in the treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice were reduced by 34.6% ($P < 0.001$) and 30.2% ($P < 0.05$), respectively (Table 1). However, no significant difference was observed in the fasting plasma insulin levels in the BA-treated mice compared to the corresponding control groups of the same genotypes (Table 1).

Table 1. Plasma lipid measurements of mice on a high fat diet supplemented with/without 2% BA.

Parameter	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} /GPx1 ^{-/-}	
	0% BA	2% BA	0% BA	2% BA	0% BA	2% BA
Final body weight*	37.70 ± 2.94	35.70 ± 3.46	33.40 ± 3.08	27.60 ± 1.57	30.00 ± 2.88	26.80 ± 1.16
Fasting blood glucose†	11.18 ± 0.76	11.78 ± 0.58	10.94 ± 0.92	7.15 ± 0.28	10.36 ± 1.17	7.23 ± 0.68
Insulin‡	0.50 ± 0.12	0.56 ± 0.08	0.32 ± 0.04	0.30 ± 0.05	0.39 ± 0.04	0.33 ± 0.04
Cholesterol†	4.19 ± 0.31	4.05 ± 0.11	31.56 ± 3.92	38.00 ± 3.21	38.88 ± 2.47	27.13 ± 1.96
Triglyceride†	0.41 ± 0.02	0.52 ± 0.03	1.36 ± 0.28	1.62 ± 0.17	1.97 ± 0.19	1.48 ± 0.19
VLDL-C§	0.13 (0.11, 0.19)	0.14 (0.11, 0.17)	12.24 (10.16, 17.96)	16.96 (13.65, 19.83)	13.93 (10.99, 18.98)	12.92 (11.00, 16.69)
LDL-C§	0.91 (0.59, 1.01)	0.71 (0.58, 0.83)	8.45 (6.66, 9.36)	6.90 (4.95, 10.45)	9.69 (8.06, 11.17)	5.56 (4.72, 7.27)
HDL-C§	2.59 (2.09, 2.83)	2.08 (1.86, 2.39)	1.01 (0.74, 1.17)	1.23 (0.79, 1.47)	1.29 (1.22, 1.47)	1.37 (1.14, 2.14)
Cholesteryl ester 	2.65(2.04, 2.90)	2.03 (1.77, 2.39)	14.41 (11.87, 18.51)	17.14 (12.99, 21.43)	17.65 (13.96, 22.83)	13.38 (11.16, 17.32)
Free cholesterol 	0.98 (0.75, 1.13)	0.89 (0.78, 1.01)	7.29 (5.69, 9.97)	7.94 (6.40, 10.33)	7.25 (6.31, 8.80)	6.47 (5.70, 8.78)

* final body weight on week 12. Parameter is expressed in g. Data represent mean ± SEM, N = 9 – 10/group. Data were compared between groups (0% and 2% BA) of each genotype using Student t-test. The values in bold indicate $P < 0.01$.

† parameters are expressed in mmol/L, and measured using glucometer for fasting blood glucose and COBAS blood analyser for cholesterol and triglyceride. Data represent mean ± SEM. The two dietary groups (0% and 2% BA) were analysed using Student t-test; the values in bold indicate statistical significance relative to the untreated animals ($P < 0.05$).

‡ parameter is expressed in ng/mL, and measured using an ELISA kit. Data represent mean ± SEM, N = 9 – 10/group.

§ parameter is expressed in mmol/L. and measured using mass spectrometry. Data represent median (interquartile range). Values were obtained by the sum of cholesteryl ester and free cholesterol in respective lipoproteins.

|| parameters are expressed in mmol/L, and represent the sum of the individual lipoprotein pools measured using mass spectrometry. Data represent median (interquartile range). The two dietary groups (0% and 2% BA) were analysed using Mann-Whitney U test; the values in bold indicate statistical significance relative to the untreated animals ($P < 0.05$).

Alkyl- and alkenylphospholipids in plasma and heart

There was no significant difference in the total phosphatidylcholine (PC) levels (diacyl-PC species only) between the 2% BA-treated and untreated groups in the heart and plasma of C57/BL6, ApoE^{-/-} mice, but the total PC level was significantly lower in plasma from the treated ApoE^{-/-}GPx1^{-/-} mice (Supplementary Table III), consistent with the reduced level of plasma cholesterol (Table 1), and slightly lower in the heart (Supplementary Table IV). We normalised all the lipid measurements relative to total PC level to enable comparison of relative values obtained from the plasma and heart analyses.

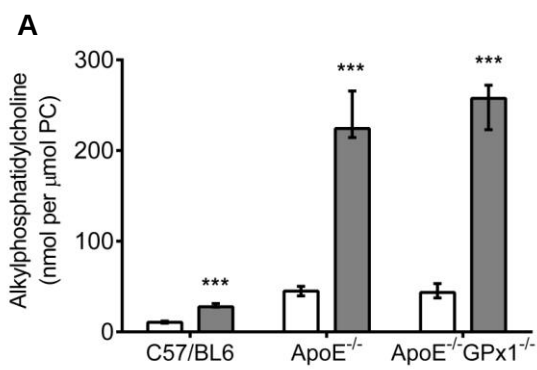
Mice treated with BA had significantly higher levels of alkyl- and alkenylphospholipids in plasma (2.60 to 16.7 fold) and heart (0.800 to 4.80 fold) relative to PC. This effect was observed in treated C57/BL6, ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice (Figure 1).

Analyses of alkyl- and alkenylphospholipid species showed that supplementation of BA increased some but not all species in plasma (Supplementary Table V) and heart (Supplementary Table VI) while some species showed a lower level in mice treated with BA. PC(O-36:1), PC(O-36:2), PC(O-38:4), PC(O-40:5), PC(P-40:5), PC(P-40:6), PE(O-38:4), PE(P-40:5), and PE(P-40:6), were all at least 10-fold higher in the plasma of the BA-treated mice (Supplementary Table V). These species were also elevated in the heart (Supplementary Table VI). While very few species were lower in plasma of the treated mice (Supplementary Table V), multiple species were significantly lower in the heart, including PC(P-30:0), PC(P-32:0), (P-34:1), PC(P-34:2), PE(P-38:5) and PE(P-38:6) (Supplementary Table VI).

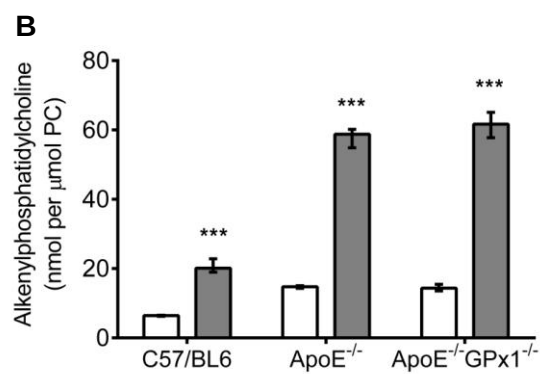
Lysophospholipids in plasma and heart

We observed a significantly higher level ($P<0.05$) of LPC and LPE in plasma and heart of all the BA-treated mice groups (data not shown). Total levels of circulating sn-2 LPC (Figure 2A) and sn-2 LPE (Figure 2B) were significantly higher in both treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice. In contrast, the total levels of sn-1 LPC (Figure 2A) and sn-1 LPE (Figure 2B) were not significantly different for all of the mice genotypes. In heart, significantly higher levels of sn-2 LPC were observed only in the treated ApoE^{-/-} mice (Figure 2C), whereas higher levels of sn-2 LPE were observed only in the treated C57/BL6 and ApoE^{-/-} mice (Figure 2D). No significant differences were observed in the level of both species of sn-1 lysophospholipids for all genotypes (Figure 2C and 2D), except for a significantly lower level of sn-1 LPE in the treated ApoE^{-/-}GPx1^{-/-} mice (Figure 2D).

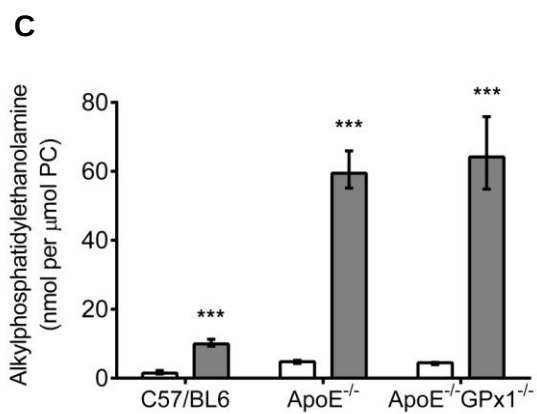
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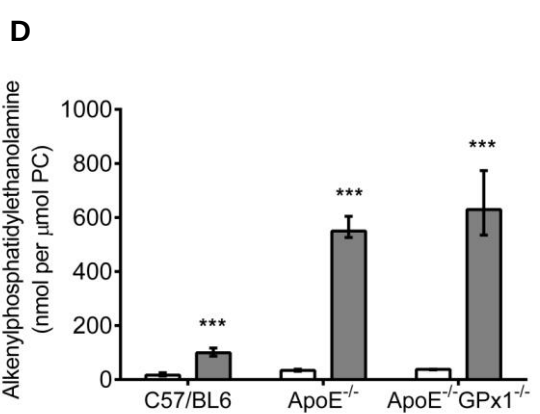
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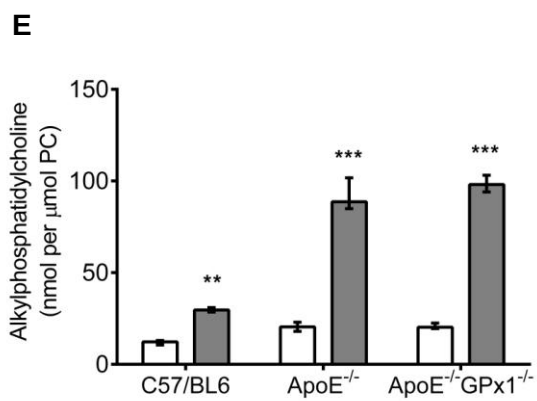
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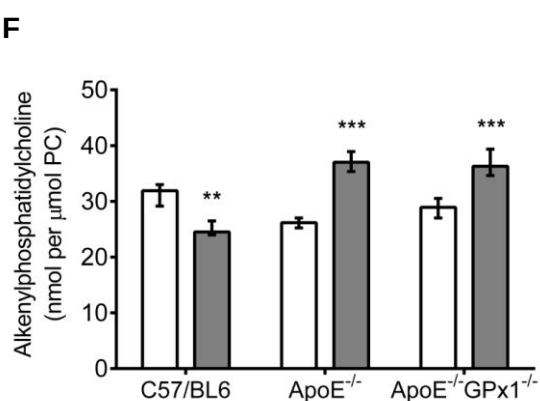
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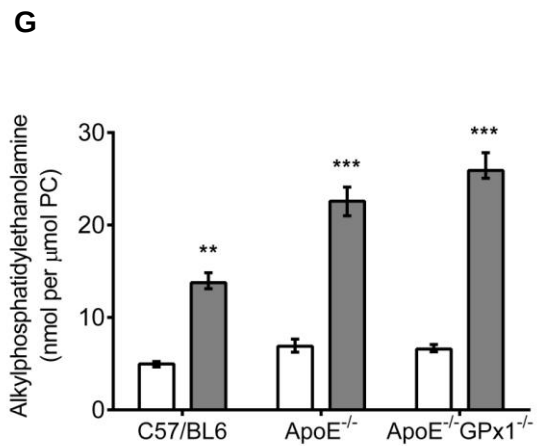
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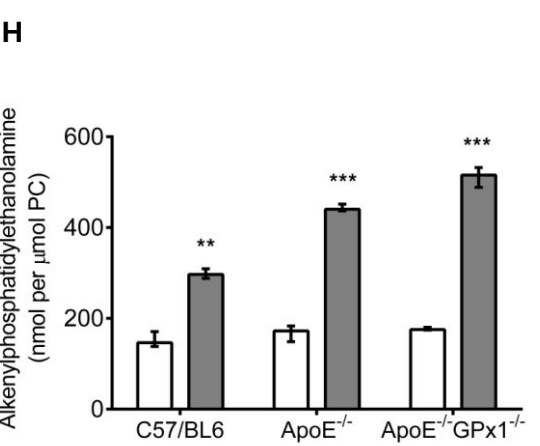


Figure 1. Level of alkyl- and alkenylphospholipids in plasma and heart of mice following 12 weeks of high fat diet supplemented with/without batyl alcohol. Total plasma level of (A) alkylphosphatidylcholine; (B) alkenylphosphatidylcholine; (C) alkylphosphatidylethanolamine; and (D) alkenylphosphatidylethanolamine; and cardiac level of (E) alkylphosphatidylcholine; (F) alkenylphosphatidylcholine; (G) alkylphosphatidylethanolamine; and (H) alkenylphosphatidylethanolamine in C57/BL6 (N=10), ApoE^{-/-} (N=10), and ApoE^{-/-}GPx1^{-/-} (N=9) mice on high fat diet supplemented with either 0% batyl alcohol (open bars) or 2% batyl alcohol (closed bars). Bars indicate median, whiskers indicate interquartile range. The two dietary groups of each genotype were analysed using the Mann-Whitney U-test; ** indicates $P<0.01$ and *** indicates $P<0.001$.

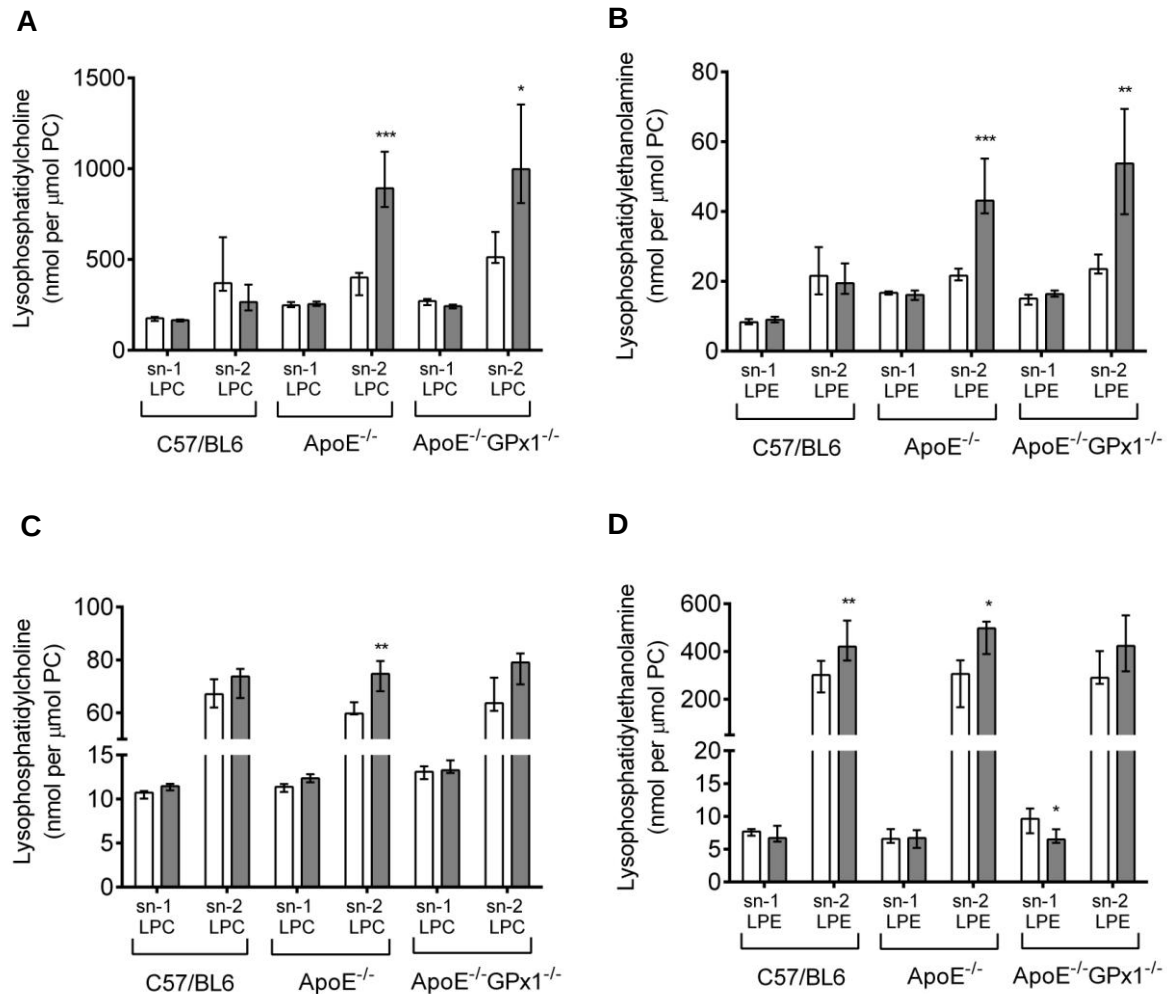


Figure 2. Level of lysophosphatidylcholine and lysophosphatidylethanolamine in plasma and heart of mice following 12 weeks of high fat diet supplemented with/without batyl alcohol. Total level of sn-1 and sn-2 lysophosphatidylcholine (sn-1 LPC and sn-2 LPC, respectively), and sn-1 and sn-2 lysophosphatidylethanolamine (sn-1 LPE and sn-2 LPE, respectively) in (A and B) plasma; and (C and D) heart of C57/BL6 (N=10), ApoE^{-/-} (N=10), and ApoE^{-/-}GPx1^{-/-} (N=9) mice on high fat diet supplemented with either 0% batyl alcohol (open bars) or 2% batyl alcohol (closed bars). Bars indicate median, whiskers indicate interquartile range. The two dietary groups of each genotype were analysed using Mann-Whitney U-test; * indicates $P<0.05$, ** indicates $P<0.01$ and *** indicates $P<0.001$.

Assessment of atherosclerotic lesions

ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice that fed on high fat diet without BA supplementation for 12 weeks developed plaques throughout the aorta (Figure 3). This plaque deposition was reduced by 70.8% ($P = 5.69\text{E-}07$) and 69.1% ($P = 2.00\text{E-}04$) in the whole aorta of the treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, respectively (Figure 3A and 3B). Similar levels of reduction in plaque deposition were observed in the aortic arch, thoracic, and abdominal regions of the aorta (Figure 3C-3E).

The high-fat diet feeding without BA supplementation resulted in the development of 0.38 mm² and 0.41 mm² lesions in the aortic sinus of ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, respectively. The lesion size was reduced by 12.3% ($P = 0.177$) and 40.3% ($P = 7.74\text{E-}03$) in the treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, respectively (Figure 4A). Compared to the untreated ApoE^{-/-} mice, BA-treated mice had a 34% reduction ($P = 1.17\text{E-}02$) in the necrotic core area. However, no difference was observed between the control and treated ApoE^{-/-}GPx1^{-/-} mice (Supplementary Figure II).

Pro-inflammatory and oxidative stress markers in atherosclerosis

BA supplementation resulted in a lower level of VCAM-1 (pro-inflammatory marker) staining (-28.3%, $P = 2.43\text{E-}02$) in the ApoE^{-/-}GPx1^{-/-} mice, but no significant difference was observed in the treated ApoE^{-/-} mice relative to the untreated control (Figure 4B). A similar trend was observed when macrophages were detected with F4/80 staining in the aortic sinus of 2%BA-treated ApoE^{-/-}GPx1^{-/-} mice. A small, albeit non-significant decrease (-22 %, $P = 0.118$) was detected, compared to the untreated mice of the same genotype (Figure 4C). No difference was noted in the ApoE^{-/-} aortic sinuses (Figure 4C). In addition, BA supplementation resulted in decreased staining of nitrotyrosine (a marker of protein nitration) by 78.3% ($P = 5.11\text{E-}06$) only in the treated ApoE^{-/-}GPx1^{-/-} mice (Figure 4D). No significant difference in 4-hydroxynonenal (4-HNE, a marker of lipid peroxidation) staining was observed between the treated and untreated groups for all genotypes (Figure 4E).

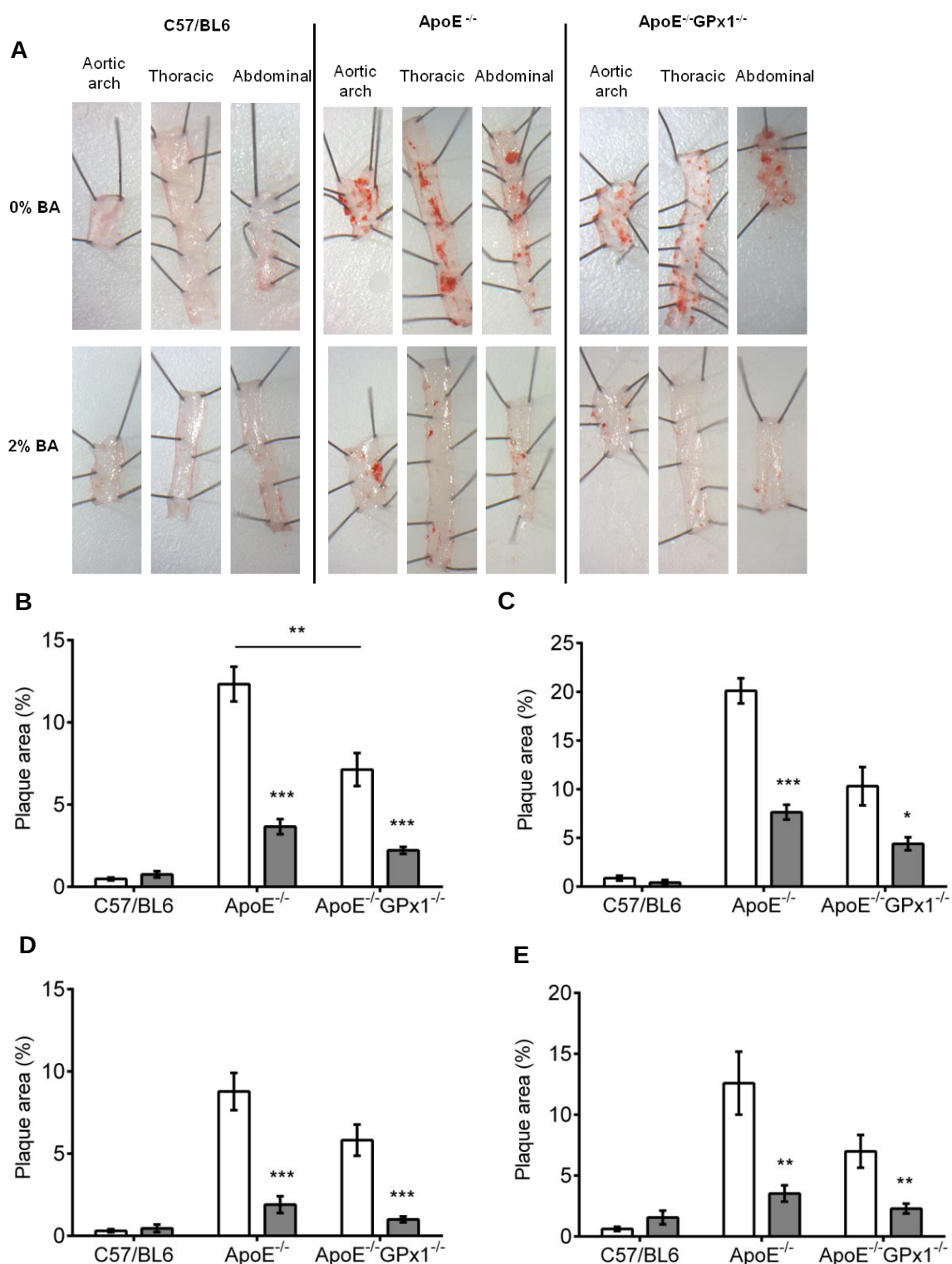
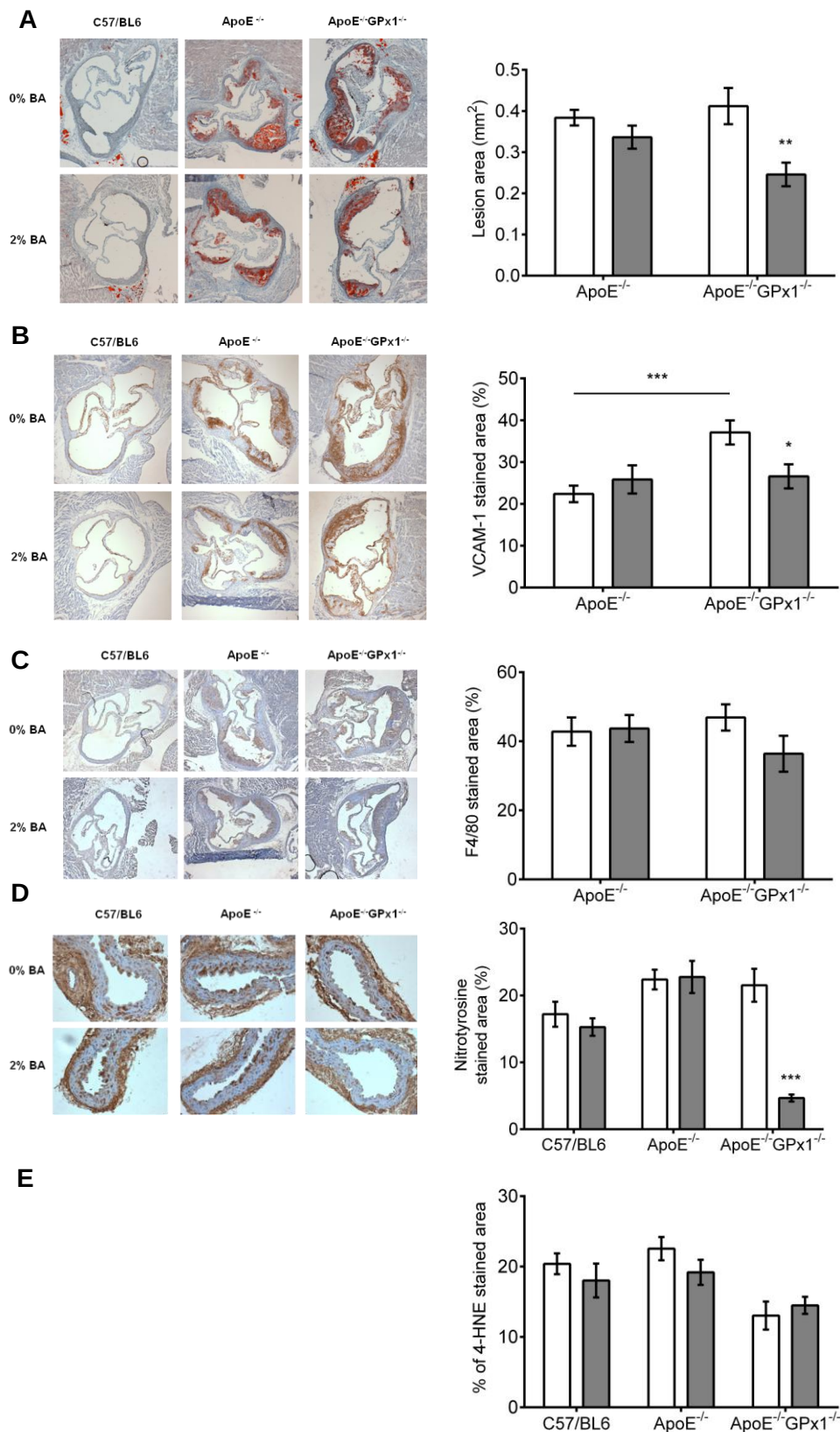


Figure 3. Atherosclerotic plaque in the aorta of mice fed a high fat diet supplemented with/without batyl alcohol. (A) Representative *en face* aortic images of C57/BL6, ApoE^{-/-}, and ApoE^{-/-}GPx1^{-/-} mice were compared within genotype between the 0% batyl alcohol (top panels) and 2% batyl alcohol (bottom panels) dietary groups. Aorta was divided into three sections; aortic arch, thoracic, and abdominal. Red staining indicates atherosclerotic plaques. Levels of atherosclerotic lesions in the (B) whole aorta; (C) aortic arch; (D) thoracic

aorta; and (E) abdominal aorta of C57/BL6 (N=10), ApoE^{-/-} (N=10), and ApoE^{-/-}GPx1^{-/-} (N=9) mice were assessed and compared between 0% batyl alcohol (open bars) and 2% batyl alcohol (closed bars) dietary groups of each genotype. Data are represented as mean $\pm$ SEM. Significant differences were assessed using Student t-tests; * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.



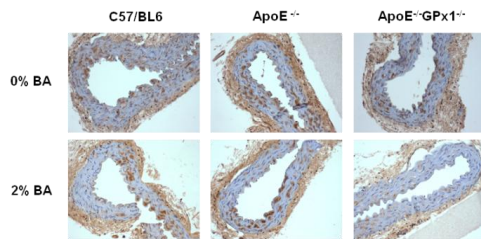


Figure 4. Aortic lesion and inflammation as well as oxidative stress in mice fed a high fat diet supplemented with/without batyl alcohol. Representative photomicrographs (left panels) of (A, B, and C) aortic sinus, magnification of X40; and (D and E) aortic vessels, magnification of x200 of C57/BL6 (N=10), ApoE^{-/-} (N=10), and ApoE^{-/-}GPx1^{-/-} (N=9) mice fed with high fat diet containing either 0% batyl alcohol (top panels) or 2% batyl alcohol (bottom panels). Aortic sinuses were stained for lesions, VCAM-1, and F4/80 whereas aortic vessels were stained for nitrotyrosine and 4-HNE. Analyses (right panels) of staining in the tissues of mice treated with 0% batyl alcohol (open bars) or 2% batyl alcohol (closed bars). Data are represented as mean $\pm$ SEM. Significant differences were assessed using Student t-tests; * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

Discussion

Multiple lines of evidence suggest that plasmalogens may be atheroprotective. We explored the effect of plasmalogen modulation in two mouse models of atherosclerosis. We report here that dietary supplementation of BA increased circulating and heart plasmalogen levels and was associated with an attenuation of atherosclerosis.

The effect of BA treatment on weight gain differed between genotypes and while lower food intake may have contributed to the observed differences in the weight gain of 0% vs. 2% BA treated- ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, we did not observe this in the control C57/BL6 mice despite a lower food intake in the treated mice. The lower weight gain the ApoE^{-/-} mice on the BA diet was not accompanied by any difference in total cholesterol or triglyceride, suggesting that these mice had equal risk of atherosclerosis resulting from their plasma lipids. In contrast, the BA-treated ApoE^{-/-}GPx1^{-/-} mice showed a lower level of total cholesterol, suggesting that BA treatment may have impacted on this lipid pathway to potentially influence atherosclerosis risk. The cholesterol lowering effect of BA supplementation in the ApoE^{-/-}GPx1^{-/-} mice suggests modulation of intracellular cholesterol transport and/or HDL-mediated cholesterol efflux where plasmalogens have been previously demonstrated to play essential roles^{7, 8}. Subsequent analysis of the free cholesterol and cholesteryl esters in the lipoprotein fractions by mass spectrometry showed that the lower levels of cholesterol in the ApoE^{-/-}GPx1^{-/-} mice were primarily the result of lower levels of LDL cholesteryl ester and so potentially influencing atherosclerosis progression.

Similar to the effect of BA on weight gain, both the treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, but not the C57/BL6, had lower fasting blood glucose levels despite showing no difference in fasting insulin levels. The effect of BA on C57/BL6 mice was similar to that in a previous report where administration of high dose of batyl alcohol (200mg/kg on high fat diet for 8 weeks) resulted in no difference in the fasting blood glucose levels²³. The mechanism whereby BA reduces weight gain and fasting blood glucose in ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice on a high fat diet may involve modulation of leptin secretion as it has been shown that administration of high dose of a related alkylglycerol (selachyl alcohol) to C57/BL6 mice led to decreased concentration of serum leptin²³. Further, mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light chain enhancer of activated B cells (NF- κ B) signalling pathways play a role in the production of inflammatory cytokines including those that induce glucose intolerance^{24, 25}. It has been demonstrated that alkylglycerol treatment to primary adipocytes resulted in the modulation of phosphorylation of key proteins, JNK and ERK in

the MAPK and NF- κ B signalling pathways²³. Hence, the lowering of fasting blood glucose levels could be attributed to the effect of BA on these pathways and the subsequent down-regulation of inflammatory genes that are associated with disease progression.

In plasma and heart, PC(P-40:5) and PC(P-40:6) showed the greatest increase in the BA-treated animals relative to the control diet, while PE(P-40:5) and PE(P-40:6) also showed similar increases and the alkyl species closely followed the same trend. These species typically contain an 18:0 alkyl/alkenyl chain with a corresponding 22:5 or 22:6 fatty acid. Clearly, there was an incorporation of BA into alkyl- and alkenylphospholipids and the preferential production of species with 18:0 alkyl and alkenyl chains. However, while species containing an 18:0 alkenyl chain represent approximately 46% of the total PE(P) class, they represent only 10% of the corresponding PC(P) class. Thus, while the up-regulation of PC(P) and PE(P) species is comparable, the relative proportion of the total class leads to considerable differences between the up-regulation at the class level with PE(P) showing a 10-15 fold up-regulation (in plasma) compared to only 2-4 fold up-regulation for PC(P). This raises an important consideration for the therapeutic potential of alkylglycerols to regulate plasmalogen synthesis as the alkyl chain present will influence the capacity to up-regulate the synthesis of particular species of plasmalogen. Based on these results, we propose that by selecting a suitable formulation of alkylglycerol species, it will be possible to modulate the level of plasmalogen and the specific profile of plasmalogens that are produced.

Lysophospholipids can be derived from oxidation of alkenylphospholipids to produce sn-2 lysophospholipids or the action of lipoprotein-associated phospholipase A₂ on diacylglycerophospholipids to produce sn-1 lysophospholipids. Lipoprotein-associated phospholipase A₂ is an emerging risk factor for coronary artery disease^{26, 27}. It was previously shown that the expression of lipoprotein-associated phospholipase A₂ was enhanced in symptomatic atherosclerotic plaques, and this was accompanied by increased levels of LPC²⁸. We observed a higher level in the total LPC and LPE in plasma and heart of the BA-treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice, compared to the corresponding control groups of the same genotypes (data not shown). However, these were due to increases in the sn-2 lysophospholipids with no effect on the sn-1 lysophospholipids, suggesting that the increase in the total levels of lysophospholipids is likely to be the result of oxidation of plasmalogen, rather than catalytic action of lipoprotein-associated phospholipase A₂. However, it is possible that sn-2 lysophospholipids were also produced by phospholipase A₁^{20, 29}.

Plasmalogen enrichment via supplementation with BA significantly attenuated atherosclerotic plaque formation in the aorta of both the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice. This plasmalogen-mediated effect was comparable to the attenuation reported in vitamin E-supplemented ApoE^{-/-} mice³⁰; in their study, lesions in the whole aorta were reduced by approximately 66%, with 39% lesion reduction in the aortic arch after 16 weeks of intervention³⁰. Similar to our findings, the reduction in atherosclerosis was not accompanied with differences in the total plasma cholesterol and triglyceride levels in the ApoE^{-/-} mice³⁰. Interestingly in our study, plasmalogen supplementation also resulted in a significant reduction in plaque formation in the aortic sinus region, albeit that this reduction was only observed in the treated ApoE^{-/-}GPx1^{-/-} mice, possibly due to the lower level of circulating cholesterol in these mice. The BA treatment also resulted in significantly lower necrotic core area only in the ApoE^{-/-} mice (Supplementary Figure II), suggesting a decrease in macrophage death, which contributes to necrotic core formation without affecting the size of the lesion; whereas in the ApoE^{-/-}GPx1^{-/-} mice, treatment with BA reduced both plaque size and necrotic core proportionately. In the untreated mice, we observed significant levels of plaque formation in the aorta of both the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice with higher levels in the ApoE^{-/-} compared to the ApoE^{-/-}GPx1^{-/-} mice (Figure 3B). While increased levels of oxidative stress and inflammation in the ApoE^{-/-}GPx1^{-/-} mice might be expected to result in increased plaque size relative to the ApoE^{-/-}GPx1^{-/-} mice. Torzewski *et al.*³ demonstrated

that high-fat feeding of ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice for 12 weeks resulted in no significant difference in the aortic plaque accumulation between the two genotypes. However, high-fat feeding for 24 weeks resulted in a higher increase in plaques in the ApoE^{-/-}GPx1^{-/-} mice compared to that in ApoE^{-/-}. This suggests that the difference between these genotypes may only become apparent at a latter time-point in the ApoE^{-/-}GPx1^{-/-} mouse model of atherosclerosis.

Consistent with a previous report ³, we observed an increased level of inflammation as indicated by VCAM-1 staining in the untreated ApoE^{-/-}GPx1^{-/-} compared to the ApoE^{-/-} mice (Figure 4B). This inflammation was attenuated in the BA-treated ApoE^{-/-}GPx1^{-/-} model, but not in the ApoE^{-/-} model, suggesting that plasmalogen exerts a greater effect in an environment of heightened inflammation and oxidative stress. This is further supported by a decreased level of aortic F4/80 (macrophage content) staining in the BA-treated compared to untreated ApoE^{-/-}GPx1^{-/-}, albeit the difference was not statistically significant. **However, it remains to be determined whether such a decrease in macrophage content results solely from the reduced endothelial inflammation or whether a decrease in monocyte levels also contributed to this effect.**

The level of nitrotyrosine was significantly reduced in the treated ApoE^{-/-}GPx1^{-/-} mice, but not in the ApoE^{-/-} mouse. Although it may have been predicted that plasmalogen treatment would lessen oxidative stress and therefore the nitrotyrosine marker in both models, a deeper examination of compensatory antioxidant mechanisms may offer an explanation. Indeed, previous studies in diabetic mice have not only revealed a significant increase in glutathione peroxidase-1 mRNA in the ApoE^{-/-} model, but also significant increases in the expression of the glutathione peroxidase isoforms, glutathione peroxidase-3 and glutathione peroxidase-4, as well as superoxide dismutase (SOD) 1 and SOD2 (cytosolic and mitochondrial isoforms of the SOD family) in the ApoE^{-/-}GPx1^{-/-} model ². Despite these "compensatory mechanisms", peroxynitrite levels were only increased in the ApoE^{-/-}GPx1^{-/-} model following the onset of the disease ², suggesting that glutathione peroxidase-1 was acting in its capacity as a peroxynitrite reductase to lessen peroxynitrite ^{2, 31} in the ApoE^{-/-} model. It has been well established that peroxynitrite is produced by superoxide and nitric oxide. However, studies have also shown that nitrite, which is a by product of peroxynitrite reduction, contributes to nitrotyrosine formation by reacting with hydrogen peroxide in the presence of myeloperoxidase ³²⁻³⁴. In light of this observation, the earlier findings by Lewis *et al.* suggest that the production of nitrotyrosine in the ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} models may not follow the same pathways. Furthermore, the production of ROS has been shown to upregulate nicotinamide adenine dinucleotide-phosphate oxidase, which in turn, increases the production of superoxide, which is a substrate of the SOD enzyme family. SOD converts superoxide to hydrogen and lipid peroxide, which then feeds forward to produce more ROS ². Placing our results in context with earlier findings of Lewis *et al.*, we propose that the increase in SOD observed in the ApoE^{-/-}GPx1^{-/-} model leads to a greater cyclical production of lipid peroxides and ROS in the ApoE^{-/-}GPx1^{-/-} model that ultimately feeds back to the production of peroxynitrite and then to nitrotyrosine formation. When plasmalogens are increased in the ApoE^{-/-}GPx1^{-/-} model, this serves to stall the cyclical production of ROS via SOD and lipid peroxides thereby reducing the peroxynitrite and nitrotyrosine production. The same effect was not observed in the ApoE^{-/-} mice because of the differential mechanism(s) driving ROS production and nitrotyrosine formation.

Plasmalogen enrichment resulted in no significant differences in the level of 4-HNE staining across all groups studied. 4-HNE is a by-product of plasmalogen oxidation. The effect of the accumulation of fatty aldehydes such as 4-HNE on cellular toxicity remains controversial. However, previous studies have provided evidence of the quick metabolism of fatty aldehydes into their respective fatty acid and fatty alcohol and their export out of the cells ^{35, 36}. In addition, Cheng *et al.* ³⁷ demonstrated that along with the production of 4-HNE, heat shock and oxidative stress treatment of human leukemic cells resulted in an induction of

glutathione S-transferase isozyme, hGST5.8 and a Ral binding GTPase-activating protein, RLIP76 that catalyse the conjugation of glutathione and 4-HNE (ie.GS-HNE) and the exportation of the GS-HNE, respectively. In light of these studies, we suggest that although increased plasmalogen levels in the treated mice likely led to increased production of 4-HNE, this in turn could have induced its increased metabolism and export from the cells and so no difference was observed between the treated dietary groups and their respective controls.

In conclusion, we demonstrate that alkylglycerol (batyl alcohol) dietary supplementation resulted in elevated levels of alkyl- and alkenylphospholipids in plasma and heart of all 2% BA-treated mice, compared to the corresponding genotype controls. The increase in plasmalogens was associated with attenuation in the formation of atherosclerosis in ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice with additional site-specific effects in the latter model. While our study suggests a pleiotropic role for plasmalogens, affecting body weight and blood glucose in both the treated ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice with additional cholesterol lowering, anti-inflammatory and anti-oxidant actions in the treated ApoE^{-/-}GPx1^{-/-} mice, further investigations are now required that will assist with an understanding of glucose metabolism and the particular inflammatory/oxidative stress pathways that plasmalogens act upon. Overall, the data presented here indicate that plasmalogen enrichment may represent a viable therapeutic strategy to prevent atherosclerosis and alleviate cardiovascular disease risk, particularly under conditions of elevated oxidative stress and inflammation.

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Disclosures:

None.

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PLASMALOGEN MODULATION ATTENUATES ATHEROSCLEROSIS IN APOE- AND APOE/GPX1-DEFICIENT MICE.

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Supplemental Material

Supplementary Table I. Internal standards used in lipid extraction and mass spectrometry analysis.

Internal standards	pmol*
7-ketocholesterol (d7)	100
Cardiolipin 14:0/14:0/14:0	100
Ceramide 17:0	100
Cholesterol (d7)	10000
Cholesterol ester 18:0 (d6)	1000
Diacylglycerol 15:0/15:0	200
Dihexosylceramide 16:0 (d3)	50
Dihydroceramide 8:0	50
Glyceryl triheptadecanoate 17:0/17:0/17:0	100
Lysophosphatidylcholine 13:0	100
Lysophosphatidylethanolamine 14:0	100
Monohexosylceramide 16:0 (d3)	50
Phosphatidylcholine 13:0/13:0	100
Phosphatidylethanolamine 17:0/17:0	100
Phosphatidylglycerol 17:0/17:0	100
Phosphatidylserine 17:0/17:0	100
Sphingomyelin C12:0	200
Trihexosylceramide 17:0	50

*Amount of internal standard per sample

Supplementary Table II. Conditions for tandem mass spectrometry analysis of lipid species identified in mice plasma and tissue.

Lipid class*	No. of species	Internal standard	Parent ion	Experiment†	Voltage settings (V)‡			
					DP	EP	ColIE	CXP
CE	26	Cholesterol ester 18:0 (d6)	[M+NH ₄] ⁺	PIS, <i>m/z</i> 369.3	30	10	20	12
COH	1	Cholesterol (d7)	[M+NH ₄] ⁺	PIS, <i>m/z</i> 369.3	55	10	17	12
LPC	22	Lysophosphatidylcholine 13:0	[M+H] ⁺	PIS, <i>m/z</i> 184.1	100	10	45	11
LPE	6	Lysophosphatidylethanolamine 14:0	[M+H] ⁺	NL, 141 Da	80	10	31	7
PC	51	Phosphatidylcholine 13:0/13:0	[M+H] ⁺	PIS, <i>m/z</i> 184.1	100	10	45	11
PC(O)	19	Lysophosphatidylcholine 13:0	[M+H] ⁺	PIS, <i>m/z</i> 184.1	100	10	45	11
PC(P)	14	Phosphatidylcholine 13:0/13:0	[M+H] ⁺	PIS, <i>m/z</i> 184.1	100	10	45	11
PE(O)	11	Phosphatidylethanolamine 17:0/17:0	[M+H] ⁺	NL, 141 Da	80	10	31	7
PE(P)	11	Phosphatidylethanolamine 17:0/17:0	[M+H] ⁺	NL, 141 Da	80	10	31	7

*CE – cholesteryl ester, COH – free cholesterol, LPC – lysophosphatidylcholine, LPE – lysophosphatidylethanolamine, PC(O) – alkylphosphatidylcholine, PC(P) – alkenylphosphatidylcholine, PE(O) – alkylphosphatidylethanolamine, PE(P) – alkenylphosphatidylethanolamine.

† NL – neutral loss, PIS – precursor ion scan.

‡ ColIE – collision energy, CXP – collision cell exit potential, DP – declustering potential, EP – exit potential.

Supplementary Table III. Differences in plasma levels of phosphatidylcholine in mice on a high fat diet supplemented with/without batyl alcohol.

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡
PC 28:0	175	0.650	2162	0.520	1545	0.450
PC 29:0	103	0.570	1316	0.460	1189	0.370
PC 30:0	3085	0.560	23365	0.670	22377	0.500
PC 31:0	1677	0.620	14121	0.730	12640	0.570
PC 31:1	1166	0.630	5006	1.13	5698	0.790
PC 32:0	19778	0.530	90465	0.770	87420	0.640
PC 32:1	52850	0.840	125698	0.910	141248	0.740
PC 32:2	5552	0.730	12325	0.910	13216	0.730
PC 32:3	385	0.720	819	0.970	1046	0.630
PC 33:0	2246	0.600	13066	0.830	13399	0.710
PC 33:1	21665	0.850	40903	0.860	40306	0.820
PC 33:2	4530	0.590	7787	0.700	7132	0.620
PC 33:3	277	0.580	582	0.570	550	0.470
PC 34:0	4049	0.500	14295	0.900	20189	0.410
PC 34:1	389290	1.01	492293	1.11	553855	0.880
PC 34:2	296850	0.94	435017	0.950	479756	0.720
PC 34:3	31422	0.670	44692	0.760	44565	0.580
PC 34:4	2241	0.580	2701	0.670	3099	0.470
PC 34:5	194	0.690	314	0.640	295	0.480
PC 35:1	33090	0.860	55143	1.01	62878	0.730

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡
PC 35:2	30604	0.740	40048	0.750	40655	0.660
PC 35:3	4087	0.670	4653	0.750	5089	0.520
PC 35:4	1679	0.530	1943	0.710	1935	0.550
PC 35:5	649	1.19	2705	1.81	2924	1.58
PC 36:0	625	0.700	2019	1.96	3422	0.850
PC 36:1	192574	1.04	297101	1.03	339779	0.780
PC 36:2	294899	0.920	356920	0.980	429749	0.670
PC 36:3	258222	0.920	234385	1.02	285006	0.690
PC 36:4a	10193	0.850	10835	0.950	12455	0.700
PC 36:4b	108421	0.810	106869	1.06	117514	0.810
PC 36:5	32352	1.01	39283	1.07	54502	0.680
PC 36:6	2791	0.760	2427	0.810	3159	0.550
PC 37:4	12088	0.540	10456	0.770	13000	0.540
PC 37:5	2549	0.680	2400	0.810	2773	0.610
PC 37:6	2849	0.630	2707	0.620	2742	0.470
PC 38:2	5825	1.05	11140	1.28	18810	0.650
PC 38:3	60437	1.14	49835	1.25	77070	0.760
PC 38:4	68470	0.870	51618	1.34	89948	0.680
PC 38:5	108434	0.810	79952	1.05	106703	0.730
PC 38:6a	11231	0.810	6030	1.20	12247	0.490
PC 38:6b	181292	0.850	108961	1.17	151909	0.730
PC 38:7	11644	0.790	5125	1.11	8063	0.610
PC 39:5	3521	0.650	2614	0.920	3599	0.520
PC 39:6	12048	0.630	7319	1.07	10129	0.610

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mL)†	Fold difference with 2% BA group‡
PC 39:7	1611	0.760	646	0.960	1082	0.480
PC 40:4	3726	0.850	2599	1.18	3346	0.930
PC 40:5	16325	0.900	10862	1.24	16959	0.780
PC 40:6	49714	0.900	25864	1.53	43378	0.850
PC 40:7	55369	0.760	20915	1.13	33519	0.610
PC 40:8	6644	0.710	2904	0.990	4639	0.530
Total PC	2489133	0.900	2845413	1.05	3460819	0.730

*PC – phosphatidylcholine

†Median level of lipids in mice which were on high fat diet supplemented with 0% batyl alcohol.

‡Fold difference of median level of lipids in mice fed with high fat diet containing 2% batyl alcohol as compared to those fed with the 0% batyl alcohol containing diet. Data were analysed using Mann-Whitney U test, corrected for multiple comparisons by Benjamini Hochberg. Values in bold indicate statistical significance (corrected $P < 0.05$).

Supplementary Table IV. Differences in levels of phosphatidylcholine in heart of mice on a high fat diet supplemented with/without batyl alcohol.

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group †	Fold difference with 2% BA group‡
PC 28:0	22	1.23	24	0.960	30	0.870
PC 29:0	17	1.12	21	0.860	22	0.730
PC 30:0	520	1.02	482	0.870	542	0.670
PC 31:0	174	0.880	164	0.960	181	0.750
PC 31:1	9	1.11	10	1.00	9	1.11
PC 32:0	2133	0.750	1452	0.970	1844	0.610
PC 32:1	1143	1.11	1056	1.14	1140	1.08
PC 32:2	129	1.19	142	0.980	155	0.980
PC 32:3	6	1.33	7	0.860	6	1.17
PC 33:0	150	0.970	159	1.05	187	0.840
PC 33:1	398	1.20	449	1.04	454	1.09
PC 33:2	54	1.24	72	0.830	67	1.03
PC 34:0	397	1.06	326	0.980	387	0.710
PC 34:1	11830	1.27	13714	1.08	14295	1.05
PC 34:2	3128	1.22	4035	0.860	3897	0.970
PC 34:3	243	1.22	282	0.890	281	1.04
PC 34:4	51	1.25	46	1.15	64	0.910
PC 34:5	3	1.67	4	1.00	4	1.25
PC 35:1	487	1.27	578	0.990	578	1.09
PC 35:2	324	1.14	398	0.830	381	0.930
PC 35:3	39	1.33	56	0.730	52	0.870

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group †	Fold difference with 2% BA group‡
PC 35:4	85	1.09	75	1.13	101	0.790
PC 36:0	47	1.28	55	0.960	54	0.870
PC 36:1	5166	1.59	7681	1.07	6672	1.15
PC 36:2	3847	1.46	5583	0.890	5426	0.930
PC 36:3	4173	1.42	5398	0.920	5744	1.01
PC 36:4a	120	1.39	320	0.430	154	1.05
PC 36:4b	6502	1.06	5790	1.06	7963	0.600
PC 36:5	499	1.54	555	1.13	657	0.980
PC 36:6	183	1.34	99	1.74	143	1.16
PC 37:4	318	1.03	286	0.880	357	0.660
PC 37:5	88	1.15	83	0.710	90	0.770
PC 37:6	237	1.02	144	1.29	158	1.04
PC 38:2	492	1.35	576	1.20	567	1.13
PC 38:3	1052	1.29	1527	0.560	1570	0.560
PC 38:4	7478	0.960	7332	0.790	8878	0.510
PC 38:5	8262	1.07	7376	0.920	8011	0.780
PC 38:6a	307	1.25	375	0.660	420	0.660
PC 38:6b	15495	0.950	11018	1.24	12764	0.850
PC 38:7	534	0.960	293	1.28	427	0.780
PC 39:5	231	0.970	215	0.76	238	0.710
PC 39:6	643	0.900	483	1.02	539	0.830
PC 40:4	282	0.950	215	0.770	297	0.740
PC 40:5	6172	1.07	6527	0.770	6482	0.700
PC 40:6	13985	0.900	11937	0.960	11132	0.700

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mg)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/mg) †	Fold difference with 2% BA group‡
PC 40:7	3918	0.910	2855	0.940	2953	0.690
PC 40:8	1161	1.04	1093	0.790	1116	0.690
Total PC	103124	1.09	101717	1.00	101880	0.880

*PC – phosphatidylcholine

†Median level of lipids in mice which were on high fat diet supplemented with 0% batyl alcohol.

‡Fold difference of median level of lipids in mice fed with high fat diet containing 2% batyl alcohol as compared to those fed with the 0% batyl alcohol containing diet. Data were analysed using Mann-Whitney U test, corrected for multiple comparisons by Benjamini Hochberg. Values in bold indicate statistical significance (corrected $P < 0.05$).

Supplementary Table V. Differences in plasma levels of alkyl- and alkenylphospholipids in mice on a high fat diet supplemented with/without batyl alcohol.

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PC(O-32:0)	506	1.19	5222	1.17	4645	1.47
PC(O-32:1)	290	1.18	1605	1.71	1625	1.93
PC(O-32:2)	22	1.32	115	1.43	101	1.60
PC(O-34:1)	1680	1.60	10165	1.49	10139	1.76
PC(O-34:2)	417	1.47	2340	1.49	1847	2.12
PC(O-34:3)	44	1.41	200	1.81	171	2.22
PC(O-34:4)	39	0.870	109	1.24	138	0.890
PC(O-35:4)	98	1.39	338	1.02	257	1.37
PC(O-36:0)	14	6.50	533	2.66	389	3.58
PC(O-36:1)	222	29.4	2112	32.5	1967	38.5
PC(O-36:2)	127	23.5	1807	13.6	1686	17.3
PC(O-36:3)	1983	1.31	3722	2.26	3847	2.27
PC(O-36:4)	1633	1.21	6895	1.59	6457	1.75
PC(O-36:5)	412	0.780	1179	0.920	1148	0.910
PC(O-38:4)	344	13.7	1737	32.3	1963	30.9
PC(O-38:5)	1400	1.03	4068	1.40	4160	1.45
PC(O-40:5)	211	11.5	986	21.4	999	25.4
PC(O-40:6)	220	0.450	613	1.77	598	1.94
PC(O-40:7)	840	0.940	1382	1.85	1574	1.51
PC(P-30:0)	298	0.900	1835	1.67	2178	1.23
PC(P-32:0)	89	0.520	881	0.57	799	0.570
PC(P-32:1)	50	0.860	268	0.880	220	1.14

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PC(P-34:1)	146	1.24	670	1.15	616	1.32
PC(P-34:2)	137	1.12	560	1.01	442	1.77
PC(P-34:3)	14	1.07	76	0.830	66	1.05
PC(P-36:2)	146	2.57	176	6.20	169	6.33
PC(P-36:4)	3390	1.12	3869	1.15	3652	1.26
PC(P-36:5)	61	1.23	211	1.20	219	1.21
PC(P-38:4)	0	n/a	71	37.1	82	27.4
PC(P-38:5)	1517	1.02	4069	1.68	3982	1.80
PC(P-38:6)	220	0.910	558	1.15	558	1.30
PC(P-40:5)	254	36.9	1093	24.5	1079	27.2
PC(P-40:6)	78	50.1	236	44.6	243	41.0
PE(O-34:1)	207	3.89	1111	4.08	944	6.04
PE(O-34:2)	45	1.29	193	1.63	117	3.95
PE(O-36:2)	184	8.09	808	13.9	674	18.3
PE(O-36:3)	41	7.83	131	16.9	94	24.7
PE(O-36:4)	80	1.44	145	3.80	150	4.37
PE(O-36:5)	25	2.24	51	5.31	61	6.26
PE(O-38:4)	298	13.2	570	38.3	605	33.9
PE(O-38:5)	334	6.17	979	12.8	911	13.3
PE(O-40:5)	181	5.75	678	9.06	610	11.6
PE(O-40:6)	136	1.17	43	0.330	41	0.560
PE(O-40:7)	62	1.74	104	3.38	139	1.94
PE(P-34:1)	250	1.76	1230	1.33	1180	1.42
PE(P-34:2)	0	n/a	300	0.530	270	1.26

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PE(P-36:1)	170	3.94	1240	3.49	1010	4.10
PE(P-36:2)	150	4.53	840	2.13	480	4.23
PE(P-36:4)	390	0.620	600	1.02	680	1.07
PE(P-38:4)	230	7.22	640	11.5	800	8.14
PE(P-38:5)	5750	0.770	10490	1.80	10850	2.16
PE(P-38:6)	1320	0.700	2400	1.23	2660	1.11
PE(P-40:4)	540	2.50	1780	6.56	1580	7.82
PE(P-40:5)	7230	10.5	12340	36.6	16130	32.6
PE(P-40:6)	670	19.2	1740	25.8	2220	19.3

* PC(O) – alkylphosphatidylcholine, PC(P) – alkenylphosphatidylcholine, PE(O) – alkylphosphatidylethanolamine, PE(P) – alkenylphosphatidylethanolamine.

†Median level of lipids in mice which were on high fat diet supplemented with 0% batyl alcohol.

‡Fold difference of median level of lipids in mice fed with high fat diet containing 2% batyl alcohol as compared to those fed with the 0% batyl alcohol containing diet. Data were analysed using Mann-Whitney U test, corrected for multiple comparisons by Benjamini Hochberg. Values in bold indicate statistical significance (corrected $P < 0.05$).

Supplementary Table VI. Differences in level of alkyl- and alkenylphospholipids in heart of mice on a high fat diet supplemented with/without batyl alcohol.

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PC(O-32:0)	649	2.21	2845	0.670	2382	0.920
PC(O-32:1)	209	2.64	938	1.46	840	2.12
PC(O-32:2)	13	1.46	47	0.910	33	1.42
PC(O-34:1)	1105	2.85	2768	2.51	2544	3.34
PC(O-34:2)	211	1.78	657	1.30	569	1.91
PC(O-34:3)	18	2.44	81	1.42	75	1.79
PC(O-34:4)	15	0.800	24	1.00	23	1.04
PC(O-35:4)	19	0.790	26	0.960	32	0.660
PC(O-36:0)	289	2.47	387	5.86	388	6.36
PC(O-36:1)	126	33.2	828	22.2	706	27.0
PC(O-36:2)	133	11.8	438	14.5	342	22.0
PC(O-36:3)	456	1.26	568	1.03	611	1.20
PC(O-36:4)	1502	0.910	2329	1.04	2712	0.930
PC(O-36:5)	122	1.36	441	0.860	465	0.790
PC(O-38:4)	314	10.5	695	24.3	991	17.5
PC(O-38:5)	4381	0.980	4530	1.59	5027	1.51
PC(O-40:5)	263	8.93	520	15.3	597	14.7
PC(O-40:6)	857	5.47	1157	13.3	1257	13.1
PC(O-40:7)	1311	0.650	792	1.28	1104	0.760
PC(P-30:0)	49	0.450	82	0.130	79	0.100
PC(P-32:0)	240	0.380	282	0.280	322	0.240

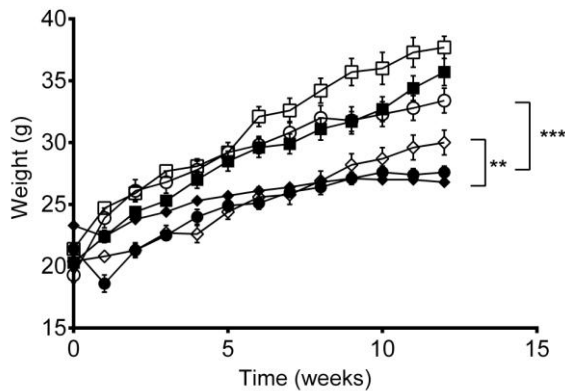
Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PC(P-32:1)	66	0.580	85	0.610	86	0.590
PC(P-34:1)	851	0.500	709	0.460	707	0.460
PC(P-34:2)	401	0.560	468	0.370	453	0.440
PC(P-34:3)	17	0.710	24	0.540	24	0.500
PC(P-36:2)	131	1.15	199	1.77	258	1.45
PC(P-36:4)	6015	0.790	5884	0.730	6608	0.700
PC(P-36:5)	131	0.740	133	0.440	172	0.400
PC(P-38:4)	975	1.93	1265	2.62	1260	2.71
PC(P-38:5)	11446	0.570	8700	0.600	9628	0.500
PC(P-38:6)	9772	0.360	6314	0.360	6596	0.290
PC(P-40:5)	857	5.47	1157	13.3	1257	13.1
PC(P-40:6)	807	2.77	704	6.82	878	4.77
PE(O-34:1)	99	3.14	219	2.29	210	3.29
PE(O-34:2)	35	1.80	97	0.550	66	1.21
PE(O-36:2)	37	3.03	63	3.35	59	0.00
PE(O-36:3)	23	6.83	54	5.76	38	11.5
PE(O-36:4)	251	1.15	483	0.740	447	1.04
PE(O-36:5)	37	1.95	154	0.480	103	0.930
PE(O-36:6)	56	0.680	63	0.460	75	0.370
PE(O-38:4)	97	46.7	659	14.7	544	20.7
PE(O-38:5)	1681	1.24	2182	1.61	2281	1.93
PE(O-40:5)	712	6.31	1202	5.48	1244	6.46
PE(O-40:6)	1199	0.800	1138	0.530	1103	0.670
PE(O-40:7)	647	0.800	480	0.670	584	0.540
PE(P-34:1)	900	0.710	980	0.670	880	1.00

Lipid species*	C57/BL6		ApoE ^{-/-}		ApoE ^{-/-} GPx1 ^{-/-}	
	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡	Median level for 0% BA group (pmol/μmol PC)†	Fold difference with 2% BA group‡
PE(P-34:2)	770	1.09	1010	0.00	900	0.00
PE(P-36:1)	530	13.6	1650	12.5	1690	15.7
PE(P-36:2)	870	3.51	1520	3.12	1430	4.26
PE(P-36:4)	6240	0.750	6590	0.550	8000	0.540
PE(P-38:4)	4270	4.32	5070	6.21	7120	5.24
PE(P-38:5)	37390	0.740	40740	0.480	42120	0.570
PE(P-38:6)	53480	0.630	47290	0.450	49340	0.390
PE(P-40:4)	7270	6.18	11560	5.75	12160	6.68
PE(P-40:5)	15930	4.90	24990	6.09	26370	7.06
PE(P-40:6)	22020	3.26	23560	5.38	27280	4.42

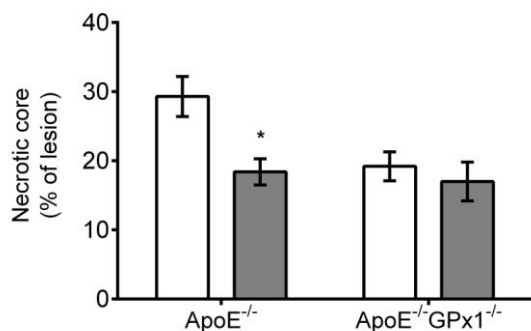
* PC(O) – alkylphosphatidylcholine, PC(P) – alkenylphosphatidylcholine, PE(O) – alkylphosphatidylethanolamine, PE(P) – alkenylphosphatidylethanolamine.

†Median level of lipids in mice which were on high fat diet supplemented with 0% batyl alcohol.

‡Fold difference of median level of lipids in mice fed with high fat diet containing 2% batyl alcohol as compared to those fed with the 0% batyl alcohol containing diet. Data were analysed using Mann-Whitney U test, corrected for multiple comparisons by Benjamini Hochberg. Values in bold indicate statistical significance (corrected $P < 0.05$).



Supplementary Figure I. Weight gain of mice on a high fat diet supplemented with/without batyl alcohol. Data represent mean $\pm$ SEM of C57/BL6 (squares) (N=10), ApoE^{-/-} (diamonds) (N=10), and ApoE^{-/-}GPx1^{-/-} (circles) (N=9) mice on high fat diet supplemented with either 0% batyl alcohol (open symbols) or 2% batyl alcohol (closed symbols). Final weight on week 12 were compared between groups of each genotype using Student t-test; ** indicates $P<0.01$ and *** indicates $P<0.001$.



Supplementary Figure II. Necrotic core in aortic sinus lesions of mice on a high fat diet supplemented with/without batyl alcohol. Data represent mean $\pm$ SEM. Data is expressed as % of lesion area, of ApoE^{-/-} and ApoE^{-/-}GPx1^{-/-} mice on high fat diet supplemented with either 0% batyl alcohol (open bars) or 2% batyl alcohol (closed bars). Significant differences were assessed using Student t-tests; * indicates $P<0.05$.