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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 plaque in the aorta was reduced by 70% ($P = 5.69E-07$) and 69% ($P = 2.00E-04$) in treated ApoE- and ApoE/GPx1-deficient mice, respectively. A 40% reduction in plaque ($P = 7.74E-03$) 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%, $P = 2.43E-02$) in the aortic sinus and nitrotyrosine staining (−78%, $P = 5.11E-06$) 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.

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

Nonstandard abbreviations and acronyms: 4-HNE, 4-hydoxynonenal; $ApoE^{-/-}$, apolipoprotein E-deficient mouse; $ApoE^{-/-}GPx1^{-/-}$, apolipoprotein E/glutathione peroxidase-1- deficient mouse; BA, batyl alcohol; CE, cholesteryl ester; LPC, lysophophatidylcholine; LPE, lysophoshatidylethanolamine; 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.

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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 transport [7] and in high-density lipoprotein (HDL)-mediated cholesterol efflux [8], and recently, the inclusion of plasmalogen into reconstituted HDL improved the lipoprotein anti-apoptotic activity on endothelial cells [9].

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 rodents [11], 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 milk [12]. 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.

2. Materials and methods

2.1. Animal groups and diet study

Six-week old male C57/BL6 (Animal Resources Centre, WA, Australia), ApoE$^{-\backslash-}$ (Animal Resources Centre, WA, Australia), and ApoE$^{-\backslash-}$GPx1$^{-\backslash-}$ (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 h 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 h, 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).

2.2. Clinical measurements

Fasting blood glucose was measured by tail bleed using a glucometer (Accu-Chek, Roche Diagnostics, Australia) following 3 h 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 1485 × *g*, 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).

2.3. 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 μL, pH 7.6) containing 100 μmol/L butylated hydroxytoluene using a Polytron electric homogeniser for 10 s and then with a mini probe homogeniser for 15 s at amplitude 23. The homogenate was stored at −80 °C.

2.4. 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 μL) 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 × g, 16 °C, 3 h) in a TLA 120.2 rotor and Optima MAX-TL ultracentrifuge (Beckman Coulter, NSW, Australia). The top layer (400 μL) of the sample, corresponding to the VLDL fraction was aspirated. The density of the remaining mixture was the adjusted to 1.063 g/mL and overlayed with the same density solution to a final volume of 1.0 mL. The sample was centrifuged (435,680 × *g*, 16 °C, 3 h) and the top layer (400 μL) 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 × g, 16 °C, 16 h) and the top layer (400 μL) corresponding to HDL fraction was aspirated.

2.5. Lipid extraction

Prior to lipid extraction, samples were randomised to reduce bias. Lipids were extracted as previously described [18]. 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