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Methyl-γ-butyrobetaine decreases levels of acylcarnitines and attenuates the development of atherosclerosis

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ABSTRACT

Objective: The elevation of the levels of L-carnitine and its fatty acid esters, acylcarnitines, in tissue or plasma has been linked to the development of atherosclerosis. Recently, a potent inhibitor of L-carnitine biosynthesis and transport, methyl-γ-butyrobetaine (methyl-GBB), was discovered. In this study, we evaluated the effects of γ-butyrobetaine (GBB), L-carnitine and methyl-GBB administration on the progression of atherosclerosis.
Methods: Apolipoprotein E knockout (apo$E^{-/-}$) mice were treated with methyl-GBB, L-carnitine or GBB for 4 months. Following the treatment, the amount of atherosclerotic lesions, the number of immune cells in atherosclerotic lesions and the plasma lipid profile were analysed. The L-carnitine and acylcarnitine levels were determined in the aortic tissues of CD-1 outbred mice 2 weeks after treatment with methyl-GBB at the dose of 10 mg/kg.
Results: Treatment with methyl-GBB decreased the acylcarnitine and L-carnitine levels in the aortic tissues by seventeen- and ten-fold, respectively. Methyl-GBB treatment at a dose of 10 mg/kg reduced the size of atherosclerotic plaques by 36%. Neither L-carnitine nor GBB treatment affected the development of atherosclerosis.
Conclusions: Methyl-GBB administration significantly attenuated the development of atherosclerosis in apo$E^{-/-}$mice. Our results demonstrate that decreasing the acylcarnitine pools can attenuate the development of atherosclerosis.

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1. Introduction

Atherosclerosis and its complications are the main causes of morbidity and mortality worldwide [23]. The development of atherosclerotic lesions is a complex, long-term process that involves many endogenous and exogenous factors. Recently, a link between the supplementation of L-carnitine and the accelerated development of atherosclerosis has been described [9]. L-Carnitine is involved in cellular energy metabolism, where it drives long-chain fatty acid transport into the mitochondria for β-oxidation to occur. L-Carnitine can be obtained through the diet or synthesised in the liver and kidneys from its precursor γ-butyrobetaine (GBB) by GBB dioxygenase [22,27].

The effects of administering of L-carnitine or its short-chain fatty acid derivative, propionyl-L-carnitine, on the development of atherosclerosis in experimental models have been widely studied. It was shown that the administration of L-carnitine to rabbits that received cholesterol-enriched diet attenuated the development of atherosclerosis, but the administration of D-carnitine accelerated the development of vascular lesions [25]. Thus, it was suggested that a deficiency or depletion in L-carnitine should be viewed as a risk factor for atherosclerosis [25]. Similarly, treating hypercholesteraemic rabbits with propionyl-L-carnitine decreased the amount of atherosclerotic lesions [26]. In an experimental mouse model of atherosclerosis, we demonstrated that the administration of mildronate, a biosynthesis and reabsorption inhibitor of L-carnitine, decreased the amount of L-carnitine in vascular tissues and simultaneously attenuated the development of atherosclerosis [29]. More recently, a link between the dietary supplementation of L-carnitine and the accelerated development of atherosclerosis in apolipoprotein E knockout (apo$E^{-/-}$) mice has been shown [9]. The pro-atherogenic mechanism of L-carnitine is based on the increased formation of trimethylamine N-oxide (TMAO), which inhibits the reversal of cholesterol transport [9] and thus promotes the development of vascular lesions. In addition, the accumulation of L-carnitine and its acyl-derivatives, acylcarnitines, has been observed in experimental animals developing atherosclerosis [6]. Elevation in the concentration of acylcarnitines has been linked to the development of insulin resistance and type 2 diabetes mellitus [19], a disease known to accelerate the development of atherosclerosis [34]. Although the results linking L-carnitine and the development of atherosclerosis are still contradictory, the latest studies have concluded that decreasing of the pools of L-carnitine and its derivatives might present a way to attenuate the development of atherosclerosis. In addition, there is experimental

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evidence that the elevation of GBB induces vasoprotective effects in experimental models of endothelial dysfunction [30,31]. However, there is still no experimental evidence linking the administration of GBB to the development of atherosclerosis.

Recently, a series of compounds that inhibit the biosynthesis and transport of L-carnitine were synthesised and characterised [15,28]. The newly synthesised compounds effectively decreased the L-carnitine pools in the plasma and heart tissues and possessed marked cardioprotective activities [15,16]. The best cardioprotective effect in the rat experimental heart infarction model was observed after treatment with 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB), a methyl-derivative of GBB that effectively inhibits GBB dioxygenase (IC_{50} 2.8 μM) and organic cation transporter 2 (IC_{50} 3.0 μM) [16].

This study was performed to test the effects of administering methyl-GBB, GBB and L-carnitine on the development of atherosclerosis in $apoE^{-/-}$ mice, as a previous study showed that lowering the L-carnitine tissue and plasma pools attenuated the development of atherosclerosis [29].

Our results demonstrate that the administration of methyl-GBB significantly attenuated the development of atherosclerosis in $apoE^{-/-}$mice. The anti-atherosclerotic mechanism of methyl-GBB treatment could be mediated by decreasing the amount of acylcarnitines and simultaneously inhibiting the activation of immune cells.

2. Materials and methods

2.1. Chemicals

Methyl-GBB or its less hygroscopic form, methyl-GBB phosphate, were used as a source of methyl-GBB. Both substances were provided by JSC Grindeks (Riga, Latvia). Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Sodium pentobarbital (Dorminal 20%) solution was purchased from Alfasan (Woerden, Holland). Heparin sodium was purchased from Panpharma (Fougeres, France). All other chemicals were obtained from Sigma Aldrich (Schnelldorf, Germany).

2.2. Animals and housing

All animal care and experimental procedures were performed in accordance with the guidelines of the European Community, local laws and policies, and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia.

Sixty female $apoE^{-/-}$ mice at the age of 7 weeks weighing 18–20 g were obtained from Taconic (Ejby, Denmark). Twelve male CD-1 outbred mice at the age of 8 weeks weighting 18–20 g were obtained from Harlan (Horst, Netherlands). Experimental animals were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22.5 ± 0.5 °C, 50 ± 5% humidity) with unlimited access to food (R70 diet, Lantmännen Lantbruk, Sweden) and water. Mice were adapted to local conditions for one week before the beginning of the study.

2.3. Treatment and experimental protocol

At 8 weeks of age, forty $apoE^{-/-}$ mice were randomly assigned to four equally sized groups (n = 10). The experimental animals from all of the groups were switched to a Western RD (P) diet that contained 21% fat and 0.15% cholesterol from Special Diets Services (Essex, Great Britain). The mice from the different experimental groups received following treatment for 4 months: mice in the first group received drinking water (control group); mice in the second group received GBB at a dose of 10 mg/kg dissolved in the drinking water (GBB group); mice in the third group received L-carnitine at a dose of 100 mg/kg (carnitine group); and mice in the fourth group received methyl-GBB phosphate at a dose of 16.8 mg/kg dissolved in the drinking water (methyl-GBB group), which corresponds to 10 mg/kg of pure methyl-GBB. The dosing of the GBB, L-carnitine and methyl-GBB was confirmed by measuring the consumption of the drinking water every 2 days and adjusting the concentration of supplemented substances.

After 4 months of treatment, the $apoE^{-/-}$ mice were intraperitoneally (ip) injected with 1000 UI of heparin and sacrificed under anaesthesia (sodium pentobarbital, 60 mg/kg, ip). Afterwards, the thorax was longitudinally opened and the blood was collected from the right ventricle. The plasma was separated by centrifugation at 1000 g at 4 °C for 10 min and stored at −80 °C until further analysis.

To study molecular mechanisms of anti-atherosclerotic mechanisms of methyl-GBB, twenty $apoE^{-/-}$ mice were randomly divided into two groups (n = 10). At the age of 8 weeks the experimental animals from both groups were switched to a Western RD (P) diet that contained 21% fat and 0.15% cholesterol from Special Diets Services (Essex, Great Britain). Experimental animals from the first group received drinking water, but mice from the second group received methyl-GBB at the dose of 10 mg/kg for 4 months together with drinking water. After the treatment experimental animals were sacrificed as described above. In addition, piece of the heart muscle (~100 mg) from the apex of the heart was frozen in liquid nitrogen for the qRT-PCR analysis.

Concentrations of triglycerides (TG), HDL- and LDL-cholesterol in the plasma samples were measured using an enzymatic kit from IL Laboratories (Lexington, USA) and concentration of tumour necrosis factor alpha (TNFα) in plasma was measured using TNFα ELISA Kit from Millipore (Billerica, USA). Activity of alanine aminotransferase (ALT) and aspartate transaminase (AST) in plasma samples was measured using kits from IL Laboratories (Lexington, USA).

Twelve CD-1 outbred mice at 9-weeks-old were divided into two groups (n = 6). The mice in the first group received drinking water, and the experimental animals in the second group received methyl-GBB phosphate at a dose of 16.8 mg/kg mixed with the drinking water. Following two weeks of treatment, the mice were sacrificed under anaesthesia (sodium pentobarbital, 60 mg/kg ip). Then, the aortas were cut out from the arch to the bifurcation, cleaned from the surrounding tissues and prepared for the characterisation of their acylcarnitine profile.

2.4. Quantification of the atherosclerotic lesions in the aortic sinus

After the blood was taken, the right ventricle was incised, and the heart was perfused with 10 ml of phosphate buffered saline through the apex of the left ventricle. Next, the heart and the whole aorta were cleaned from the surrounding fatty and connective tissues and dissected. Afterwards the heart was cut along a plane between the tips of 2 atria, and the top half was embedded in OCT Tissue-Tek embedding medium (Sakura, The Netherlands) and snap-frozen. Nine 10 μm thick cryosections were cut from the appearance of the aortic valves with 100 μm intervals. Sections were mounted on poly-L-lysine coated slides and dried. After fixation in neutral 4% paraformaldehyde solution, sections were stained with Oil Red O (ORO). Finally, the ORO-stained sections were examined under a Leica DM IL microscope (Wetzlar, Germany) and used for quantitative evaluation. Images of the aorta were recorded using a Leica DFC490 digital camera (Wetzlar, Germany). The total area of the lesion was measured using Image-Pro Plus 6.3 software, as previously described [14].

2.5. Quantification of the infiltration of macrophages and monocytes in the atherosclerotic lesions

To determine the number of macrophages and monocytes in atherosclerotic lesions of the aortic sinus, 10 μm sections from aortic roots were prepared similarly as described before (Section 2.4.). Sections were mounted on poly-L-lysine coated slides, air-dried and fixed in cold acetone. Following fixation, the sections were stained with rat anti-MOMA-2 antibodies from Abcam (Cambridge, UK), as previously described [7].

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