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Butyrate from pectin fermentation inhibits intestinal cholesterol absorption and attenuates atherosclerosis in apolipoprotein E-deficient mice☆

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Abstract

Short-chain fatty acids (SCFAs), the major products of dietary fiber fermentation by intestinal microflora, exert beneficial effects on pathogenesis of multiple metabolic diseases. The aim of this study was to determine whether SCFAs from fermentation of pectin (PE), a soluble dietary fiber, prevent the development of atherosclerosis in apolipoprotein E-deficient (apoE$^{-/-}$) mice. Male apoE$^{-/-}$ mice (8-week-old) were fed a high-fat, high-cholesterol diet (HCD; 21% wt/wt fat, 0.15% wt/wt cholesterol) or HCD supplemented with 20% wt/wt PE (HCD+PE) alone or with antibiotics (HCD+PE + A) in drinking water for 12 weeks. Serum lipids and SCFAs concentrations, atherosclerotic lesion area, and intestinal morphology and function were measured. Caco-2 cells were treated with SCFAs to determine whether they affected the expression of genes involved in cholesterol absorption. HCD+PE-treated mice exhibited decreased serum total and low-density lipoprotein cholesterol, and reduced atherosclerotic lesion area compared with HCD mice. These beneficial effects of PE were not observed in the HCD+PE+A group. Incubation of Caco-2 cells with butyrate, but not acetate and propionate, down-regulated the expression of Niemann-Pick C1-Like 1 but up-regulated the ATP-binding cassette transporters G5 and G8 (ABCG5 and G8) at the mRNA level. Butyrate treatment also increased transcriptional activity of liver X receptor in Caco-2 cells. Our data suggest that butyrate from PE intestinal fermentation protects mice from the progression of diet-induced atherosclerosis in apoE$^{-/-}$ mice. These findings suggest a novel mechanism by which dietary fiber may prevent the development of atherosclerosis.

Keywords: Atherosclerosis; Dietary fiber; Pectin; Short-chain fatty acid; Cholesterol absorption

1. Introduction

Cardiovascular diseases (CVDs) constitute the leading cause of mortality and morbidity worldwide and are caused primarily by complications of atherosclerosis [1]. Atherosclerosis is a slow and progressive disease, which is characterized by abnormal accumulation of lipids in the large and medium-sized arteries [2,3]. Epidemiological studies have revealed numerous risk factors, including dietary pattern, physical inactivity, dyslipidemia, diabetes mellitus, smoking, obesity and psychological stress associated with the initiation and development of atherosclerosis and its clinical manifestations [4]. A growing number of studies have shown that higher dietary fiber intake was associated with a reduced risk of atherosclerotic CVD incidence and maintenance of whole-body cholesterol homeostasis; however, the related molecular mechanism is not fully elucidated. Dietary fiber comprises the indigestible complex carbohydrates mainly derived from plants, which can be divided into two categories: insoluble and soluble fibers. Insoluble fibers are only marginally fermented with the properties of bulking action, whereas soluble fibers, which can be dissolved in water, are easily fermented by a wide variety of anaerobic intestinal microbiota in the human colon, leading to an increase in physiologically active byproducts such as short-chain fatty acids (SCFAs) [5,6]. The most commonly occurring SCFAs are acetate, propionate and butyrate, which are present in an approximate molar ratio of 60:20:20 in the colon and stool [7]. Previous study has shown that the total concentration of SCFAs was ranging from 70–140 mmol/L in the proximal colon to 20–70 mmol/L in the distal colon depending on the different dietary patterns [8].

Previous studies have documented that SCFAs may elicit effects on lipid metabolism via de novo synthesis and transport. Acetate is not only used as an energy source, but is also constituted as a substrate for the cholesterol synthesis in the liver through acetyl-CoA, thus affecting plasma cholesterol levels [9]. In addition, propionate was shown to reduce plasma cholesterol concentrations in rodents and humans by inhibiting de novo synthesis of cholesterol through inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) [10,11]. Furthermore, butyrate has been shown to stimulate the ApoA-IV-containing lipoprotein secretion and therefore regulate reverse cholesterol transport [12,13]. Thus, microbiota-dependent

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SCFA generations plays an essential role in modifying lipid metabolism and thus may partly account for the beneficial impacts of dietary fiber on atherosclerotic CVD prevention.

The small intestine is a unique organ that provides dietary and reabsorbed biliary cholesterol to the body and plays a critical role in the regulation of whole-body cholesterol balance [14,15]. Intestinal cholesterol absorption has recently been considered as an important procedure in the modulation of cholesterol homeostasis [16]. Several key regulators were found to play a crucial role in the modulation of cholesterol absorption in intestine. Niemann-Pick C1-Like 1 (NPC1L1) protein was discovered as an intestinal phytosterol and cholesterol transporter to regulate intestinal cholesterol absorption and hepatobiliary cholesterol excretion [17]. Moreover, NPC1L1 also functions as the molecular target of ezetimibe, a potent cholesterol absorption inhibitor, and is widely used for the treatment of hypercholesterolemia [18]. In addition, the family of ATP-binding cassette (ABC) transporters G5 and G8 (ABCG5 and ABCG8) form an obligate heterodimer that limits cholesterol and plant sterol absorption by effectively transporting these sterols from enterocytes into the intestinal lumen [19]. However, the impact of the SCFAs on the intestinal cholesterol absorption is still unknown.

The objective of this study was to investigate the potential effect of SCFAs by fiber intestinal fermentation on atherosclerosis in high-fat and high-cholesterol-fed apolipoprotein E-deficient (apoE$^{-/-}$) mice. Furthermore, the underlying mechanism involved in the effects of SCFAs was determined in vitro.

2. Materials and methods

2.1. Materials

Analytical standards of sodium acetate, sodium propionate, sodium butyrate and chromatographic grade acetic, propionic and butyric acids were all provided by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals protocol and diets

The animal experiments protocol was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Apolipoprotein E-deficient mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, MA, USA) and housed in stainless-steel at 22–24°C with the light-cycle illumination from 08:00 to 20:00. The high-fat, high-cholesterol diet (HCD) containing 21.0% (wt/wt) fat (with 20% from anhydrous milk fat and 1% from corn oil), 19.5% (wt/wt) casein and 0.15% (wt/wt) cholesterol was obtained from Medicience Ltd (Jiangsu, China), and stored with a seal at 4°C until used. Pectin (PE) was provided by Louis Francois (Marne-la-Vallée, France). The antibiotics including ampicillin (Sigma-Aldrich), vancomycin (Sandoz), metronidazole (Sanofi-Aventis) and neomycin (Sigma) were in drinking water. Food and water were provided ad libitum during the duration of the experiment.

Thirty male apoE$^{-/-}$ mice (8 weeks old) were randomly divided into three groups and received the following diets: (1) HCD, (2) HCD supplemented with 20% wt/wt PE (HCD+PE) and (3) HCD+PE with antibiotics (1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole and 0.5 g/L vancomycin) in drinking water (HCD+PE+A) for 12 weeks. The detailed components of the diet tested are shown in Supplemental Table 1. Food intake, water consumption and body weight gain were recorded daily. After 12 weeks of feeding, mice were anesthetized with 10% (w/v) chloral hydrate by intraperitoneal injection in a dose of 3.5 ml/kg and tissues were sampled from anesthetized mice. Blood was collected with the use of an orbital vein puncture just before killing. Gut tissue specimens were collected and frozen immediately in liquid nitrogen and then fixed in neutral-buffered formalin.

2.3. Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test were performed at 12 weeks according to the previous report [20]. Briefly, mice were feed-deprived for 6 h and then received a 20% glucose solution by intraperitoneal injection (2 mg/g body weight). Blood samples were taken from the saphenous vein before (time=0 min) and 15, 30, 60, 90 and 120 min after the glucose injection. Blood glucose levels were determined using a glucometer (Accu-Chek Integra, Roche, Mannheim Germany).

2.4. Serum lipid analysis

Serum concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides were measured by enzymatic colorimetric methods at the end of experiment as previously described [21].

2.5. Oil Red O staining

The whole aorta was thoroughly cleaned of adventitial fat and cut open longitudinally and the upper half of the heart with aortic root was embedded in OCT compound (Tissue-Tek, Torrance, CA, USA). The detection of atherosclerotic plaque area was determined by Oil Red O staining method as described in our previous study [22]. For quantification of lesion area, images of the stained preparation were taken by using a dissection microscope with a standard digital camera and quantified by LAS software (Leica Application Suite, Nussloch, Germany). The lesion area of whole aorta was expressed as a percentage of the total surface area covered by Oil Red O staining and aortic root lesion size was calculated.

2.6. Gut permeability assays

Gut permeability analysis was performed as previously described [23]. The 4000-FITC-dextran (Sigma-Aldrich) concentration in serum was measured using a fluorescence spectrophotometer (Multimode Microplate Reader, Tecan, Switzerland) with excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.7. Serum SCFA concentrations

Serum SCFAs were determined by gas chromatography according to the previous study [24] with minor modifications regarding the pretreatment of serum samples. Briefly, 100 μl serum sample was diluted with 400 μl water. After oscillation blending for 10 min, the samples were then loaded via a Poly-Sery Max SPE Cartridge (CNW Technologies GmbH, Düsseldorf, Germany). Finally, the collected samples were derived using 20 μl pentafluorobenzyl bromide (Anpel, Shanghai, China) derivative agent for analysis.

2.8. Cell culture

The human epithelial colorectal carcinoma cell line Caco-2 (American Tissue Culture Collection, ATCC, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle medium with high glucose (GibcoBRL, Grand Island, NY, USA) containing 20% fetal bovine serum, 1% penicillin–streptomycin mixture and 1% nonessential amino acids at 37°C under a humidified atmosphere with 95% air and 5% CO_2. Upon reaching 80% confluence, the cells were treated with various concentrations of SCFAs including sodium acetate (0, 0.1, 1 and 10 mmol/L), sodium propionate (0, 0.01, 0.1 and 1 mmol/L) and sodium butyrate (0, 0.01, 0.1 and 1 mmol/L) for 24 h prior to analysis.

2.9. Intestinal cholesterol absorption assay

Cholesterol absorption was measured in Caco-2 cells by using a cholesterol uptake cell-based assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. Caco-2 cells were incubated with various concentrations of SCFAs in serum-free culture medium to assess the effects of SCFAs on cellular absorption of cholesterol.

2.10. RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from the intestinal mucosa tissue or Caco-2 cells with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and then cDNA was synthesized by using a reverse transcription kit (TaKaRa, Tokyo, Japan). The resulting cDNA samples containing SYBR Green mixture were used to assess the transcript expression level with a Real-Time PCR System (ABI ViiA7, Foster City, CA, USA) with values presented as $2^{-\Delta\Delta CT}$. The thermal cycling conditions were as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 2 min, and 40 cycles of amplification at 95°C for 1 s and 60°C for 30 s. The primer sequences for each specific gene are listed in Supplemental Table 2.

2.11. Plasmid constructs and luciferase activity assay

The liver X receptor response element (LXRE) Luciferase Reporter Plasmid (pGMLXRE-Lu; Genomeditech, Shanghai, China) was used to measure the LXRE luciferase activity. Caco-2 cells were seeded in 96-well plates, and then 0.4 μg pGMLXRE-Lu and 0.02 μg pRL-SV40 (Promega, Madison, WI, USA) were co-transfected with *Renilla* luciferase activity used as the endogenous control. After transfection, cells were incubated with various concentrations of butyrate (0.01, 0.1 and 1 mmol/L) for another 24 h. The amount of LXRE and *Renilla* luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.12. Statistical analysis

All values are expressed as the means±S.E.M. If homoscedastic, One-way analysis of variance was used for comparisons between multiple experimental groups, followed by post hoc analysis using Dunnett's *t* test. If heteroscedastic, the Kruskal–Wallis method followed by Dunn's multiple comparisons test was used in the comparison between