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Voluntary wheel running increases bile acid as well as cholesterol excretion and decreases atherosclerosis in hypercholesterolemic mice

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ABSTRACT

Objective: Regular physical activity decreases the risk for atherosclerosis but underlying mechanisms are not fully understood. We questioned whether voluntary wheel running provokes specific modulations in cholesterol turnover that translate into a decreased atherosclerotic burden in hypercholesterolemic mice.
Methods: Male LDLR-deficient mice (8 weeks old) had either access to a voluntary running wheel for 12 weeks (RUN) or remained sedentary (CONTROL). Both groups were fed a western-type/high cholesterol diet. Running activity and food intake were recorded. At 12 weeks of intervention, feces, bile and plasma were collected to determine fecal, biliary and plasma parameters of cholesterol metabolism and plasma cytokines. Atherosclerotic lesion size was determined in the aortic root.
Results: RUN weighed less (~13%) while food consumption was increased by 17% ($p = 0.004$). Plasma cholesterol levels were decreased by 12% ($p = 0.035$) and plasma levels of pro-atherogenic lipoproteins decreased in RUN compared to control. Running modulated cholesterol catabolism by enhancing cholesterol turnover: RUN displayed an increased biliary bile acid secretion (68%, $p = 0.007$) and increased fecal bile acid (93%, $p = 0.009$) and neutral sterol (33%, $p = 0.002$) outputs compared to control indicating that reverse cholesterol transport was increased in RUN. Importantly, aortic lesion size was decreased by ~33% in RUN ($p = 0.033$).
Conclusion: Voluntary wheel running reduces atherosclerotic burden in hypercholesterolemic mice. An increased cholesterol turnover, specifically its conversion into bile acids, may underlie the beneficial effect of voluntary exercise in mice.

1. Introduction

Atherosclerosis is a complex vascular disease, which is characterized by major abnormalities in systemic factors, such as circulating lipids and lipoproteins, and concomitant inflammation of the vascular wall.

It has long been known that exercise is a deterrent of atherosclerosis. Numerous clinical and experimental studies report on the beneficial effects of physical activity on atherosclerosis [1–7] and various effects of physical activity on different processes involved in the pathogenesis and progression of atherosclerosis have been reported. For example, it has been shown that physical activity improves the antioxidant system [4], plaque composition as well as plaque stability [3,6] and favorably modulates the inflammatory response [1]. However, despite these recent efforts it remains unclear how exactly physical activity decreases the atherosclerotic process. We hypothesize that the enterohepatic system, which plays a critical role in several aspects of cholesterol metabolism, may be of great relevance herein.

Increasing cholesterol excretion into feces as neutral sterols or bile acids represents an efficient strategy in the amelioration of atherosclerosis, as it improves the pro-atherogenic state by modulating lipid content in plasma [8,9]. The liver secretes free cholesterol into bile, which is released into the intestine upon ingestion of a meal. In the small intestine, biliary cholesterol mixes with dietary cholesterol and is partially reabsorbed. The remainder is lost in the feces within the neutral sterol fraction. Bile acids are synthesized from cholesterol exclusively in the liver and enter

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the intestinal lumen after a meal. Bile acids are important for the emulsification and absorption of dietary fats in the intestine [10]. About 95% of the bile acids are reabsorbed from the terminal ileum, transported back to the liver for re-secretion into bile (enterohepatic circulation). The fraction of bile acids that escapes reabsorption is lost in feces and constitutes an important part of cholesterol turnover, since fecal bile acid loss is compensated for by *de novo* synthesis from cholesterol to maintain the bile acid pool size [11]. Under steady state conditions, fecal bile acid loss equals hepatic *de novo* bile acid synthesis.

We have recently shown that exposing healthy chow-fed mice to a voluntary running wheel for two weeks enhanced fecal neutral sterol and bile acid excretion with specific changes in biliary, plasma and intestinal parameters contributing to an increased cholesterol turnover upon running [12]. To our knowledge, no previous studies have examined the effects of exercise on cholesterol and bile acid metabolism in a hypercholesterolemic mouse model. Thus, the purpose of this study was to investigate whether the recently observed effects of voluntary running wheel exercise on whole body cholesterol turnover in healthy chow-fed mice [12] extend to the hypercholesterolemic LDLR-deficient mouse model. We hypothesized that voluntary wheel running beneficially modulates cholesterol and bile acid metabolism in hypercholesterolemic mice and thereby mediates a reduction in atherosclerotic burden.

2. Methods

All experiments were approved by the Animal Care and Use Committee of the University of Groningen, The Netherlands. The University of Groningen is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

2.1. Animals and voluntary cage-wheel exercise

Sixteen 5-week-old male LDLR deficient (B6.129S7-*LDLR*^{tm1Her}/J) mice were purchased from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME, USA). Upon arrival, mice were singly housed in a cage ($47 \times 26 \times 14.5$ cm) in a temperature-controlled room with a 12:12 h light–dark cycle and had access to standard commercial pelleted laboratory chow (RMH-B, ABDiets, Woerden, The Netherlands). At 8 weeks of age, mice were switched to a western-type diet (0.25% cholesterol, 16% fat, Purified Western Diet, 4021.06, ABDiets, Woerden, The Netherlands) and were randomly selected to either voluntary cage wheel running (RUN, $n = 9$) or to remain sedentary (CONTROL, $n = 7$) for 12 weeks. Throughout the study, mice had *ad libitum* access to food and water. The voluntary running wheel set-up has been described previously [12]. Twice a week, mice were weighed and food intake was recorded. Two mice in the running group were excluded from all analyses because they showed no activity on the running wheel.

2.2. Experimental procedures

Fecal, plasma, biliary, hepatic and intestinal parameters were collected at the endpoint of the experiment after 12 weeks of RUN or CONTROL, i.e., at 20 weeks of age.

2.3. Fecal parameters

Forty-eight hours feces were collected before and at 12 weeks running wheel exposure. Feces were dried, weighed and homogenized to a powder. Aliquots of fecal powder were used for analysis of total bile acids by an enzymatic fluorimetric assay [13]. Neutral sterols and bile acid profiles were determined according to Arca et al. [13] and Setchell et al. [14], respectively [15].

2.4. Determination of biliary parameters of cholesterol and bile acid metabolism

After 12 weeks of CONTROL or RUN, all mice underwent gallbladder cannulation for continuous collection of bile [16]. Briefly, mice were anaesthetized by intraperitoneal injection with Hypnorm® ($1\,ml\,kg^{-1}$) and diazepam ($10\,mg\,kg^{-1}$). During the 30 min bile collection period, mice were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of $1\,g\,ml^{-1}$ for bile. Bile was stored at −20 °C until analysis. Total biliary bile acids were determined by an enzymatic fluorimetric assay [17]. Biliary cholesterol and phospholipids levels were measured as described by Kuipers et al. [18].

2.5. Determination of plasma markers of cholesterol metabolism

Immediately after bile collection, blood was drawn via the orbital sinus. Plasma was collected by centrifugation and stored at −20 °C until analyzed. Plasma total cholesterol, free cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially available assay kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma pro- and anti-inflammatory makers were analyzed using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA). Utilizing gaschromatography, as described by Windler et al. [19], we analyzed plasma plant sterols (campesterol and sitosterol) relative to plasma cholesterol levels as marker of intestinal cholesterol absorption in pooled plasma samples of each group. Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) in a superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium)

2.6. Tissue collection

Mice were opened immediately after blood collection. The heart was slowly perfused with PBS at physiological pressure. Then, the liver was excised, weighed and snap frozen in liquid nitrogen. The small intestine was excised, flushed with ice cold PBS (4 °C) and divided into three sections of equal lengths and subsequently snap-frozen in liquid nitrogen. Lastly, the thoracic aorta was excised and epididymidal fat pads were removed and weighed. Thoracic aorta, liver and intestine were stored at −80 °C for later analysis. Hearts were flushed with PBS to remove the excess of blood before fixation in formaldehyde 1% (Formal-Fixx, Thermo Electron Corporation, Pittsburgh, PA) for 24 h, cut in an angle eventually revealing the aortic sinus and stored at −80 °C embedded in OCT (Tissue-Tek O.C.T., Sakura, Zoeterwoude, The Netherlands).

2.7. Determination of atherosclerotic lesion size and aortic cholesterol content

Frozen sections from the aortic sinus were prepared according to Paigen et al. [20]. Surface lesion area was measured after Oil Red O staining by computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 video camera. At least 5 sections per mouse were examined for each staining. Due to technical difficulties, we were able to analyze atherosclerotic lesion size in 4 of 7 running mice and 4 of 7 sedentary mice. Aortic cholesterol content was utilized as an alternative method to assess atherosclerotic