Atherosclerosis 228 (2013) 117–123

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Bile acid sequestration normalizes plasma cholesterol and reduces atherosclerosis in hypercholesterolemic mice. No additional effect of physical activity

Maxi Meissner [a], Henk Wolters [a], Rudolf A. de Boer [b], Rick Havinga [a], Renze Boverhof [a], Vincent W. Bloks [a], Folkert Kuipers [a,c], Albert K. Groen [a,c,*]

[a] Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[b] Department of Cardiology, Center for Liver, Digestive, and Metabolic Diseases, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[c] Department of Laboratory Medicine, Center for Liver, Digestive, and Metabolic Diseases, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 15 July 2012
Received in revised form
14 January 2013
Accepted 11 February 2013
Available online 26 February 2013

Keywords:
Atherosclerotic lesions
Cholesterol
Bile acid metabolism
Bile acid sequestration
ECHO
Running wheel
Mice
LDLRKO mice

ABSTRACT

Aims: Bile acid sequestrants (BAS) and physical activity (RUN) decrease incidence of cardiovascular events. Both treatments are often prescribed, yet it is not known whether their beneficial effects are additive. We assessed the effects of BAS treatment alone and in combination with RUN on cholesterol metabolism, heart function and atherosclerotic lesion size in hypercholesterolemic mice.
Methods: Male Ldlr-deficient mice remained either sedentary (CONTROL), were treated with Colesevelam HCl (BAS), had access to a running wheel (RUN), or were exposed to BAS and RUN (BAS RUN). All groups were fed a high cholesterol diet for 12 weeks. Then, feces, bile and plasma were collected. Atherosclerotic lesion size was determined in the aortic arch and heart function by echocardiography.
Results: BAS RUN ran more than RUN (6.4 ± 1.4 *vs.* 3.5 ± 1.0 km/day, $p < 0.05$). BAS and BAS RUN displayed ~3-fold reductions in plasma cholesterol levels ($p < 0.001$), ~2.5-fold increases in fecal neutral sterol ($p < 0.001$) and bile acid ($p = 0.01$) outputs, decreases in biliary secretions of cholesterol (~6-fold, $p < 0.0001$) and bile acids (~2-fold, $p < 0.001$) *vs.* CONTROL while no significant effects were observed in RUN. Compared to CONTROL, lesion size decreased by 78% in both BAS and BAS RUN, ($p < 0.0001$).
Conclusion: BAS reduce atherosclerosis in Ldlr-deficient mice, coinciding with a switch from body cholesterol accumulation to cholesterol loss. RUN slightly modulated atherosclerotic lesion formation but the combination of BAS and RUN had no clear additive effects in this respect.
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1. Introduction

Cholesterol has a central role in the pathogenesis of atherosclerosis. Strategies that increase fecal cholesterol excretion as neutral sterols or bile acids, i.e., accelerate whole body cholesterol turnover, improve the pro-atherogenic state by modulating plasma lipid content and thus represent attractive strategies in the amelioration of atherosclerosis.

Conversion of cholesterol into bile acids quantitatively represents a major route of cholesterol removal from the body. Bile acids are synthesized exclusively in the liver and enter the intestinal lumen after a meal to stimulate solubilization and uptake of dietary lipids and fat soluble vitamins [1]. About 95% of the bile acids is reabsorbed from the terminal ileum, transported back to the liver for resecretion into bile (enterohepatic circulation). Besides their role in lipid uptake, bile acids have emerged as important metabolic regulators of glucose, lipid and energy metabolism [2] all implicated in the pathogenesis of atherosclerosis.

Bile acid sequestrants (BAS) have long been utilized for treatment of hypercholesterolemia [3]. BAS are positively charged indigestible resins. In the intestinal lumen BAS bind negatively charged bile acids forming non-absorbable complexes, thus increasing fecal bile acid excretion which subsequently stimulates the liver to convert cholesterol into bile acids. A compensatory increase in hepatic LDL receptor activity clears LDL-cholesterol

* Corresponding author. Laboratory of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, Hanzeplein 1, 9713 EZ Groningen, The Netherlands. Tel.: +31 (0)50 3611262; fax: +31 (0)50 3611746.
E-mail address: a.k.groen@umcg.nl (A.K. Groen).

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http://dx.doi.org/10.1016/j.atherosclerosis.2013.02.021

from the circulation, thereby reducing plasma LDL-cholesterol [3,4]. Additionally, human studies have shown that colestipol and cholestyramine, two older generation BAS [3], induced reductions in total and LDL-cholesterol that were not only accompanied by reductions in relative coronary heart disease death [5] but also by increased regression and decreased progression in coronary artery lesion [6–9]. Colesevelam HCL is a more recent BAS that was specifically engineered for more specificity [3]. However, its effect on atherosclerosis in humans has not been reported so far.

Physical activity has long been known as a beneficial strategy for cardiovascular risk reduction [10–17]. We have recently shown that voluntary wheel running enhances cholesterol turnover into bile acids in healthy chow-fed mice [18] as well as in hypercholesterolemic atherosclerotic mice [17]. Furthermore, the running-induced increase in fecal bile acid secretion coincided with a reduction in atherosclerotic lesion size development in hypercholesterolemic mice [17], demonstrating that physical activity beneficially modulates atherosclerosis by enhancing cholesterol turnover in mice. However, despite the recognized benefits of physical activity in the prevention and management of cardiovascular diseases, regular physical activity programs are frequently reported to be underutilized, adherence to these programs proofs poor or engagement in physical activity is not frequent enough [19,20]. Moreover, physical activity will not result in adequate improvements in individuals presenting with more aggressive cardiovascular derangements. In these situations, pharmacological intervention becomes necessary and is prescribed either alone or in combination with physical activity.

Thus, the aim of this study was to evaluate the effects of BAS-induced disruption of bile acid metabolism on parameters of cholesterol metabolism, atherosclerosis development and parameters of heart function in hypercholesterolemic mice. Since pharmacological intervention and lifestyle changes are often co-prescribed in cardiovascular disease risk management we further evaluated whether a combination treatment of BAS and voluntary wheel running would result in additional benefits to BAS treatment alone.

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 (Local University approval number: 5599C). Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

2.1. Animals, voluntary cage wheel exercise and BAS treatment

Seventy-four 5-week-old male LDLR-deficient (B6.129S7-*Ldlr*^{tm1Her}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were singly housed ($47 \times 26 \times 14.5$ cm cage) in a temperature-controlled room with a 12:12 light–dark cycle and had access to standard pelleted laboratory chow (RMH-B, ABDiets Woerden, the Netherlands). At 8 weeks of age, all mice were switched to a western diet (0.25% cholesterol, 16% fat, Purified Western Diet, 4021.06, ABDiets, Woerden, The Netherlands) and randomly selected to either of the following treatment groups: Sedentary on western-type diet only (CONTROL, $n = 17$), BAS treatment (BAS, western diet supplemented with 2% (wt/wt) Colesevelam HCL (Daiichi Sankyo, Inc., Parsippany, NJ, USA, $n = 17$)), western-type diet exposed to a voluntary running wheel (RUN, $n = 20$) and BAS exposed to a voluntary running wheel (BAS RUN, $n = 20$). All treatments lasted for 12 weeks, mice had *ad libitum* access to food and water throughout the study.

The voluntary running wheel set-up utilized has previously been described [21]. Briefly, the cage of RUN mice was equipped with a hamster-sized metal cage wheel with a diameter of 11 cm which was fitted with a cycle computer containing a digital magnetic counter (Art NO.: K-13-TL SET-P3-NL, Xiron, Netherlands). Each morning, total distances ran were recorded. Once a week, bodyweight and food intake were recorded. Exposing mice to a voluntary running wheel for two weeks has previously been shown to result in cardiac and skeletal muscle adaptations consistent with those of endurance exercise [21]. Six mice with access to a voluntary running wheel were excluded from all analyses because they showed no running wheel activity.

2.2. Experimental procedures

To examine the effect of BAS treatment and the combination of BAS and voluntary wheel running on cholesterol and bile acid metabolism fecal, plasma, biliary, hepatic and intestinal parameters were collected at the endpoint of the experiment after 12 weeks of respective intervention when mice were 20 weeks of age and analyzed as described in the Supplement.

2.3. 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).

2.4. Determination of atherosclerotic lesion size and aortic cholesterol content

Atherosclerotic lesion size was determined as described earlier. In brief, surface lesion area in the aortic sinus 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 DFC420 video camera. At least 5 sections per mouse were examined for each staining.

2.5. Heart function

Under 2% isoflurane anesthesia and maintenance of body temperature mice were subjected to transthoracal echocardiography utilizing a Vivid 7 machine (GE Healthcare, Diegem, Belgium) equipped with special rodent software and a 14 MHz transducer as described [22]. Cardiac dimensions were measured using parasternal short-axis view and M-mode tracings to determine end-diastolic and end-systolic LV internal diameter, posterior wall thickness (LVPW), and interventricular septal thickness (IVS). Left ventricular mass was calculated as described [23]: LV mass = 1.05 $([\text{LVIDd} + \text{LVPWd} + \text{IVSd}]^3 - [\text{LVIDd}]^3)$ g. The cardiac output was calculated by measuring the left ventricular outflow tract (LVOT)

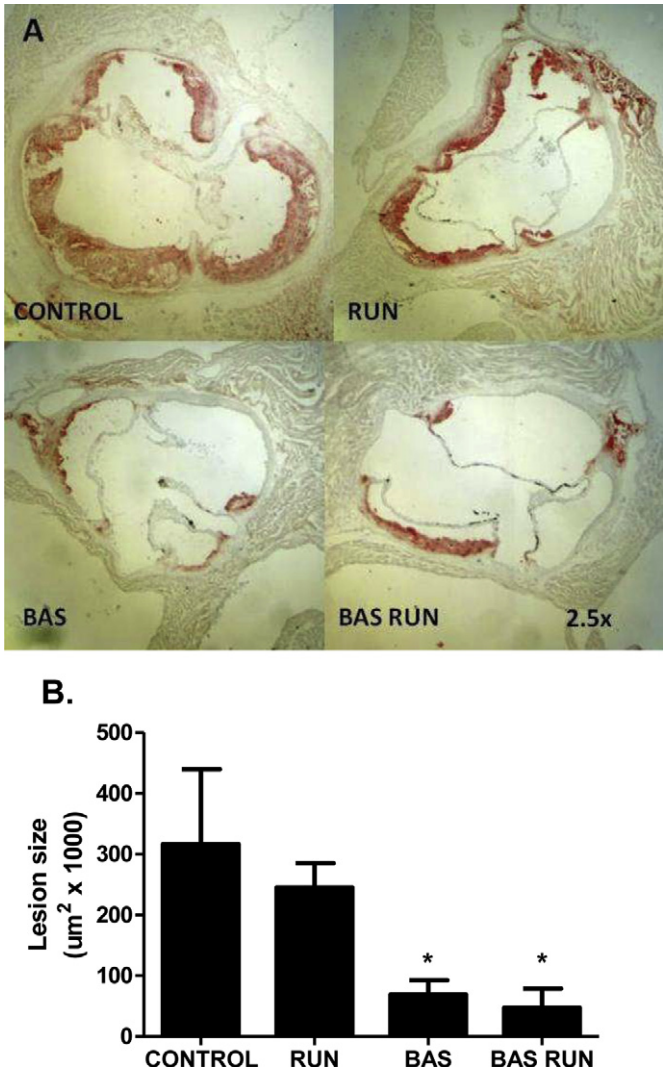

Fig. 1. BAS treatment reduces atherosclerotic lesion size. (A) Representative morphological sections of aortic sinus stained with Oil Red Oil of CONTROL, RUN, BAS and BAS RUN mice, (B) Quantification of lesion size in aortic sinus of CONTROL ($n = 9$), RUN ($n = 9$), BAS ($n = 10$) and BAS RUN ($n = 7$) mice at 12 weeks of intervention. $^{*}p < 0.05$ *vs.* CONTROL.

diameter and measuring a PW doppler signal over the LVOT (using the formula: LVOT radius 2× π (3.14) LVOT VTI× Heart rate).

2.6. Statistics

Multiple group comparisons were done by the Kruskal–Wallis *H* test, followed by Posthoc Conover test, using BrightStat software [24] unless stated otherwise. The Wilcoxon Signed Rank test was utilized for Supplemental Figure 1, comparing each week to week 1 separately. All data are expressed as means ± SD. A *P*-value of <0.05 was accepted as statistically significant.

For more extended description of Methods see Supplement.

3. Results

3.1. Effect of BAS treatment on morphometric parameters and running wheel activity

Despite an increased food intake compared to control mice, BAS-treated running and sedentary mice both displayed a ~14% decrease in bodyweight which was paralleled by a decrease in liver weights. (Table S1). All mice exposed to a voluntary running wheel progressively ran less during the 12-week running wheel intervention. While a daily average running distance of ~10 km was observed at the beginning of the experiment, it dropped to 6.4 km/day in BAS-treated mice and to 3.5 km/day in non-BAS-treated running mice ($p < 0.05$) (Supplemental Figure 1). Furthermore, we observed a significant decrease in running wheel activity in non-BAS-treated mice compared to BAS-treated mice starting at 11 weeks of running wheel exposure ($p < 0.05$, Supplemental Figure 1).

Table 1
Cardiac function.

	CONTROL	RUN	BAS	BAS RUN
LV mass (mg)	95.2 ± 16.5	96.3 ± 24.7	88.4 ± 17.2	86.1 ± 15.4
% Fractional shortening	43 ± 4	43 ± 3	45 ± 4	47 ± 5
Heart rate (bpm)	443 ± 49	415 ± 56	395 ± 41	417 ± 26
Cardiac output (ml/min)	31.7 ± 4.7	28.5 ± 5.0	29.4 ± 4.9	28.5 ± 5.8

Values represent mean ± SD at 12 weeks of running in CONTROL, RUN, BAS and BAS RUN, ($n = 8$ for each group), LV = left ventricular, bpm = beats per minute.

3.2. BAS treatment decreases atherosclerotic lesion size development in LDLR-deficient mice

Quantification of atherosclerotic lesion size in aortic sinus showed a 78% reduction in BAS-treated mice compared to CONTROL (Fig. 1). The combination treatment of BAS and voluntary wheel exercise yielded no additional effects on reducing atherosclerotic lesion size. Running mice not treated with BAS, showed a 23% reduction in atherosclerotic lesion size compared to CONTROL. Atherosclerosis can over time lead to hemodynamic impairments in the heart and thus to acute myocardial events. Impairments in cardiac function have previously been reported for 36-week-old LDLR-deficient mice fed a western diet [25,26]. Thus, we evaluated whether the BAS-mediated reduction in atherosclerotic lesion size would impact on cardiac function in the 20-week-old LDLR-deficient mice of this study. However, no differences were observed in left ventricular mass, percent of fractional shortening, heart rate or cardiac output upon BAS and combination treatment (Table 1).

3.3. BAS treatment beneficially affects lipid metabolism in LDLR-deficient mice

Elevated lipid levels are key in the development of atherosclerosis. We evaluated the effect of BAS treatment alone and in combination with voluntary wheel running on plasma lipid levels, plasma lipoprotein profiles as well as on hepatic parameters involved in lipoprotein and lipid metabolism. BAS-treated mice displayed significant reductions in plasma levels of total cholesterol (2.8-fold), cholesterol-ester (2.5-fold), free cholesterol (3.7-fold) and triglycerides (3.9-fold) compared to CONTROL (Table 2), while no additional improvements were observed in the combination treatment. Importantly, we found improved plasma lipoprotein profiles with reduced levels of VLDL- and LDL-sized lipoproteins in all BAS-treated mice compared to CONTROL (Figure S2).

Further beneficial effects of BAS treatment were observed on hepatic lipid storage. BAS-treated mice displayed 1.8-fold reductions in hepatic triglyceride stores compared to control mice (Table 2), which was paralleled by a 40% reduction in hepatic expression of the lipogenic gene Srebp1c (Table 3). The combination treatment of BAS and voluntary wheel running led to further reductions in hepatic triglyceride stores and hepatic Srebp1c levels

Table 2
Plasma and liver lipids.

	CONTROL	RUN	BAS	BAS RUN
Plasma lipids (mmol/L)				
Total cholesterol	30.4 ± 3.4	30.2 ± 4.3	10.7 ± 1.2*	12.5 ± 3.0*
Free cholesterol	10.8 ± 1.0	10.8 ± 1.5	2.9 ± 0.6*	3.8 ± 1.4*
Cholesterol esters	19.6 ± 2.4	19.4 ± 2.9	7.8 ± 0.7*	8.7 ± 1.7*
Triglycerides	11.9 ± 1.3	10.9 ± 1.3	3.0 ± 0.5*	3.3 ± 1.2*
Liver lipids (nmol/mg liver)				
Triglycerides	40.6 ± 7.7	23.3 ± 5.5*	23.4 ± 3.4*	13.6 ± 1.2*,†
Total cholesterol	18.0 ± 3.3	21.1 ± 4.0	8.2 ± 0.8*	8.1 ± 0.8*
Free cholesterol	8.2 ± 0.7	8.2 ± 0.7	5.6 ± 0.4*	5.8 ± 0.3*
Cholesterol-ester	10.0 ± 3.1	13.0 ± 3.4	2.6 ± 0.5*	2.3 ± 0.5*
Phospholipids	26.7 ± 1.3	28.2 ± 1.3*	26.1 ± 3.1	27.1 ± 1.3

Values represent mean ± SD at 12 weeks of running in CONTROL, RUN, BAS and BAS RUN, ($n = 8$ for each group). *$p < 0.05$ *vs.* CONTROL. †$p < 0.05$ *vs.* BAS.

compared to BAS treatment alone. Moreover, all BAS-treated mice displayed reductions in hepatic total cholesterol (2.2-fold), free cholesterol (1.5-fold) and cholesterol-ester contents (3.8-fold) compared to control mice. Despite the BAS-induced decrease in hepatic cholesterol stores, we found increased hepatic levels of *Hmgcr* (4.3-fold induction), which encodes the rate-limiting enzyme in cholesterol biosynthesis, and *Srebp2* (2-fold induction), a protein implicated in control of cholesterol synthesis upon cellular cholesterol depletion, in BAS-treated mice (Table 3). Collectively, these data show that BAS provokes favorable changes in lipid metabolism. To investigate whether the decrease in TG stores induced on BAS treatment may have been caused by an increased rate of FFA oxidation in muscle we have determined mRNA expression levels of Cpt1 and2; Mcad and Pparα and γ, no changes were observed (data not shown).

Table 3
Hepatic and intestinal genes of cholesterol and bile acid metabolism.

	CONTROL	RUN	BAS	BAS RUN
Liver				
lipogenesis				
Srebf1	1.0 ± 0.2	0.7 ± 0.2*	0.6 ± 0.1*	0.5 ± 0.1*,†
Cholesterol synthesis				
Hmgcr	1.0 ± 0.3	0.6 ± 0.2*	4.3 ± 1.0*	5.0 ± 1.0*
Srebf2	1.0 ± 0.1	0.9 ± 0.2	2.0 ± 0.3*	1.9 ± 0.2*
Cholesterol excretion				
Abcg5	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.1*	0.7 ± 0.2*
Abcg8	1.0 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.8 ± 0.2
Bile acid metabolism				
Cyp7a1	1.0 ± 0.6	0.7 ± 0.4	3.8 ± 1.6*	5.3 ± 2.0*
Cyp8b1	1.0 ± 0.2	0.8 ± 0.2	2.1 ± 0.4*	2.1 ± 0.3*
Cyp27a1	1.0 ± 0.2	0.9 ± 0.2	1.3 ± 0.2*	1.3 ± 0.2*
Abcb11 (Bsep)	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.1*
Intestine				
Cholesterol metabolism				
Jejunal Abcg5	1.0 ± 0.2	1.3 ± 0.2	0.7 ± 0.1*	0.7 ± 0.2*
Jejunal Abcg8	1.0 ± 0.3	1.3 ± 0.2	0.6 ± 0.1*	0.6 ± 0.2*
Jejunal Abca1	1.0 ± 0.2	0.8 ± 0.2	0.3 ± 0.1*	0.3 ± 0.1*
Bile acid metabolism				
Ileal slc10a2 (Asbt)	1.0 ± 0.4	1.0 ± 0.4	2.6 ± 0.9*	2.3 ± 0.4*
Ileal Fabp6 (Ibabp)	1.0 ± 0.3	1.1 ± 0.2	0.6 ± 0.1*	0.6 ± 0.1*
Ileal Fgf15	1.0 ± 0.5	1.8 ± 0.9	0.02 ± 0.01*	0.02 ± 0.02*

Hepatic and intestinal mRNA expression levels for sterol regulatory element-binding protein 1 and 2 (Srepbf1/f2), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (Hmgcr), ATP-binding cassette transporter g5 (Abcg5) and G8 (Abcg8), cholesterol 7 alpha-hydroxylase (Cyp7a1), sterol 12-alpha-hydroxylase (Cyp8b1), sterol 27-hydroxylase (Cyp27a1), bile salt export pump (Abcb11, Bsep), ATP-binding cassette transporter A1 (Abca1), apical sodium-dependent bile acid transporter (Slc10a2, Asbt), ileal bile acid-binding protein (Fabp6, Ibabp), fibroblast growth factor 15 (Fgf15) in CONTROL ($n = 8$), RUN ($n = 8$), BAS ($n = 8$) and BAS RUN ($n = 8$); Hepatic values are relative to b-actin, Intestinal values are relative to cyclophyllin and Rplp0 (36b4) all values represent mean ± SD. *$p < 0.05$ *vs.* CONTROL. †$p < -0.05$ *vs.* BAS.

Table 4
Parameters of bile acid and cholesterol metabolism.

	CONTROL	RUN	BAS	BAS RUN
Bile flow (μl/min/100 gBW)	5.47 ± 0.90	6.00 ± 0.49	4.86 ± 0.62*	5.12 ± 0.82
Bile acids				
Bile (μmol/day/ 100g BW)	302 ± 32	361 ± 151	136 ± 53*	146 ± 52*
Feces (μmol/day/ 100 gBW)	9 ± 2	13 ± 5	40 ± 4*	53 ± 14*
Synthesis (μmol/day/ 100 gBW)	9 ± 2	13 ± 5	40 ± 4*	53 ± 14*
Reabsorption (μmol/day/ 100 gBW)	293 ± 31	312 ± 121	96 ± 55*	93 ± 61*
Absorption (%)	97 ± 1	96 ± 3	65 ± 18*	59 ± 20*
Feces (%)	3 ± 1	4 ± 3	35 ± 18*	41 ± 20*
Cholesterol				
Dietary intake (μmol/day/ 100 gBW)	83 ± 4	97 ± 8*	135 ± 7*	156 ± 14*
Bile (μmol/day/ 100 gBW)	3.25 ± 0.40	2.69 ± 1.17	0.58 ± 0.54*	1.08 ± 0.76*
Dietary cholesterol balance (μmol/day/ 100 gBW)	21 ± 6	17 ± 5*	−36 ± 10*	−22 ± 14*
Feces (μmol/day/ 100 gBW)	62 ± 8	79 ± 8	171 ± 12*	178 ± 19*

This table represents measured and calculated values of fluxes involved in the enterohepatic system and parameters of cholesterol metabolism Values for bile acid synthesis were assumed to equal the measured values for bile acids excreted via the feces. Daily bile acid reabsorption was calculated by the difference in measured bile acids secreted in the bile and feces. Dietary cholesterol balance was calculated by the difference between dietary cholesterol intake and fecal cholesterol excretion.
Values represent mean ± SD at 12 weeks of interventions for CONTROL ($n = 6$), RUN ($n = 8$), BAS (n = 8) and BAS RUN ($n = 8$); *$p < 0{,}05$ vs. CONTROL.

3.4. Effects of BAS treatment on sterol metabolism

Next, we assessed whether the BAS-induced beneficial changes in atherosclerotic lesion size and the improvements in plasma and hepatic lipid levels were accompanied by BAS-induced modulation of cholesterol and bile acid metabolism.

3.5. BAS treatment increased fecal sterol output in LDLR-deficient mice

BAS-treated mice had significantly higher neutral sterol and bile acid excretions (2.3-fold and 4.2-fold, respectively; Table 4). As fecal bile acid excretion is reflective of *de novo* synthesis, BAS-treated mice displayed an increased *de novo* bile acid synthesis. This was paralleled by a BAS-induced increase in hepatic expression of genes encoding bile acid synthesizing enzymes; Cyp7a1, Cyp8b1 and Cyp27a1 (Table 3) and by decreased ileal expression of fibroblast growth factor 15 (Fgf15), a gene which acts to inhibit bile acid synthesis (Table 3).

3.6. BAS treatment promotes a negative cholesterol balance

Because accumulation of excess cholesterol in vascular macrophages leads to formation of atherosclerotic plaques, we calculated whole body cholesterol balances. The difference between dietary cholesterol intake and fecal cholesterol excretion was positive in non-BAS-treated mice (Table 4), demonstrating cholesterol accumulation in the body. However, the cholesterol balance was markedly decreased and became negative in BAS-treated mice, even more in BAS-only-treated mice than in mice receiving the combination treatment, showing that BAS-treated mice excreted

their accumulated cholesterol and when in a new steady state probably compensated by an increase in *de novo* synthesis (Table 4).

3.7. BAS treatment decreased biliary sterol secretions

Subsequently, we assessed biliary parameters. Mice were subjected to gallbladder cannulation for collection of hepatic bile at 12 weeks of interventions. BAS-treated mice had an 11% decreased bile flow, and major reductions in biliary secretions of cholesterol (82% decreased, Table 4), bile acids (55% decreased, Table 4) and phospholipids (50% decreased, *data not shown*) compared to CONTROL. The BAS-induced decrease in biliary cholesterol secretion was paralleled by a modest decrease in expression of hepatic ATP-binding cassette transporter Abcg5 and a slight, albeit not significant, reduction in Abcg8 (Table 3), which both act to transport cholesterol across the canalicular membrane into the bile. BAS had no effect on hepatic Abcb11 (Bsep) expression, which acts to transport bile acids across the canalicular membrane (Table 3). The combination treatment did not result in differential or stronger effects on fecal and biliary parameters than BAS treatment alone. As the fecal cholesterol loss was by far higher than the sum of biliary and dietary cholesterol input upon BAS treatment, a non-hepatobiliary cholesterol excretion pathway must have increased considerably. This increase did not coincide with enhanced expression of the cholesterol exporters Abcg5 and g8 because jejunal expression actually decreased in the BAS-treated groups (Table 3). Surprisingly, expression of the basolateral cholesterol efflux mediator Abca1 decreased substantially as well (Table 3).

3.8. BAS treatment decreased bile acid reabsorption

From the fecal and biliary data we calculated other parameters of *in vivo* bile acid and cholesterol turnover. As expected, daily reabsorption of bile acids decreased upon BAS treatment (97 ± 1% in CONTROL *vs.* 65 ± 18% in BAS and 59 ± 20% in BAS RUN). A compensatory upregulation of the ileal apical sodium-dependent bile acid transporter (Slc10a2, Asbt), was observed. However, expression of ileal bile acid binding protein (Fabp6, Ibabp), was decreased with BAS treatment (Table 3).

4. Discussion

In the present study, we describe the effects of disrupting the enterohepatic circulation of bile acids by Colesevelam HCL, a new generation bile acid sequestrant (BAS) in an established mouse model of atherosclerosis. Since lifestyle interventions, such as physical activity, are often co-prescribed to pharmacological intervention, we furthermore evaluated whether a combination treatment of BAS and voluntary wheel running would yield additional benefits over BAS treatment alone. We were able to show, for the first time, that treatment with Colesevelam HCL indeed provokes specific changes in cholesterol metabolism, specifically switching from a positive to a negative cholesterol balance and increasing cholesterol removal by a non-hepatobiliary route. These favorable BAS-induced modulations translated into improved plasma as well as liver lipid metabolism and ultimately into a dramatic (78%) reduction in atherosclerotic lesion size development in western-type diet fed, hypercholesterolemic LDLR-deficient mice. We also demonstrate that a combination treatment of BAS and physical activity had no additional benefits herein. Physical exercise alone modestly reduced lesion size (23%). Surprisingly, heart function was normal in all groups studied.

Our data demonstrate that under severe hypercholesterolemia as in LDLR deficiency, BAS treatment clearly induces stronger metabolic alterations that altogether lead to a much reduced progression of atherosclerotic lesion size development compared to the running treatment by itself. In general the changes in sterol metabolism induced by the running intervention by itself where on average ~2- to 3-fold less compared to the changes induced by the BAS treatment. The combination treatment of BAS and running most likely had no further benefits compared to the BAS treatment alone, possibly because BAS stimulated and exerted maximal possible change.

Intriguingly, running mice not treated with BAS ran ~2-times less than running mice treated with BAS during the last two weeks of the experiment. To our surprise, they also ran significantly less than LDLR-deficient mice fed a western-type diet in a previous study [17]. Interestingly the decrease in atherosclerotic lesion size was also less. To investigate whether the physical activity correlated to the effect on lesion size we have plotted these parameters. A significant negative correlation was observed (Spearman $rs = -0.63$, $P = 0.044$) for running wheel activity the area under the curve for average km/day from week 1 to week 12 was determined. A dose–response relationship between amount of physical activity and cardiovascular risk reduction has previously been observed [27,28] and the decrease in physical activity in running only mice observed here is most likely the underlying factor for the discrepancy between this and the previous study. We have no explanation for the difference between the current and previous study because all conditions were kept identical. There was also no difference in inflammatory status of the mice as monitored by levels of plasma cytokines such as IFNγ, IL6/10 and TNFα (data not shown).

The differences in lesion size between groups did, in contrast to our expectations, not associate with differences in cardiac function in any group of the LDLR-deficient mice. All echocardiographic parameters were comparable to previously published reference values for C57BL/6J and CD1 mice [22,29]. Previous studies showed impaired heart function in 36-week-old LDLR-deficient mice [25,26]. Other reports suggested that LDLR-deficient mice are prone to cardiac dysfunction, even on normal diet [30] or on very short-term (15 days) western-type diet [31]. We could not confirm these observations. It may be that our intervention was too short lasting to elicit cardiac ischemia and subsequent damage. Thus, we suspect that the control mice in the present study were too young and atherosclerosis thereby not advanced enough for abnormalities in heart function to manifest. Alternatively, although the LDLR-deficient mouse has resemblance with human coronary artery disease, the sequence of events is rather different from man and cardiac dysfunction and heart failure does not seem as prevalent in this mouse model as in human disease. It thus remains to be determined whether BAS treatment benefits heart function in more advanced atherosclerosis.

Numerous studies have previously shown that BAS-induced interruption of the enterohepatic circulation of bile acids leads to an enhanced hepatic cholesterol demand for bile acid synthesis [32–34].

Our data demonstrate a BAS-induced response for the increased demand for cholesterol biosynthesis in several ways. First, we found a more than ~4-fold induction of the rate-limiting enzyme of cholesterol synthesis, Hmgcr, in BAS-treated mice. This is inline with the increased Hmgcr expression and fractional cholesterol synthesis we recently reported in Colesevelam HCL-treated lean and *db*/*db* mice on normal chow [32] but also supports an older study showing that cholestyramine increased HmgCR activity ~6-fold in gallstone patients [33]. Secondly, we found BAS-induced reductions in plasma LDL-sized lipoproteins paralleling earlier human studies using cholestyramine [34,35]. In contrast to results in human studies, BAS treatment strongly reduced plasma triglyceride as well as hepatic TG levels in the Ldlr mice on western-type diet. Apparently, in this extreme model the pull on cholesterol metabolism is adequate to largely normalize plasma lipoprotein levels.

Importantly, our data show that 12 weeks of Colesevelam HCL treatment induces a new steady state in the enterohepatic flux of bile acids in hypercholesterolemic atherosclerotic mice. For example within the model we studied, the calculated bile acid reabsorption was reduced to 65% in BAS-treated mice compared to control mice, which parallels the BAS-induced reduction in bile acid reabsorption observed in lean and *db*/*db* mice [32]. Additionally, while previous studies report no effects of BAS on biliary lipid secretions [32,35], and bile acid pool size [32], we observed profound reductions in biliary lipid secretion upon BAS here. These differences might be attributable to duration of treatment, diets or mouse strain studied. Additionally, BAS treatment exerted substantial effects on the cholesterol balance. Under steady state conditions the amount of cholesterol from dietary input and endogenous synthesis equals fecal excretion. In this situation there is no accumulation of cholesterol in the body. A positive cholesterol balance leads to accumulation of excess cholesterol within the body which can lead to formation of atherosclerotic plaque. The LDLR-deficient mouse has been long utilized as a murine model for atherosclerosis due to its inability of hepatic LDL-lipoprotein uptake which subsequently results in atherosclerosis. However, the actual whole body cholesterol balance upon Ldlr deficiency has not been reported. We show that BAS treatment is effectively interfering with body accumulation of cholesterol leading to a substantial net loss of the sterol which coincided with a reduction in atherosclerosis. Interestingly, BAS-treated mice displayed a substantial deficit between fecal sterol excretion and the sum of dietary cholesterol intake and biliary cholesterol secretion. This demonstrates that BAS treatment strongly enhances cholesterol excretion from a non-hepatobiliary route. Since the origin of this cholesterol was not determined in the present study we can not differentiate whether this was caused by increased transintestinal cholesterol excretion (TICE) or enhanced shedding of intestinal epithelial cells.

In summary, the present study shows that disruption of the enterohepatic circulation by BAS treatment substantially reduced atherosclerotic lesion size in hypercholesterolemic mice which coincided with profound changes in sterol metabolism, specifically leading to a massive cholesterol loss. A combination treatment of physical activity and BAS had no additive effects on atherosclerosis development. Now that a new generation BAS compound has been developed, with a much better safety and side effects profile [36,37], it may be imperative to re-evaluate the usefulness of BAS in fighting atherosclerosis in humans.

Acknowledgements

Funding statement: This study was funded by an unrestricted research grant of the Groningen Expert Center for Kids with Obesity (GECKO) and Daiichi Sankyo (Pirsipanny, NJ). Neither GECKO nor Daiichi Sankyo took part in the data collection nor the preparation of the manuscript.

Disclosure: Authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.02.021.

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