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Atorvastatin accelerates clearance of lipoprotein remnants generated by activated brown fat to further reduce hypercholesterolemia and atherosclerosis

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ABSTRACT

Background and aims: Activation of brown adipose tissue (BAT) reduces both hyperlipidemia and atherosclerosis by increasing the uptake of triglyceride-derived fatty acids by BAT, accompanied by formation and clearance of lipoprotein remnants. We tested the hypothesis that the hepatic uptake of lipoprotein remnants generated by BAT activation would be accelerated by concomitant statin treatment, thereby further reducing hypercholesterolemia and atherosclerosis.

*Methods: APOE*3-Leiden.CETP* mice were fed a Western-type diet and treated without or with the selective β3-adrenergic receptor (AR) agonist CL316,243 that activates BAT, atorvastatin (statin) or both.

Results: β3-AR agonism increased energy expenditure as a result of an increased fat oxidation by activated BAT, which was not further enhanced by statin addition. Accordingly, statin treatment neither influenced the increased uptake of triglyceride-derived fatty acids from triglyceride-rich lipoprotein-like particles by BAT nor further lowered plasma triglyceride levels induced by β3-AR agonism. Statin treatment increased the hepatic uptake of the formed cholesterol-enriched remnants generated by β3-AR agonism. Consequently, statin treatment further lowered plasma cholesterol levels. Importantly, statin, in addition to β3-AR agonism, also further reduced the atherosclerotic lesion size as compared to β3-AR agonism alone, without altering lesion severity and composition.

Conclusions: Statin treatment accelerates the hepatic uptake of remnants generated by BAT activation, thereby increasing the lipid-lowering and anti-atherogenic effects of BAT activation in an additive fashion. We postulate that, in clinical practice, combining statin treatment with BAT activation is a promising new avenue to combat hyperlipidemia and cardiovascular disease.

Abbreviations: ApoE, apolipoprotein E; β3-AR, β3-adrenergic receptor; (i)BAT, (interscapular) brown adipose tissue; (T)C, (total) cholesterol; CETP, cholesteryl ester transfer protein; [^{14}C]CO, [^{14}C]cholesteryl oleate; (F)FA, (free) fatty acids; [^{3}H]TO, glycerol tri[^{3}H]oleate; (V)LDL, (very-) low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; PCSK9, proprotein convertase subtilisin/kexin 9; SQRT, square root; TRL, triglyceride-rich lipoprotein; UCP1, uncoupling protein 1; (g, s)WAT, (gonadal, subcutaneous) white adipose tissue..

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1. Introduction

Cardiovascular diseases (CVD) are the number one cause of death in the Western society [1]. The main underlying pathology of CVD is atherosclerosis, for which hyperlipidemia is a major risk factor [2]. Statins are currently the main strategy to lower plasma nonHDL-C levels in patients with elevated risk for CVD. Although statins reduce total cholesterol (TC) levels by approximately 30% [3], they only prevent 25-45% of all cardiovascular events [1], urging the need for additional therapies.

Brown adipose tissue (BAT) was discovered in rodents as an important player in lipid metabolism [4], and was more recently shown to also contribute to lipid metabolism in humans [5]. BAT is physiologically activated by cold, resulting in release of noradrenalin from sympathetic nerve endings within BAT, which subsequently binds to the β3-adrenergic receptor (β3-AR), present on the membrane of brown adipocytes [6]. Upon activation of brown and beige adipocytes, TG-derived fatty acids (FA) are released from the numerous intracellular lipid droplets and directed towards the mitochondria. Here, they are either oxidized and used for oxidative phosphorylation [6] or to allosterically activate uncoupling protein 1 (UCP1) [7], which uncouples the oxidative phosphorylation from ATP synthase, resulting in the generation of heat instead of ATP. As a consequence of intracellular FA combustion, the intracellular lipid stores become depleted and are subsequently replenished by the uptake of TG-derived FA after lipoprotein lipase (LPL)-mediated lipolysis of TG-rich lipoproteins (TRL) (*i.e.* VLDL and chylomicrons) [8]. In fact, activated BAT can take up such large amounts of FA that it normalizes plasma TG levels in a hypertriglyceridemic mouse model [4].

We have recently reported that BAT activation using the specific β3-AR agonist CL316,243 reduces both hyperlipidemia and atherosclerosis development by increasing the uptake of TG-derived FA from TRL by BAT, and accelerating subsequent formation and hepatic uptake of the cholesterol-enriched lipoprotein remnants in APOE*3-Leiden.CETP (*E3L.CETP*) mice, a well-established model of human-like lipoprotein metabolism [9]. Of note, an intact apolipoprotein E (apoE)-hepatic LDL receptor (LDLR) pathway is crucial for hepatic uptake of cholesterol-enriched remnants [9]. Since statins increase the LDLR-mediated uptake of lipoproteins remnants [10], we hypothesized that statin treatment increases the lipid-lowering and anti-atherogenic effects of BAT activation by accelerating the clearance of TRL remnants generated by BAT activation.

To test our hypothesis, we treated hyperlipidemic *E3L.CETP* mice without or with the β3-AR agonist CL316,243, atorvastatin, or a combination of both. We showed that statin treatment accelerated the hepatic uptake of cholesterol-enriched lipoprotein remnants as generated by β3-AR agonism. Consequently, statin treatment further increased the lipid-lowering and anti-atherogenic effects induced by β3-AR agonism.

2. Materials and methods

2.1. Animals and treatment

Hemizygous *APOE*3-Leiden* (*E3L*) mice were crossbred with homozygous human cholesteryl ester transfer protein (CETP) transgenic mice to generate heterozygous *E3L.CETP* mice [11]. In all studies described below, 10-12 week old female *E3L.CETP* mice were housed under standard conditions in conventional cages with a 12-hour light/dark cycle, at room temperature (22 °C), and with *ad libitum* access to food and water.

During all studies, mice were fed a Western-type diet (WTD; AB diets, Woerden, The Netherlands) containing 15% cacao butter, 1% corn oil and 0.15% (w/w) cholesterol (composition of the diet is listed in Supplemental Table 1). After a run-in period of 3 weeks, mice were randomized into 2 groups that received WTD supplemented without or with atorvastatin (statin; 0.0036%, w/w). After an additional run-in period of 3 weeks, mice in each treatment group were randomized into 2 groups and additionally treated with vehicle (PBS) or the β3-adrenergic receptor (AR) agonist CL316,243 (Tocris Bioscience Bristol, United Kingdom; 20 μg/mouse; subcutaneous injections between 14.00 and 16.00 h). This resulted in the following 4 treatment groups: (1) vehicle (symbol: -), (2) CL316,243 (β), (3) statin (s), and (4) statin + CL316,243 (s+β).

For the atherosclerosis study, mice were treated with CL316,243 5 times weekly for 9 weeks. For the other (short-term) studies, mice were treated with CL316,243 daily for 2 weeks. Food intake was monitored 3 times a week. Both body weight and body composition (body fat and lean mass; EchoMRI-100; EchoMRI, Houston, TX, USA) were monitored weekly. All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and have received approval from the Animal Ethical Committee (Leiden University Medical Center, Leiden, The Netherlands).

2.2. Indirect calorimetry and physical activity

Indirect calorimetry was performed in fully automatic metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany) during the fourth week of treatment. After 1 day of acclimatization, O_2 consumption (VO_2), CO_2 production (VCO_2) and caloric intake were measured for 4 consecutive days [12]. Total energy expenditure (EE) was calculated from the VO_2 and VCO_2, and carbohydrate and fat oxidation rates were calculated from VO_2 and VCO_2 as described previously [13]. Physical activity was measured using infrared sensor frames.

2.3. Plasma lipid parameters

Blood was collected from the tail vein of 4 h fasted mice into sodium heparinized capillaries. Capillaries were placed on ice and centrifuged, and plasma was assayed for TG and TC using enzymatic kits from Roche Diagnostics (Mannheim, Germany). To measure HDL-C levels, apoB-containing lipoproteins were precipitated from plasma with 20% polyethylene glycol 6000 in 200 mmol/L glycine buffer (pH 10), and TC was measured in the supernatant as described above. Plasma nonHDL-C levels were calculated by subtraction of HDL-C from TC levels.

2.4. Plasma PCSK9

Plasma PCSK9 concentration was measured using a commercially available ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.5. In vivo plasma decay and organ uptake of TRL-like particles

TRL-like particles (45 nm), double-labeled with glycerol tri [^{3}H] oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO), were prepared and characterized as described previously [14]. Mice were fasted for 4 h and injected ($t = 0$) intravenously with 200 μL of VLDL-like particles (1.0 mg TG per mouse). Blood samples were taken from the tail vein at $t = 2$, 5, 10 and 15 min after injection to determine the plasma clearance of [^{3}H]TO and [^{14}C]CO. Plasma volumes were calculated as 0.04706 × body weight (g) as previously determined from ^{125}I-BSA clearance studies [15]. After taking the last blood sample, mice were killed by cervical dislocation and perfused for 5 min with ice-cold PBS via the heart to remove blood