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EP 80317, a CD36 selective ligand, promotes reverse cholesterol transport in apolipoprotein E-deficient mice

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ABSTRACT

Aims: The CD36 selective ligand, EP 80317, features potent anti-atherosclerotic and hypocholesterolemic effects that are associated with an increase in macrophage cholesterol efflux through the activation of the peroxisome proliferator-activated receptor γ—liver X receptor α (LXRα)—ATP-binding cassette (ABC) transporter pathway. Cholesterol efflux is the first step of reverse cholesterol transport (RCT). However, whether EP 80317 exerts its hypocholesterolemic and anti-atherosclerotic activity through RCT *in vivo* has yet to be determined. In the present study, we investigated the effects of EP 80317 on RCT, in particular on macrophage-to-feces RCT and the expression of selected genes associated with hepatic cholesterol metabolism and intestinal cholesterol transport.

Methods and results: Reverse cholesterol transport was assessed following the intraperitoneal injection of [^{3}H]-cholesterol-labelled J774 macrophages to hypercholesterolemic apoE- and apoE/CD36 double-deficient mice that had been treated for 12 weeks with EP 80317. Forty-eight hours after the administration of [^{3}H]-cholesterol-labelled cells, blood, liver, intestines and feces were harvested. The radioactivity recovered in the feces (cholesterol and bile acid combined) was significantly increased by 311% ($P = 0.0259$) in EP 80317-treated mice compared with that found in vehicle-treated mice despite no significant change in [^{3}H]-tracer recovery in plasma between groups. Whereas the mRNA levels of LXRα in the gut were significantly upregulated, mRNA and protein levels of the Niemann–Pick C1-like 1 protein (NPC1L1) transporter, a LXRα target which regulates intestinal cholesterol absorption, were downregulated in EP 80317-treated mice. In contrast, neither mRNA nor protein levels of investigated transporters and receptors were modulated in the small intestine of double-deficient mice, nor was the fecal recovery of radioactivity. No change was observed in targeted genes in liver of either apoE- or apoE/CD36 double-deficient mice after a chronic treatment with EP 80317.

Conclusion: This study shows that EP 80317 elicits macrophage-to-feces reverse cholesterol transport in a manner dependent on CD36 expression. This effect is associated with the upregulation of LXRα and the downregulation of NPC1L1 expression.

1. Introduction

The macrophage CD36 receptor has been shown to play a prominent role in scavenging oxidatively modified low density lipoprotein (oxLDL) and generating foam cells [1]. OxLDL catabolism within macrophages has been associated with the generation of bioactive lipids which activate peroxisome proliferator-activated receptor γ (PPAR-γ) leading to the upregulation of a number of genes involved in lipid metabolism, including CD36 [2]. Along that line, our previous studies showed that EP 80317, a synthetic peptide ligand of CD36, presents potent anti-atherosclerotic effects associated with reduced vascular inflammation and hypocholesterolemia

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following a long-term (>6 weeks) treatment at a dose of 300 μg/kg per day in apoE-deficient (apoE$^{-/-}$) mice [3,4]. At the cellular level, we showed that murine macrophages incubated with EP 80317 exhibited increased cholesterol and phospholipid efflux through PPARγ activation [5]. The latter was shown to raise 15d-PGJ$_2$ production through an ERK1/2-dependent COX-2 upregulation, thereby promoting cholesterol and phospholipid efflux from macrophages [5]. In this scheme of events, PPARγ signalling in macrophages is expected to upregulate ATP-binding cassette (ABC) transporters and increase the efflux of cholesterol to extracellular acceptors along the HDL pathway. This is the first step of a multi-step process called reverse cholesterol transport (RCT), resulting in the relocation of cholesterol from the peripheral tissues to the liver, with subsequent hepatobiliary cholesterol secretion and excretion into feces [6–8]. Alternatively, cholesterol may be cleared from the periphery by a liver-independent, transintestinal cholesterol excretion (TICE) pathway, implying a direct cholesterol transport from blood to the intestinal lumen [8–10]. Although detailed mechanisms of TICE remain to be elucidated, key players in cholesterol absorption/secretion by the apical membrane of enterocytes lining the small intestine include Niemann–Pick C1-like 1 protein (NPC1L1), the ABC transporters G5 and G8 and the CD36 receptor, the latter also involved in chylomicron formation [11].

The aim of the present study was to determine whether enhanced macrophage cholesterol efflux associated with prolonged administration of EP 80317 promoted RCT in a CD36-dependent manner. To this aim, apoE$^{-/-}$ and apoE/CD36 double-deficient (apoE$^{-/-}$/CD36$^{-/-}$) mice were fed with a high fat high cholesterol (HFHC) diet from 6 weeks of age and pretreated with EP 80317 by daily s.c. injection for 6–12 weeks. Mice were then injected intraperitoneally with [^{3}H]-cholesterol-labelled J774 murine macrophages and the appearance of [^{3}H]-cholesterol in the plasma, liver, and feces over the next 48 h was quantitated. The expression levels of key cholesterol transporters in liver and small intestine have been assessed.

Our results show that a chronic treatment with EP 80317 promotes macrophage-to-feces RCT, an effect associated with the upregulation of genes and proteins involved in intestinal cholesterol transport and a downregulation of those associated with intestinal cholesterol absorption.

2. Materials and methods

2.1. Materials

[1α, 2α (*n*)-^{3}H]-Cholesterol (35–50 Ci/mmol) was purchased from Amersham Biosciences. EP 80317 (Haic–D-2MeTrp–D-Lys–Trp–D-Phe–LysNH$_2$) was provided by Ardana Bioscience (Edinburgh, UK). All solutions for parenteral administration were sterile.

2.2. Animals

All experimental procedures were approved by the Institutional Animal Ethics Committee of the Université de Montréal, in accordance with the Canadian Council on Animal Care guidelines and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (A5213-01). ApoE$^{-/-}$ and apoE$^{-/-}$/CD36$^{-/-}$ mice and their littermate controls were backcrossed six times to C57BL/6 mice [4]. Six-week-old male mice were fed an HFHC diet (D12108, cholate-free AIN-76A semi-purified diet, Research Diets Inc.) containing 40% wt/wt fat and 1.25% wt/wt cholesterol and water ad libitum. Mice were treated with EP 80317 (300 μg/kg) or vehicle (0.9% NaCl) administered by daily s.c. injection for 12 weeks.

2.3. Plasma lipid analysis

Total plasma cholesterol and HDL cholesterol were assayed using the Infinity™ cholesterol reagent from Thermo Fisher Scientific according to the manufacturer's instructions. QUANTOLIP® HDL (HDL$_2$/HDL$_3$) precipitation reagent from Technoclone was used to precipitate non-HDL cholesterol from plasma.

2.4. Cell culture

J774 murine macrophages were obtained from the American Type Culture Collection. J774 macrophages were cultured in Dulbecco's minimal essential media (DMEM) supplemented with 10% FBS, 100 UI/ml penicillin, and 100 μg/ml streptomycin. J774 cells were radiolabelled by incubating the cells with 5 μCi/ml of [1α, 2α (*n*)-^{3}H]-cholesterol for 48 h at 37 °C. The cells were washed three times, equilibrated in DMEM containing 0.2% BSA overnight and resuspended in PBS.

2.5. In vivo reverse cholesterol transport

ApoE$^{-/-}$ and apoE$^{-/-}$/CD36$^{-/-}$ mice were injected i.p. with [^{3}H]-cholesterol-labelled J774 cells (typically 5×10^6 cells containing between 3 and 4×10^6 counts per min (cpm) in 0.5 ml PBS). Blood samples (50 μl) were collected from the saphenous vein at 2, 6, 24 and 48 h and plasma radioactivity was quantitated by liquid scintillation counting (Hionic Fluor scintillation fluid, Perkin Elmer) using a beta counter (Perkin Elmer). After 48 h, mice were euthanized by CO_2 asphyxiation and exsanguinated, perfused with ice-cold PBS and the organs (liver and jejunum) were removed. Aliquots (100 mg) of liver and jejunum were dissolved in 2 ml of Solvable™ reagent (Perkin Elmer), incubated at 50 °C overnight, and treated with 0.2 ml of 30% hydrogen peroxide. Feces were collected at 24 and 48 h and weighed. Radioactivity content in plasma, liver, intestine and feces was expressed as percent counts relative to total injected tracer.

2.6. Real-time PCR analysis of hepatic and intestinal mRNA levels

In additional experiments, total mRNA was extracted from liver and jejunum (100 mg aliquots) in Trizol (Invitrogen Life Technologies) and reverse transcribed to cDNA using oligo dT 12–18 random primers and SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using Platinum® SYBR® green qPCR supermix (Invitrogen) and gene-specific oligonucleotides (Supplemental materials Table 1) with the Rotor-Gene 3000 instrument (Montreal Biotech). mRNA levels were normalized to the housekeeping gene GAPDH and the relative gene expression (vs. the mean Ct of the vehicle group) was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot of intestinal NPC1L1 and ABCG8 transporters

Jejunum enterocytes isolated from 6 weeks treated apoE$^{-/-}$ and apoE$^{-/-}$CD36$^{-/-}$ mice were weighted and resuspended in ice-cold Tris buffer containing complete protease inhibitor cocktail (PIC) (50 mM Tris–HCl pH 7.4). The samples were homogenized using a Potter–Elvehjem glass-Teflon homogenizer. Crude membrane isolation was obtained after centrifugation at 35,000× *g* for 20 min at 4 °C. The pellet fraction was then resuspended in RIPA lysis buffer (50 mM Tris–HCL pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 25 mM NaF) containing complete PIC and the protein content solubilized by incubation of membrane pellets at 4 °C for 1 h followed by a 10 s sonication. Lysates were centrifuged at 17,000× *g* at 4 °C for 1 h. The supernatants were assayed for protein concentration and