Intervention in hepatic lipid metabolism : implications for atherosclerosis progression and regression

Li, Z.

Chapter 4

Niacin reduces plasma CETP levels by diminishing liver macrophage content in CETP transgenic mice

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ABSTRACT

Background & Aims: The anti-dyslipidemic drug niacin has been recently shown to reduce the hepatic expression and plasma levels of CETP. Since liver macrophages contribute to hepatic CETP expression, the aim of the current study was to investigate the role of macrophages in the CETP-lowering effect of niacin in mice.
Methods and Results: *In vitro* studies showed that niacin does not directly attenuate CETP expression in macrophages. Treatment of human CETP transgenic mice, fed with a Western-type diet, with 2% (w/w) niacin for 4 weeks significantly reduced hepatic cholesterol concentration (-20%), hepatic CETP gene expression (-20%), and plasma CETP mass (-30%). Concomitantly, niacin decreased hepatic expression of CD68 (-44%) and ABCG1 (-32%), both of which are specific markers for the hepatic macrophage content. The decrease in hepatic CETP expression was significantly correlated with the reduction of hepatic macrophage content. Furthermore, niacin treatment attenuated atherogenic diet-induced inflammation in liver, as shown in decreased expression of MCP-1 (-32%) and TNFalpha (-43%). Post hoc analysis showed that niacin also decreased the macrophage content in APOE*3-Leiden.CETP transgenic mice on a Western-type diet.
Conclusions: Niacin decreases hepatic CETP expression and plasma CETP mass by attenuating liver inflammation and macrophage content in response to its primary lipid-lowering effect, rather than by attenuating CETP expression in the macrophage.
Keywords: CETP, niacin, macrophages, lipoprotein, liver, inflammation

INTRODUCTION

The anti-dyslipidemic drug niacin, also known as nicotinic acid, lowers plasma levels of pro-atherogenic lipids/lipoproteins, including very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) as well as triglycerides (TG). The lipid-lowering effect of niacin has been widely recognized as an action on adipose tissue[1]. Niacin rapidly inhibits intracellular TG lipolysis, reduces the mobilization of free fatty acids (FFA) from adipose tissue to liver, and lowers plasma FFA levels acutely. In addition, niacin reduces hepatic synthesis and secretion of VLDL into the circulation[2]. Concomitantly, niacin increases the level of anti-atherogenic high-density lipoprotein (HDL) in normolipidemic as well as hypercholesterolemic subjects[3]. Several clinical trials have shown that niacin reduces cardiovascular disease and myocardial infarction incidence, providing an emerging rationale for the use of niacin in the treatment of atherosclerosis[4,5].

Recently, we showed that niacin increases HDL by reducing the hepatic expression and plasma levels of the pro-atherogenic cholesteryl ester transfer protein (CETP)[6]. CETP, as a lipid transfer protein, has an established role in cholesterol metabolism[7]. It modifies the arterial intima cholesterol content via altering the concentration and function of plasma lipoproteins. Human population investigations favor low CETP as atheroprotective; this is supported by animal models where overexpression of CETP increased concentration of apoB-lipoprotein-cholesterol[8] and atherosclerosis[9]. We have demonstrated, by using APOE*3-Leiden.CETP versus APOE*3-Leiden mice, that niacin markedly reduces hepatic CETP expression and plasma CETP mass. Since CETP expression is driven by liver X receptor (LXR) activation, the reduction in hepatic CETP expression may be secondary to reduced liver lipid levels. However, the exact mechanism behind the hepatic CETP-lowering effect of niacin is still unresolved.

The liver consists of several different types of cells, including hepatocytes and non-parenchymal cells such as resident macrophages, also known as Kupffer cells. Kupffer cells reside in the sinusoidal space of the liver and represent approximately 80-90% of the body's resident macrophages[10,11]. Kupffer cells are derived from monocytes that arise from bone marrow progenitors and migrate from the circulation[12]. Interestingly, Van Eck *et al*[13] have shown a 47-fold higher expression of CETP mRNA in liver Kupffer cells than in hepatocytes. CETP expression in Kupffer cells contributes approximately 50% to the total hepatic CETP expression, indicating that bone marrow-derived CETP is an important contributor to hepatic CETP expression and plasma CETP mass. As the niacin receptor GPR109A is expressed in macrophages[14,15], it is important to determine whether there is a direct action of niacin on liver macrophages. Therefore, the aim of the current study was to investigate the mechanism underlying the hepatic CETP-lowering effect of niacin in CETP transgenic mice.

MATERIALS AND METHODS

Animals

Female CETP transgenic mice expressing the human CETP transgene under the control of its natural flanking regions (CETP Tg; C57BL/6 background) were used

in the present study. The animals were fed with semi-synthetic Western-type diet (WTD) containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK) for 3 weeks (run-in), after which the diet for the treatment group was supplemented with 2% niacin (Sigma-Aldrich) for 4 weeks, while the control group was continuously fed with WTD. After euthanization, mice were bled via orbital exsanguination, and perfused *in situ* through the left cardiac ventricle with ice-cold PBS (pH 7.4) for 20 minutes. Tissues were dissected and snap-frozen in liquid nitrogen. One lobe of the liver was dissected free of fat and stored in 3.7% neutral-buffered formalin (Formal-fixx, Shandon Scientific Ltd., UK) for histological analysis. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Culture of bone marrow-derived macrophages

Bone marrow-derived macrophages were obtained from CETP transgenic mice. Single-cell suspensions were harvested as described above. Cell concentration was adjusted to 8 x 10^6 cells/mL, and cells were placed on a non-tissue culture treated Petri dish in RPMI1640 (PAA Laboratories) containing 20% (v/v) fetal calf serum (FCS), 2 mM/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% (v/v) non-essential amino acids, 1% (v/v) pyruvate, and 30% (v/v) L929-conditioned media. Cells were cultured in a humidified incubator with 5% CO_2 at 37°C for 7 days. After bone marrow cell differentiation, adherent macrophages were harvested and cultured on 12-well plate in DMEM (PAA Laboratories) containing 10% (v/v) FCS, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at a density of 0.5 x 10^6 cells/mL. After 24 hours, non-adherent cells were removed, and macrophages were incubated in the absence or presence of niacin (Sigma-Aldrich) at a concentration of 0.1 µM, 1 µM, and 10 µM for 24 hours. Total RNA was extracted and gene expression analysis was performed as described above.

Hepatic lipid analysis

Lipid analysis was performed when the animals received 4 weeks of WTD with or without 2% niacin. Mice were fasted overnight prior to euthanization. Lipids were extracted from liver using the Folch method. Briefly, 100 mg of tissue was homogenized with chloroform/methanol (1:2). The homogenate was centrifuged to recover the upper phase, which was further washed with chloroform-0.9% NaCl (1:1, pH 1.0). After centrifugation, the lower chloroform phase containing lipids was evaporated and the retained lipids were resolubilized in 2% Triton X-100 by sonification. Protein contents were analyzed by BCA assay (Pierce Biotechnology, Thermo Fisher Scientific BV, IL, USA). Total cholesterol content of lipid extracts was measured using the enzymatic colorimetric assay with 0.025 U/mL cholesterol oxidase, 0.065 U/mL peroxidase, and 15 µg/mL cholesteryl esterase (Roche Diagnostics, Mannheim, Germany). Data were expressed as milligrams of lipid per milligram of protein.

RNA isolation and gene expression analysis

Total RNA from liver was isolated using acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction. Briefly, 500 µL of GTC solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine) was added to

each sample, followed by acid phenol:chloroform extraction. The RNA in aqueous phase was precipitated with isopropanol. The quantity and purity of the isolated RNA were examined using ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA). One microgram of the isolated RNA from each sample was converted into cDNA by reverse transcription with RevertAid™ M-MuLV Reverse Transcriptase (Promega, Madison, WI, USA). Negative controls without addition of reverse transcriptase were prepared for each sample. Quantitative real-time PCR was carried out using ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. 36B4, Beta-actin, and GAPDH were used as internal housekeeping genes. Amplification curves were analyzed using 7500 Fast System SDS software V1.4 (Applied Biosystems, Foster City, CA, USA). The relative expression of each gene was expressed as comparative numerical fold changes $2^{-(\Delta\Delta CT)}$. Standard error of the mean (SEM) and statistical significance were calculated using ΔΔCt formula.

CETP mass determination in plasma
Plasma CETP mass was determined by ELISA, using a commercially available immunoturbidimetry kit (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions.

Immunohistochemistry
Macrophage content in livers of APOE*3-Leiden.CETP mice treated with or without 1% of niacin6 was analyzed by immunohistochemistry staining. The liver was embedded in O.C.T™ Compound (Tissue-Tek, Sakura finetek, Tokyo, Japan), and subsequently sectioned using a Leica CM 3050S cryostat at 8 µm intervals. After incubation with blocking solution (5% goat serum), macrophages were detected using F4/80 antibody (rat antibody directed against murine macrophages, AbD Serotec, Oxford, UK). A rabbit anti rat IgG/HRP was used as second antibody (Dako, Heverlee, Belgium). Sections were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories, Peterborough, UK) according to the manufacturer's instructions. Slides were counterstained with hematoxylin (Sigma-Aldrich).

Statistical analysis
Statistical analyses were performed by the unpaired Student's t-test for independent samples (Instat GraphPad software, San Diego, USA). Statistical significance was defined as $p<0.05$. Data are expressed as means ± SEM.

RESULTS

Niacin reduces hepatic lipid content, plasma CETP mass, and hepatic CETP expression
CETP Tg mice were utilized to analyze the effects of niacin on hepatic CETP expression and plasma levels of CETP. Four weeks of niacin treatment significantly reduced hepatic cholesterol concentration by 20% ($p=0.005$, Figure 1). In addition, in line with our previous data[6], niacin treatment resulted in a significant 30% reduction ($p=0.0002$) in plasma CETP mass (Figure 1). In parallel, gene expression of CETP in liver was also significantly reduced by 20% ($p=0.034$, Figure 1).

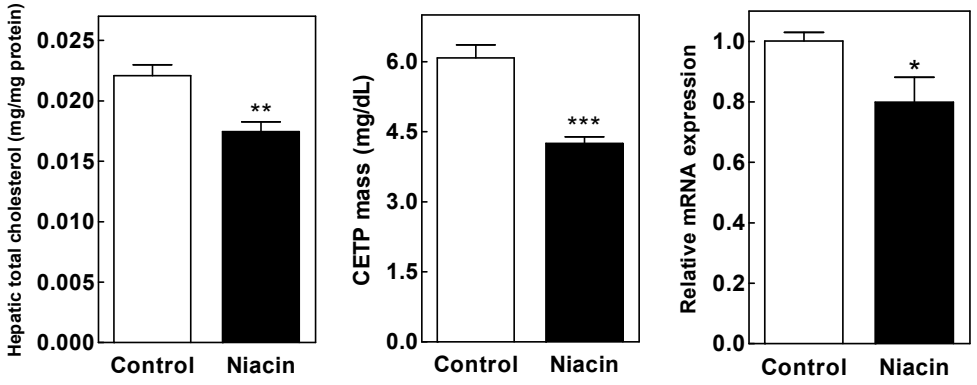

Figure 1. Effect of niacin on hepatic cholesterol concentration, plasma CETP mass, and hepatic CETP expression in CETP Tg mice. After 4 weeks of Western-type diet (WTD) feeding with or without 2% niacin supplement, mice were euthanized and lipids were extracted from liver. Total cholesterol concentration was measured. Plasma CETP mass was determined by ELISA. Total RNA was isolated from liver. Relative mRNA expression of CETP was determined by quantitative PCR and presented as fold-change relative to control group. Values are means ± SEM (6 mice per group). *P<0.05; **P<0.01; ***P<0.001.

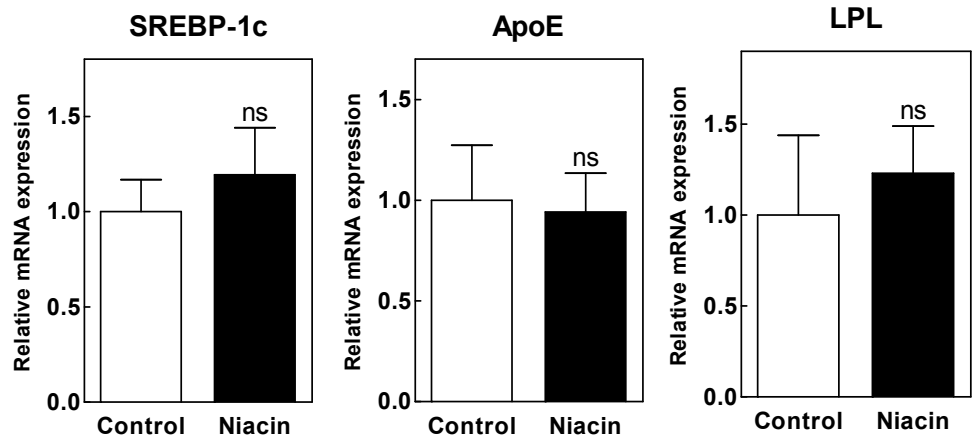

Figure 2. Effect of niacin on LXR-target genes in CETP Tg mice. Total RNA was isolated from liver. Relative mRNA expression of the LXR-target genes SREBP-1c, ApoE, and LPL were determined by quantitative PCR and presented as fold-change relative to control group. Values are means ± SEM (6 mice per group). ns, not significant.

Niacin does not directly regulate LXR activity in the liver

To evaluate whether niacin decreased hepatic CETP expression by attenuating LXR activation, we measured the effect of niacin on the LXR target genes SREPB-1c, ApoE, and LPL. The hepatic expression of these three genes remained unchanged after niacin treatment (Figure 2), indicating that the reduction of hepatic CETP expression was not due to a direct regulation of niacin on LXR.

Niacin does not directly regulate CETP expression in bone marrow-derived macrophages

To assess whether niacin directly attenuates CETP expression in macrophages, bone marrow-derived macrophages isolated from CETP Tg mice were exposed to various concentrations of niacin (0.1 μM, 1 μM, and 10 μM) for 24 hours. Niacin treatment did not alter CETP expression (Figure 3). In addition, niacin did not affect expression of the LXR-regulated targets SREBP-1c or apoE, or the cholesterol metabolism-related genes ABCA1, ABCG1, SR-B1, CD36 (data not shown).

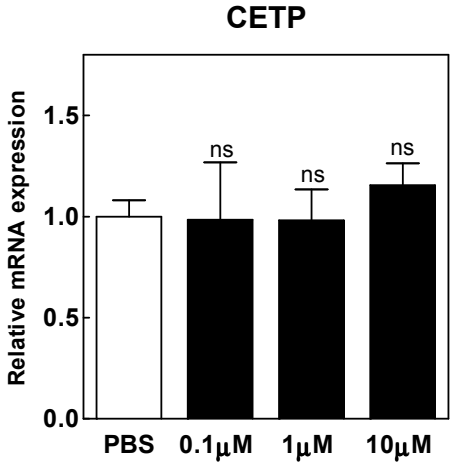

Figure 3. Effect of niacin on CETP expression in bone marrow-derived macrophages from CETP Tg mice. Bone marrow-derived macrophages isolated from CETP Tg mice were incubated in medium in the absence or presence of niacin at a concentration of 0.1 μM, 1 μM, and 10 μM for 24 hours. Total RNA was extracted and CETP mRNA expression was assessed by quantitative PCR and presented as fold-change compared to PBS-control group. Values are presented as means ± SEM (5 independent cell isolations). ns, not significant.

Niacin reduces macrophage content and inflammation in the liver

Since these data indicated that niacin may reduce hepatic CETP expression by reducing the liver macrophage content, we evaluated the effect of niacin on CD68 and ABCG1, both of which have been defined as reliable markers to assess the hepatic macrophage content[16, 17, 18]. Interestingly, niacin treatment significantly decreased hepatic expression of CD68 by 44% (p=0.027) and ABCG1 by 32% (p=0.001) (Figure 4A). In addition, niacin did not affect the CETP/ABCG1 and ABCG1/CD68 expression ratios (Figure 4A). This suggests that niacin does not directly reduce the expression of CETP or ABCG1 by on macrophages, in line with our previous results from *in vitro* study, but in fact reduces the liver macrophage content, thereby

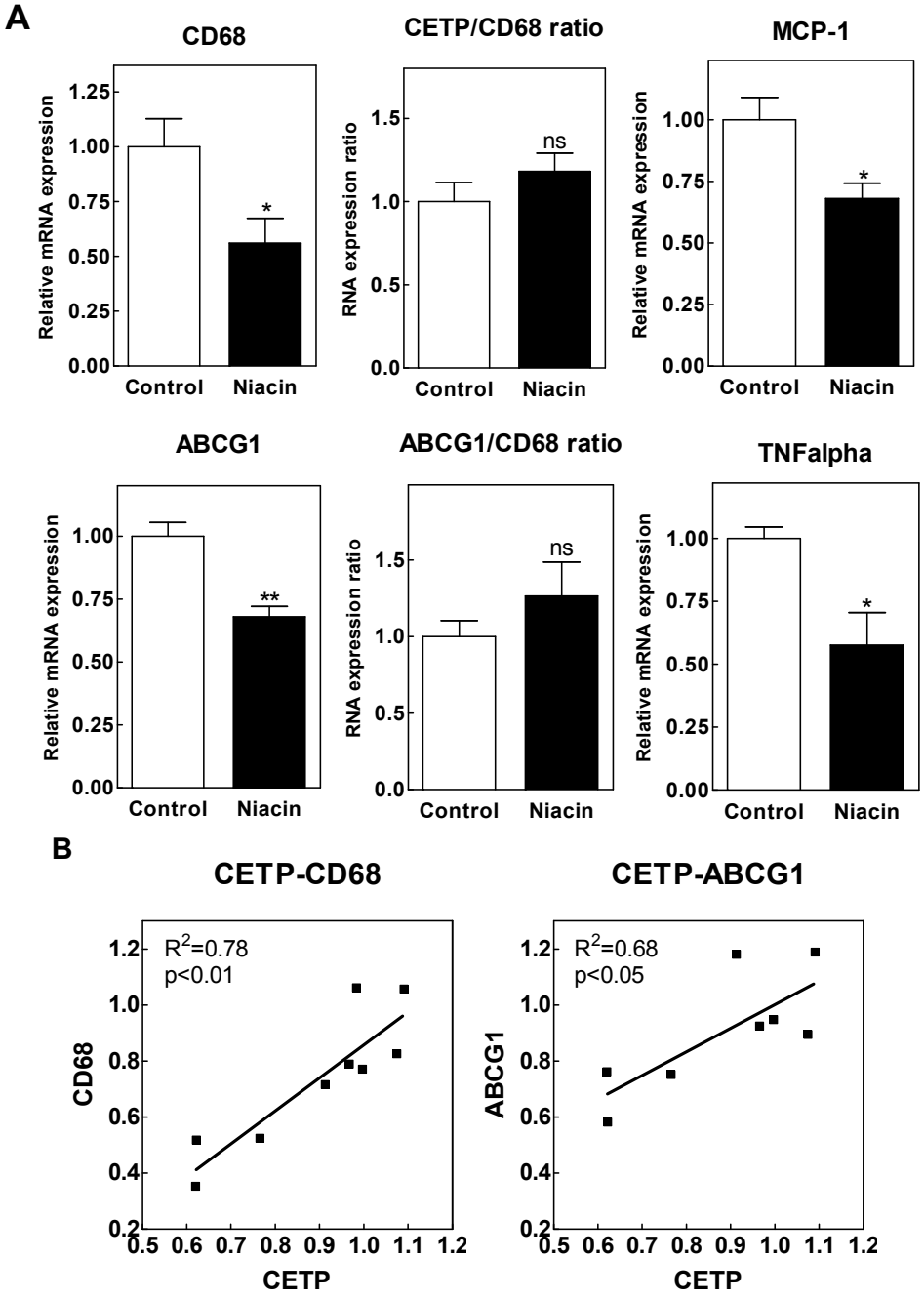

Figure 4. Effect of niacin on hepatic macrophage gene expression in CETP Tg mice. Total RNA was extracted from liver and relative mRNA expression of CD68, ABCG1, MCP-1, and TNFalpha were assessed by quantitative PCR and presented as fold-change relative to control group (A). Ratio between the expression level of CETP and CD68, ABCG1 and CD68 were calculated (A). Correlation between hepatic CETP and CD68 / ABCG1 expression upon niacin treatment was linearly plotted (B). Values are means ± SEM (6 mice per group). ns, not significant. *P<0.05; **P<0.01.

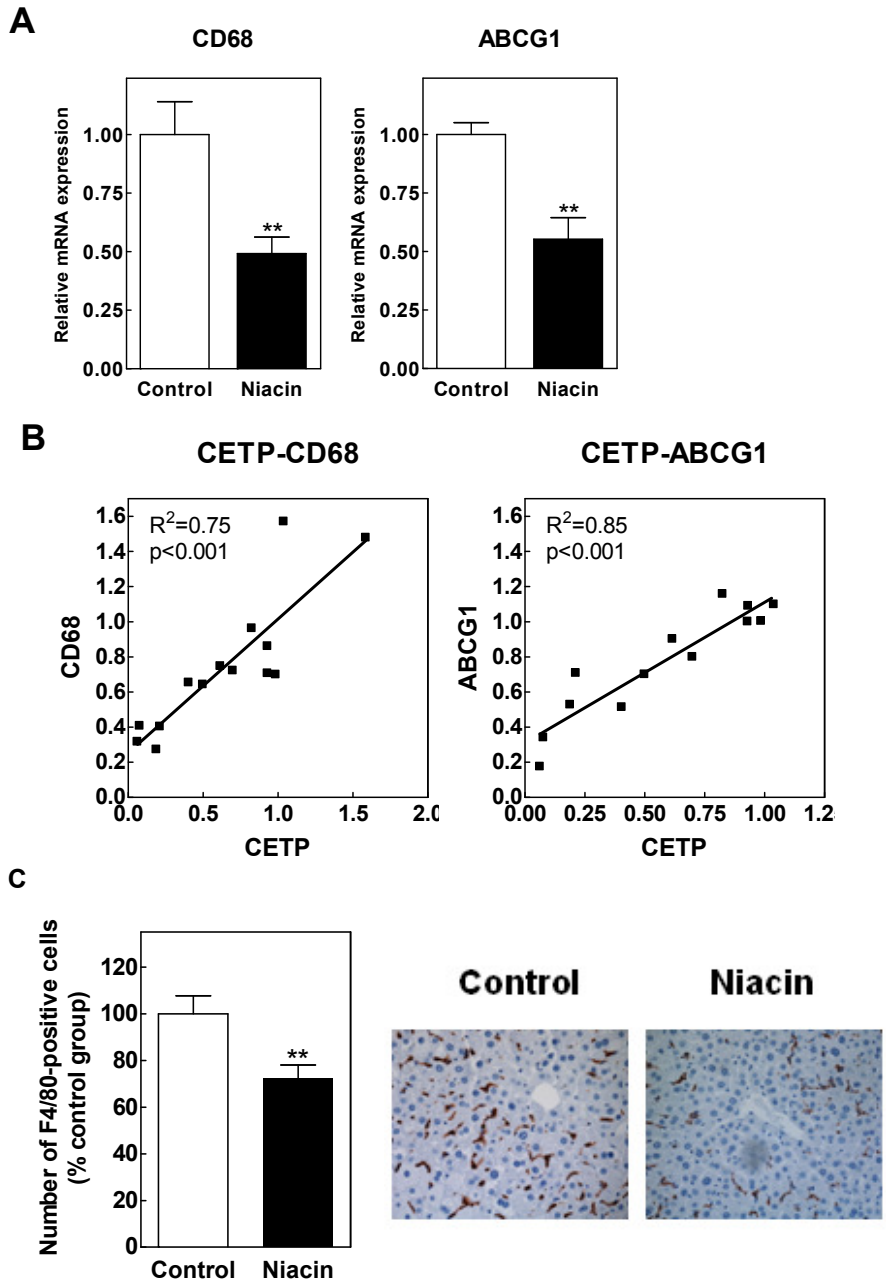

Figure 5. Effect of niacin on hepatic macrophage gene expression and number of macrophages in APOE*3-Leiden.CETP mice. Total RNA was extracted from liver and relative mRNA expression of CD68 and ABCG1 were assessed by quantitative PCR and presented as fold-change relative to control group (A). Correlation between hepatic CETP and CD68 / ABCG1 expression upon niacin treatment was linearly plotted (B). Macrophage content in the liver was visualized via immunohistochemistry staining with F4/80 antibody, and the number of positive cells were counted and expressed as percentage of control group. Representative pictures are shown (C). Values are means ± SEM (8 mice per group). ns, not significant. *P<0.05; **P<0.01.

reducing CETP expression. In accordance with the decreased liver macrophage content, gene expression of the inflammatory markers MCP-1 and TNFalpha in the liver both decreased substantially by 32% (p=0.016) and 43% (p=0.011), respectively, after niacin treatment (Figure 4A), suggesting attenuated high-fat diet-induced inflammation in liver.

The comparable reductions of hepatic CETP, liver macrophage markers, and liver inflammation markers suggest that the decrease of hepatic CETP expression is caused by a reduced amount of inflammatory macrophages in liver. Indeed, linear regression showed a very significant and strong positive correlation between hepatic CETP and CD68 expression ($p<0.01$; $R^2=0.78$), as well as between hepatic CETP and ABCG1 expression ($p<0.05$, $R^2=0.68$).

Consistent with our current results, post-hoc analysis on livers of APOE*3-Leiden.CETP mice treated with 1% niacin, from our previous study, in which the CETP-lowering effect of niacin was first observed[6], revealed similar significant reductions in hepatic gene expression of CD68 (-51%, p=0.007) and ABCG1 (-45%, p=0.001) (Figure 5A). In addition, there were also significant correlations between hepatic CETP and CD68 ($p<0.001$; $R^2=0.75$) or ABCG1 ($p<0.001$; $R^2=0.85$) expression (Figure 5B). The reduction of hepatic macrophage content was further visualized by staining of F4/80-positive cells, where niacin significantly reduced the number of macrophages in the liver by 28% (p=0.006) (Figure 5C).

DISCUSSION

To explain the CETP-lowering effect of niacin, we set out to investigate the effect of niacin on macrophages. Our observations *in vitro* showed that niacin at various concentrations did not reduce CETP expression in cultured macrophages derived from CETP Tg mice. Neither did niacin alter cholesterol metabolism-related genes, such as ABCA1, ABCG1, and SR-B1. We thus conclude that niacin does not directly regulate expression of CETP or other lipid-related genes in macrophages, albeit that secondary effects of niacin in an *in vivo* setting, e.g. related to a reduction in the fatty acid flux from adipose tissue to (liver) macrophages, can not be ruled out.

Luo *et al*[19] have previously demonstrated that CETP is trans-activated by nuclear receptor LXR, suggesting its role in regulating CETP expression *in vivo*. We also proposed in our previous study that niacin may decrease the hepatic CETP mRNA expression via LXR responsive element in the CETP promoter following decreased cholesterol content. However, our current *in vitro* data showed that niacin did not directly regulate expression of LXR-regulated target genes, such as ABCA1. Our *in vivo* data further confirmed that niacin did not regulate the expression of classical LXR targets such as SREBP-1c, apoE, or LPL in liver. In addition, although niacin treatment reduced the gene expression of ABCG1 in liver, it did not affect the ABCG1/CD68 expression ratio, indicating that niacin does not directly reduce the expression of ABCG1 on macrophages. The reduction in ABCG1 *in vivo* is thus probably not simply the consequence of reduced LXR activation. Therefore, it is suggested that either direct or indirect regulation of LXRs in the liver is not the main mechanism by which niacin reduces CETP expression.

The liver is a unique immunological site responding to inflammation. Antigen-rich blood from the gastrointestinal tract and the peripheral circulation enters the

hepatic parenchyma, passes through a network of liver sinusoids and is scanned by immune cells including macrophages and lymphocytes [20]. Thus, liver macrophages have profound implications in many aspects of the hepatic inflammatory response[21]. Plasma pro-atherogenic lipoproteins, mainly (V)LDLs, are important determinants of liver inflammation. Recent evidence has indicated an increased hepatic inflammation and macrophage content upon high-fat diet-induced hyperlipidemia. In C57Bl/6J mice fed a high-fat diet, up-regulation of hepatic expression of CD68 was found associated with increased hepatic lipid content[22]. Another study showed that in the LDLr$^{-/-}$ mice fed a high-fat diet, an increase of CD68 expression in the liver was correlated with increased plasma VLDL cholesterol levels. Omitting cholesterol from the diet rapidly reduced plasma TG and VLDL-cholesterol accumulation, associated with significantly lowered CD68 expression in liver together with other inflammatory genes[23]. In humans, a similar correlation between increased presence of CD68-positive Kupffer cells and the histological severity of human hepatic lipid content in fatty liver has been reported[24]. Such correlations between altered macrophage content and circulatory inflammatory factors define macrophage infiltration as a common response against hepatic and circulatory inflammation.

In the current study, niacin treatment reduced cholesterol content in the liver. In line with this attenuated liver fat accumulation, we further observed a significant reduction of pro-inflammatory markers MCP-1 and TNFalpha in liver. MCP-1 is one of the best characterized pro-inflammatory chemokines in liver. It has been reported that dietary fat and cholesterol, inappropriately high in the Western-type diet, are potent metabolic stress inducers of hepatic expression of the MCP-1 gene[25]. An increase in MCP-1 expression contributes to macrophage infiltration and hepatic steatosis in mice[26]. The pro-inflammatory cytokine TNFalpha is also critically involved in the pathophysiology of liver steatosis, and this cytokine is primarily secreted by Kupffer cells and liver-infiltrating macrophages[27]. Taken together, the results suggested an attenuated liver inflammation after niacin treatment.

In line with the attenuated diet-induced inflammation in the liver, the hepatic gene expression of CD68 and ABCG1 were also reduced upon niacin treatment. CD68 has been defined as a reliable macrophage marker and widely used for quantification of macrophage content in numerous studies[28,29,30]. ABCG1 has also been shown to be a good marker to assess Kupffer cell content in the liver, since ABCG1 is not expressed in hepatocytes[31,32]. In the current study, a reduction in the hepatic MCP-1 expression was associated with decreased CD68 and ABCG1 gene expression in liver, and also a reduced number of macrophages in liver, indicating an attenuated macrophage infiltration and/or an increased macrophage efflux from the liver into the liver and thus a decreased liver macrophage content. More importantly, the significant positive correlation between hepatic CETP and both CD68 and ABCG1 expression observed in both the current study and in the present post-hoc analysis of our previous study suggests that the liver macrophage is a primary contributor to hepatic and total plasma CETP mass, and that the hepatic CETP reduction induced by niacin treatment is a direct consequence of a reduced macrophage content of the liver.

Figure 6 illustrates the proposed mechanism underlying the action of niacin on hepatic CETP expression. We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated

hepatic inflammation, and thus less macrophage infiltration into and/or increased macrophage emigration out of the liver. The decreased amount of hepatic macrophages leads to an overall reduction in hepatic CETP expression and a lower plasma CETP level.

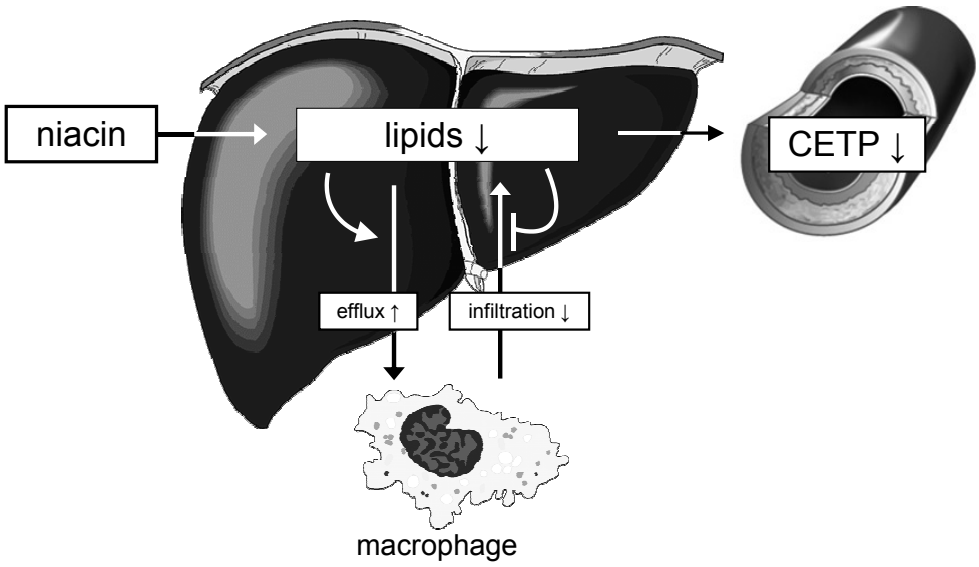

Figure 6. Proposed mechanism underlying the action of niacin on hepatic CETP expression and plasma CETP mass. We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated hepatic inflammation, and thus less macrophage infiltration into and/or increased macrophage emigration out of the liver. The decreased amount of hepatic macrophages, which are significant contributors of CETP, leads to an overall reduction in hepatic CETP expression and a lower plasma CETP level.

In conclusion, our study sheds new light on the mechanism underlying the CETP-lowering effect of niacin in CETP transgenic mice. We have shown that niacin does not directly alter macrophage CETP expression, but attenuates liver inflammation and the macrophage content in response to its primary lipid-lowering effect, which leads to a decrease in hepatic CETP expression and plasma CETP mass.

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