Novel modulators of lipoprotein metabolism : implications for steatohepatitis and atherosclerosis

Wang, Y.

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NIACIN REDUCES PLASMA CETP LEVELS BY DIMINISHING LIVER MACROPHAGE CONTENT IN CETP TRANSGENIC MICE

Zhaosha Li, Yanan Wang, Ronald J. van der Sluis,
José W.A. van der Hoorn, Hans M.G. Princen, Miranda Van Eck,
Theo J.C. van Berkel, Patrick C.N. Rensen, Menno Hoekstra

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ABSTRACT

The anti-dyslipidemic drug niacin has recently been shown to reduce the hepatic expression and plasma levels of CETP. Since liver macrophages contribute to hepatic CETP expression, we investigated the role of macrophages in the CETP-lowering effect of niacin in mice. In vitro studies showed that niacin does not directly attenuate CETP expression in macrophages. Treatment of normolipidemic human CETP transgenic mice, fed a Western-type diet with niacin for 4 weeks, significantly reduced the hepatic cholesterol concentration (-20%), hepatic CETP gene expression (-20%), and plasma CETP mass (-30%). Concomitantly, niacin decreased the 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 markers. Furthermore, niacin attenuated atherogenic diet-induced inflammation in liver, as evident from decreased expression of TNF-alpha (-43%). Niacin similarly decreased the macrophage markers and absolute macrophage content in hyperlipidemic *APOE*3-Leiden.CETP* transgenic mice on a Western-type diet. In conclusion, 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 the macrophage CETP expression level.

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, where it reduces the mobilization and flux of free fatty acids from adipocytes into the plasma by inhibiting intracellular lipolysis [1, 2]. In addition to lowering pro-atherogenic lipoprotein levels, 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].

Previously, we have shown that niacin increases HDL by reducing the hepatic expression and plasma levels of the pro-atherogenic cholesteryl ester transfer protein (CETP) in *APOE*3-Leiden.CETP* transgenic mice that exhibit a human-like lipoprotein profile [6]. Importantly, a similar effect of niacin treatment on plasma CETP has also been detected in the human clinical setting (25-30% decrease; Chapman *et al.*, unpublished data). 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 and atherosclerosis [8]. 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 [9, 10]. Kupffer cells are derived from monocytes that arise from bone marrow progenitors and migrate from the circulation [11]. Interestingly, Van Eck *et al.* [12] have shown a 47-fold higher expression of CETP mRNA in liver Kupffer cells than in hepatocytes of CETP transgenic mice. Furthermore, immunolocalization studies by Pape *et al.* [13] have also suggested that non-parenchymal cells are the primary site of CETP expression in livers from cynomolgus monkeys. Combined, these studies indicate that bone marrow-derived CETP is an important contributor to hepatic CETP expression and plasma CETP mass. Since the niacin receptor GPR109A is expressed in macrophages [14, 15] and niacin has been shown to exhibit potent anti-inflammatory

activities independent of its lipid lowering action [16-18], it is important to determine whether there is a direct action of niacin on liver macrophages. The aim of the current study was therefore to investigate whether macrophages are involved in the hepatic CETP-lowering effect of niacin, by using CETP transgenic mice on a wild-type and *APOE*3-Leiden* transgenic background.

MATERIALS AND METHODS

Animals

Twelve to fourteen week old female CETP transgenic mice expressing the human CETP transgene under the control of its natural flanking regions (*CETP* Tg; strain 5203; C57BL/6J N10) [19] were used. The animals were fed a semi-synthetic Western-type diet 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. Given the ~7-fold higher total metabolic rate [20, 21] and ~6-fold higher glomerular filtration rate [22] in mice as compared to humans, the 2% dose of niacin given to the mice corresponds to approximately 18 g/day for an average 70 kg human subject. Although this dosage is higher than the therapeutic dose of niacin used in the clinical setting [23], in pharmaceutical literature a relatively high dose of dietary niacin is commonly used dose to study the biological effect of niacin in mice [24-26]. After an overnight fast, mice were euthanized, 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.

Some of the data reported here are derived from post-hoc analyses on samples from a previous study executed in *APOE3*Leiden.CETP* mice [6]. In the indicated study, female *APOE3*Leiden.CETP* mice were fed a semi-synthetic cholesterol-rich diet for 3 weeks to obtain similar total cholesterol levels. After matching, mice received a Western-type diet without or with 1% niacin (Sigma) for 3 weeks.

Culture of bone marrow-derived macrophages

Bone marrow from female CETP Tg mice was harvested by flushing the femurs and

tibias with PBS (pH 7.4). Single-cell suspensions were prepared by passing the cells through a 70 µm cell strainer (BD, Breda, The Netherlands). 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 for 7 days to specifically induce macrophage differentiation. Optimal differentiation was confirmed microscopically by visual examination of cell morphology (i.e. shape) and using routine blood cell analysis (Sysmex XT-2000iV Veterinary Hematology analyzer; Sysmex Corporation). 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, 10 µM, and 100 µM for 24 hours.

Tissue lipid analysis

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 solubilized in 2% Triton X-100 by sonication. Protein content of the tissue homogenates was analyzed by BCA assay (Pierce Biotechnology, Thermo Fisher Scientific BV, IL, USA). Total cholesterol and triglyceride contents of the lipid extract were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). Data were expressed relative to the protein content.

RNA isolation and gene expression analysis

Total RNA 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; Sigma-Aldrich) 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 using the primers in Table 1. 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). Transcripts that showed Ct>35 were considered not detectable. The relative expression of each gene was expressed as comparative numerical fold changes 2−(ΔΔCT). Standard error of the mean (SEM) and statistical significance were calculated using ΔΔCt formula.

Table 1: Primers used for quantitative real-time PCR

Gene	Accession	Forward Primer	Reverse Primer
36B4	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCA1	NM013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG1	NM009593	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
APOA1	NM009692	ACTCTGGGTTCAACCGTTAGTCA	TCCCAGAAGTCCCGAGTCA
ATGL	NM025802	TGCCCTCAGGACAGCTCC	TTGAACTGGATGCTGGTGTTG
Beta-actin	X03672	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
CD68	NM009853	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
CETP	NM000078	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
CHOP	MMCHOP10	CTCTTGACCCTGCGTCCCTAG	TGGGATGTGCGTGTGACCT
FAS	NM007988	GGCGCGGCACCTATGGCGAGG	CTCCAGCAGTGTGCGGTGGTC
GAPDH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HSL	NM001039507	CTGACAATAAAGGACTTGAGCAACTC	AGGCCGCAGAAAAAAGTTGAC
LPL	NM008509	CCAGCAACATTATCCAGTGCTAG	CAGTTGATGAATCTGGCCACA
SREBP-1C	NM011480	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA
TNF-alpha	X02611	GCC AGC CGA TGG GTT GTA	AGGTTGACTTTCTCCTGGTATGAGA

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. Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP; Roar Biomedical, New York, NY) as described [27].

Immunohistochemistry

Macrophage content in livers of *APOE*3-Leiden.CETP* mice treated with or without niacin [6] 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 a rat anti-murine F4/80 antibody (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). Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTPbiotin nick-end labeling (TUNEL) with an in situ cell death detection kit (Roche). Nuclei were counterstained with 0.3% Methylene Green.

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.

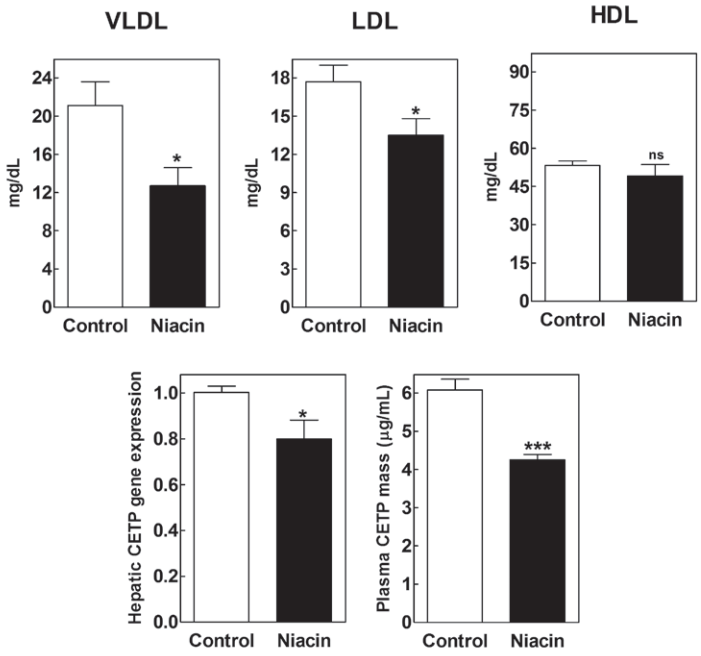


Figure 1. Effect of niacin on plasma lipoprotein concentrations, hepatic CETP expression, and plasma CETP mass in *CETP* Tg mice. *CETP* Tg mice were fed a Western-type diet with or without supplementation of niacin (w/w) for 3 weeks before analysis. Values are means±SEM. ns, not significant. *$P<0.05$; ***$P<0.001$.

RESULTS

Niacin lowers VLDL/LDL levels and reduces plasma CETP mass in *CETP* Tg mice

In agreement with its established lipid lowering capacity in the human situation, four weeks of niacin treatment induced a significant decrease in plasma levels of pro-atherogenic apoB-containing lipoproteins VLDL (-40%; $P<0.05$) and LDL (-24%; $P<0.05$) in Western-type diet fed *CETP* Tg mice (Fig. 1). Although a CETP-dependent increase in plasma HDL levels has previously been noted upon niacin treatment in our *APOE3*Leiden* mouse model [6], we did not observe a significant change in plasma HDL-

cholesterol levels in *CETP* Tg mice upon feeding the diet supplemented with niacin (Fig. 1), probably because of low (V)LDL levels as acceptor of CETP-mediated HDL-CE transfer. In line with our previous data from *APOE*3-Leiden.CETP* transgenic mice [6], niacin treatment did result in a significant reduction in hepatic CETP gene expression (-20%: P<0.05) and plasma CETP mass (-30%; P<0.001) in *CETP* Tg mice (Fig. 1). Probably due to the low amount of substrate available for CETP action, i.e. relatively low plasma VLDL/LDL levels, and associated low CETP activity already under basal (non-niacin) conditions we did not observe a concomitant decrease in the endogenous plasma CETP activity upon niacin treatment in our *CETP* Tg mouse model (0.35±0.03 mmol/mL/h for niacin vs 0.37±0.03 mmol/mL/h for controls; *P*>0.05).

Gene expression analysis on livers revealed that the relative mRNA expression level of the primary protein moiety of HDL, apolipoprotein A1 (APOA1), was as expected [28] markedly stimulated (+84%; *P*<0.05) by niacin treatment (Fig. 2). Li *et al.* [26] recently showed that activation of the niacin receptor GPR109A diminishes the hepatic expression of ABCA1 and impairs the efflux of cholesterol from hepatocytes to APOA1. In accordance, we also detected a significant decrease in hepatic ABCA1 expression (-45%; *P*<0.05) in mice subjected to niacin treatment (Fig. 2).

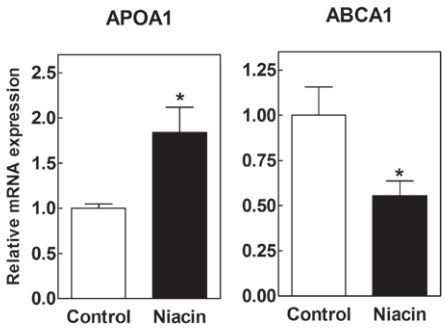


Figure 2. Effect of niacin on HDL-associated gene expression in liver of *CETP* Tg mice. Relative expression levels as fold compared to control of apolipoprotein A1 (APOA1) and ATP-binding cassette transport A1 (ABCA1). Values are means±SEM. *P<0.05.

Niacin treatment does not affect LXR activity in livers of *CETP* Tg mice

As evident from the Oil red O stainings depicted in Fig. 3A, Western-type high cholesterol/high fat diet feeding was associated with the appearance of neutral lipid stores within hepatocytes of control mice. In contrast, livers of niacin-treated mice showed virtually no lipid droplets (Fig. 3A). Quantification of intra-hepatic lipid levels revealed that the effect on neutral lipids stores upon niacin treatment coincided with a

reduction in hepatic total cholesterol levels (-20%; $P<0.01$; Fig. 3B).

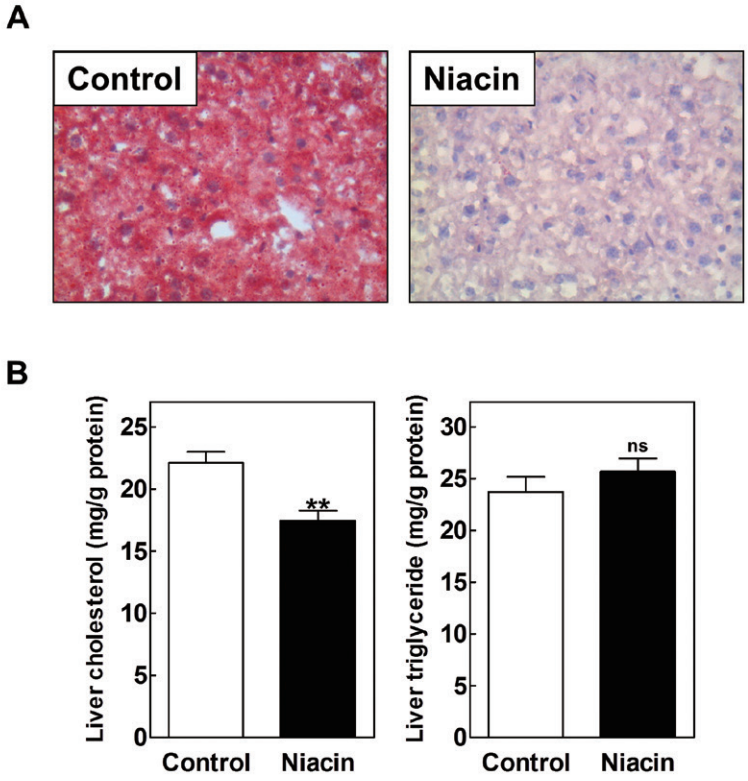


Figure 3. Effect of niacin on hepatic neutral lipid stores in *CETP* Tg mice. (A) Neutral lipid content was visualized by Oil red O staining. (B) Liver cholesterol and triglyceride concentrations corrected for cellular protein content. Values are means±SEM. ns, not significant. **$P<0.01$.

The cholesterol sensor liver X receptor (LXR) is able to directly stimulate CETP and ABCA1 transcription through specific LXR responsive elements in their promoter regions [29, 30]. To evaluate whether niacin decreased hepatic CETP expression and ABCA1 by attenuating LXR activation, we measured the effect of niacin on the expression of the other established LXR target genes SREBP-1C, APOE, and LPL. The hepatic expression of these three genes remained unchanged after niacin treatment (data not shown), indicating that the reduction of hepatic CETP expression was not due to a change in LXR activation upon niacin treatment.

Niacin does not change macrophage CETP expression in vitro

Previous studies using cultured peritoneal macrophages have indicated that niacin at a concentration of 100 μM can directly affect macrophage function by altering their calcium flux [31] or gene expression profile [32] to a similar extent as observed in vivo in mice treated with doses of 0.3-1% niacin. To assess whether niacin directly attenuates CETP expression in macrophages, bone marrow-derived macrophages from *CETP* Tg mice were exposed to various concentrations of niacin (0.1 μM, 1 μM, 10 μM, and 100 μM) for 24 hours. Niacin treatment did not significantly alter CETP expression. Furthermore, niacin did not affect relative mRNA expression levels of the LXR-regulated targets SREBP-1C or APOE, or the cholesterol metabolism-related genes ABCA1, ABCG1, SR-B1, CD36 in cultured bone marrow-derived macrophages (data not shown).

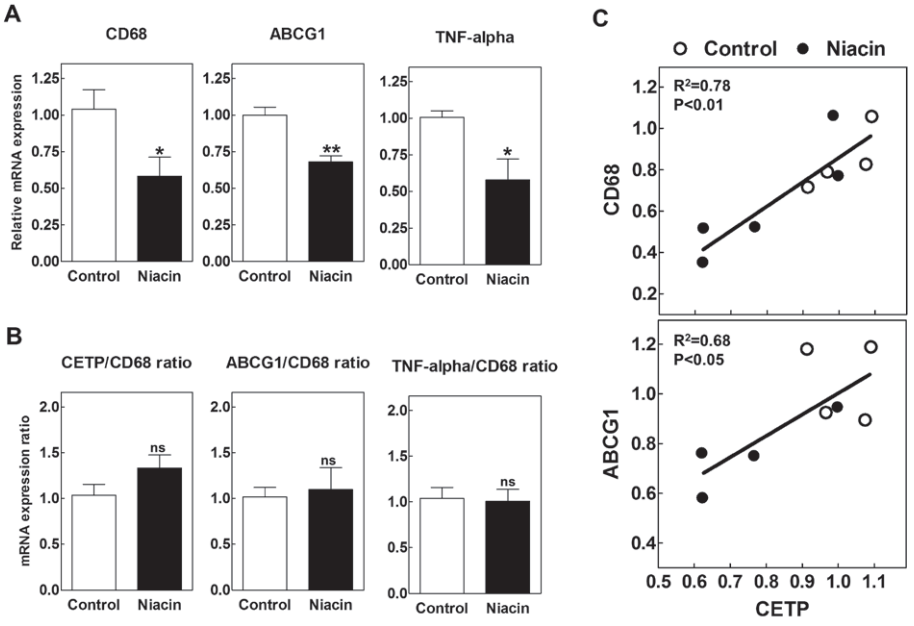


Figure 4. Effect of niacin on hepatic macrophage gene expression in *CETP* Tg mice. (A) Relative expression levels as fold compared to control of macrophage marker CD68, the ATP binding cassette transporter G1 (ABCG1), and tumor necrosis factor-alpha (TNF-alpha). (B) Ratios between the expression level of CETP and CD68, ABCG1 and CD68, TNF-alpha and CD68 were calculated. (C) Correlation between hepatic CETP and CD68/ABCG1 expression was linearly plotted. Values are means±SEM. ns, not significant. **P*<0.05; ***P*<0.01.

Niacin reduces the liver macrophage content in *CETP* Tg mice

Since these data indicate that niacin may reduce hepatic CETP expression by reducing

the liver macrophage content, we evaluated the effect of niacin on the established macrophage markers CD68 and ABCG1 [33-35]. Niacin treatment significantly decreased hepatic expression of CD68 by 44% ($P<0.05$) and ABCG1 by 32% ($P<0.01$) (Fig. 4A). However, niacin did not affect the CETP/ABCG1 and ABCG1/CD68 expression ratios (Fig. 4B). Combined, these findings suggest that niacin - in line with our in vitro data - does not directly reduce the expression of CETP on macrophages, but in fact reduces hepatic CETP expression by diminishing the liver content of (CETP-expressing) macrophages.

In accordance with a decreased liver macrophage content, gene expression of the pro-inflammatory M1 macrophage subtype marker TNF-alpha decreased by 43% ($P<0.05$) after niacin treatment (Fig. 4A). Since the TNF-alpha/CD68 ratio did not change after niacin treatment (Fig. 4B) and the anti-inflammatory M2 macrophage marker interleukin-10 (IL-10) could not be detected in either treatment group (Ct>35; data not shown), it seems that treatment of CETP Tg mice with niacin did not affect the in vivo macrophage phenotype.

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, as evident from Fig. 4C linear regression showed a 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 these results, post-hoc analysis on livers of *APOE*3-Leiden.CETP* mice treated with 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 the macrophage markers CD68 (-51%; $P<0.01$) and ABCG1 (-45%; $P<0.01$) (Fig. 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 (Fig. 5A). 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% ($P<0.01$) (Fig. 5B).

In agreement with a prominent contribution of the liver macrophage-derived CETP to total plasma CETP levels, we observed a significant positive correlation between the plasma CETP level and hepatic CD68 mRNA expression in *CETP* Tg mice treated with niacin ($P<0.05$; Supplemental Fig. 1A) and between the plasma CETP level and the number of macrophages in livers of *APOE*3-Leiden.CETP* mice treated with niacin ($P<0.05$; Supplemental Fig. 1B).

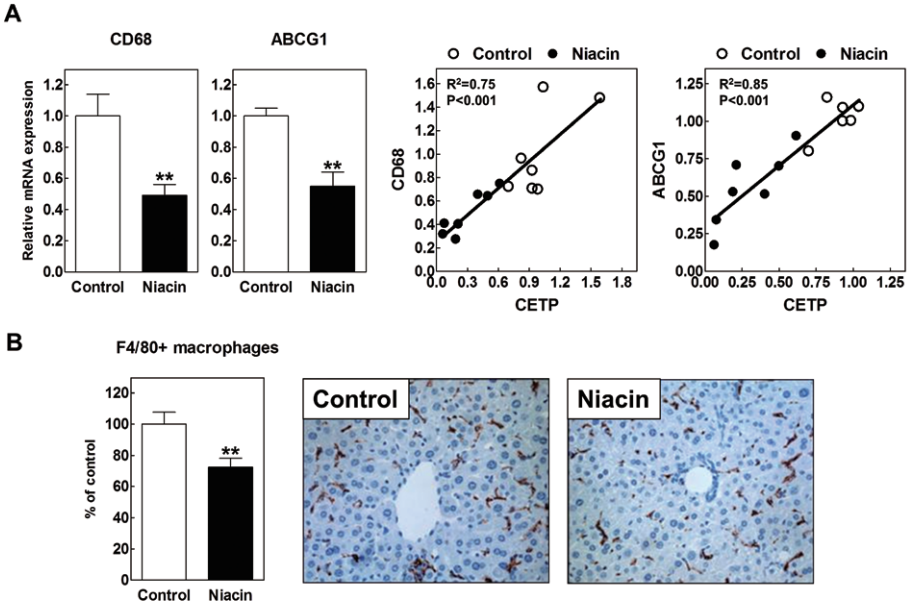
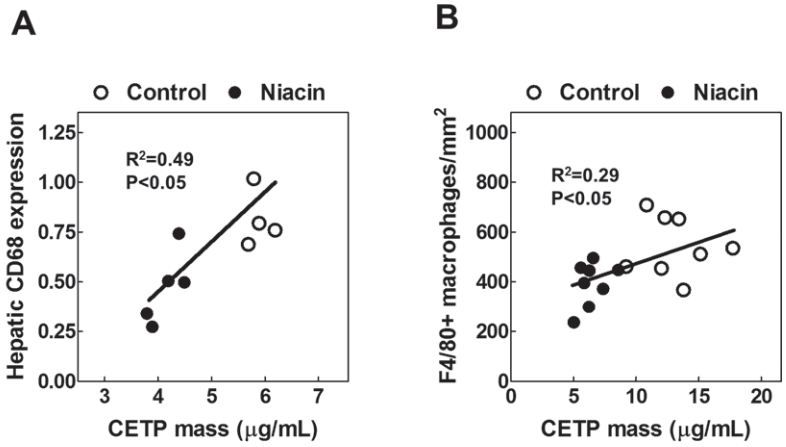


Figure 5. Effect of niacin on hepatic macrophage gene expression and number of macrophages in *APOE*3-Leiden.CETP* mice. (A) Relative expression levels as fold compared to control of macrophage marker CD68 and the ATP binding cassette transporter G1 (ABCG1). Correlation between hepatic cholesteryl ester transfer protein (CETP) and CD68/ABCG1 expression 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. Values are means±SEM. ***P*<0.01.



Supplemental Figure 1. Correlations between the plasma CETP level and hepatic CD68 mRNA expression in *CETP* Tg mice treated with 2% niacin (A) and the plasma CETP level and the number of macrophages in livers of *APOE*3-Leiden.CETP* mice treated with 1% niacin (B).

Niacin does not induce apparent liver toxicity in *CETP* Tg mice

Mild hepatic toxicity is a known side-effect of high dose niacin treatment in humans [36]. In addition, a case of severe liver toxicity, i.e. fulminant hepatic failure, upon niacin treatment has been reported [37]. We therefore evaluated possible hepatotoxic effects of niacin treatment in the current study. The liver structure of *CETP* Tg mice fed the diet without or with niacin appeared normal. In addition, no TUNEL-positive apoptotic cells were noted in livers of either treatment group (Fig. 6A). In fact, niacin decreased the hepatic mRNA expression level of the pro-apoptotic molecule C/EBP homologous protein (CHOP; -66%; $P<0.01$; Fig. 6B) that is highly sensitive to endoplasmatic reticulum stress [38]. Combined, these findings suggest that niacin may actually diminish hepatotoxicity. In line with an overall lower hepatic stress level upon niacin exposure, plasma aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) levels both tended to decrease ($P=0.08$ for both) in *APOE*3-Leiden.CETP* mice subjected to niacin treatment (Fig. 6C).

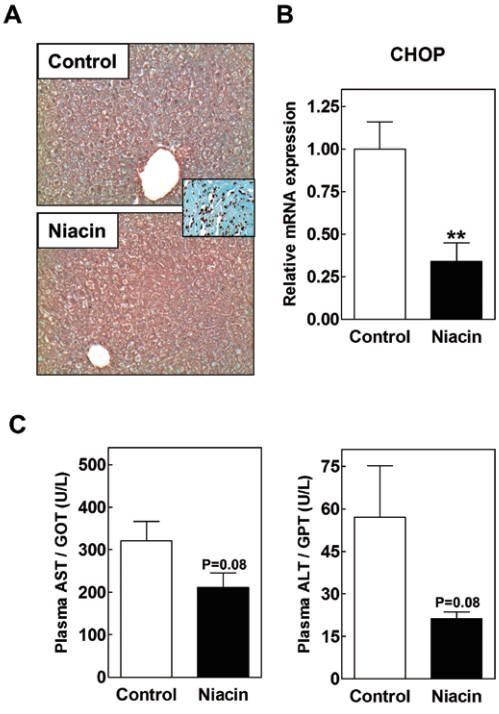


Figure 6. Effect of niacin on liver toxicity in *CETP* Tg mice and *APOE*3-Leiden.CETP* mice. (A) No TUNEL-positive apoptotic cells could be detected in livers *CETP* Tg mice treated with or without niacin, while our positive control (parallel stained vain graft material) did show TUNEL-positive staining (black nuclei; inset). (B) Relative expression levels as fold compared to control of C/EBP homologous protein (CHOP) in *CETP* Tg mice treated with niacin. (C) Plasma aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) in *APOE*3-Leiden.CETP* mice treated with niacin. Values are means±SEM. **$P<0.01$.

Niacin modulates white adipose tissue gene expression and lipids in *CETP* Tg mice

Niacin executes its primary lipid lowering action in adipocytes within white adipose tissue, where it via GPR109a-mediated modulation of intracellular signalling pathways inhibits lipolysis by decreasing the activity of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [39]. We did not detect a change in the relative mRNA expression level of ATGL and HSL in abdominal white adipose tissue of niacin-treated mice, excluding a direct transcriptional effect of niacin on the ATGL-HSL axis (Fig. 7A). In contrast to what one would expect in response to the diminished lipolytic activity, a marked decrease in the white adipose tissue triglyceride content (-70%; $P<0.01$; Fig. 7B) was noted upon niacin treatment. However, in agreement with similar observations in patients with impaired glucose tolerance treated with extended release niacin [40], a significant decrease (-38%; $P<0.01$) in the white adipose tissue expression of fatty acid synthase (FAS) was also observed upon niacin exposure (Fig. 7A). Since we subjected the mice to an overnight fast, both groups of mice contained low levels of abdominal white adipose tissue at sacrifice. As a result, we did not see an apparent change in the body weight of niacin-treated mice (data not shown).

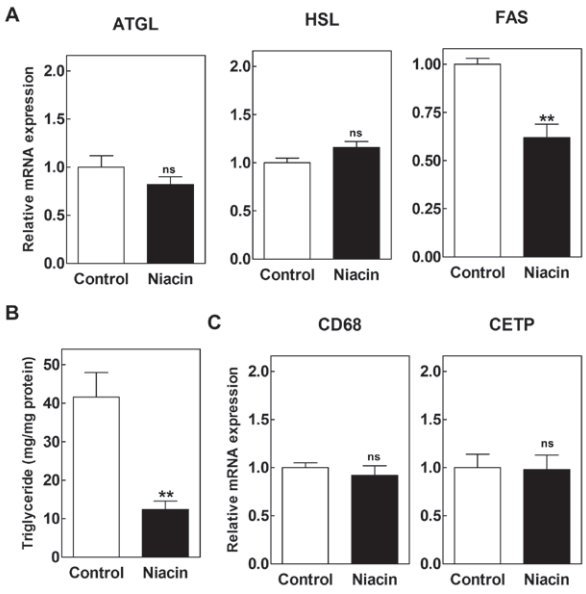


Figure 7. Effect of niacin on abdominal white adipose tissue triglyceride content and gene expression in *CETP* Tg mice. (A) Relative expression levels as fold compared to control of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and fatty acid synthase (FAS). (B) White adipose tissue triglyceride concentration corrected for cellular protein content. (C) Relative expression levels as fold compared to control of macrophage marker CD68 and CETP. Values are means±SEM. ns, not significant. $^{**}P<0.01$.

The relative expression levels of CD68 and CETP in abdominal white adipose tissue were unaffected by niacin treatment (Fig. 7C). This suggests that the effect of niacin treatment on plasma CETP levels can be attributed to attenuation of the macrophage-derived CETP expression specifically in the liver and argues against a niacin-induced general cytotoxic (i.e. apoptotic/necrotic) effect on macrophages in vivo.

DISCUSSION

Niacin lowers plasma CETP levels both in mice [6] as well as in the human clinical setting (unpublished data; Chapman et al.). However, the mechanism behind the niacin-induced decrease in CETP levels has thus far not been delineated.

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 in macrophages, 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.

Luo *et al.* [29] have previously demonstrated that CETP is trans-activated by nuclear receptor LXR, suggesting its role in regulating CETP expression in vivo. Therefore, we proposed in our previous study that niacin may decrease the hepatic CETP mRNA expression via LXR responsive element in the CETP promoter following decreased hepatic cholesterol content [6]. However, our current showed that niacin did not directly regulate expression of LXR-regulated target genes, such as ABCA1, in cultured macrophages in vitro. Our in vivo data further confirmed that niacin did not regulate the hepatic expression of classical LXR targets such as SREBP-1C, APOE, or LPL. 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 reduce the relative expression level of ABCG1 per macrophage. 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 [41]. Thus, liver macrophages have profound implications in many aspects of the hepatic inflammatory response [42]. 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, upregulation of hepatic expression of CD68 was found associated with increased hepatic lipid content [43]. Another study showed that in the LDL receptor knockout mice fed a high fat diet containing cholesterol, an increase of CD68 expression in the liver was correlated with increased plasma VLDL cholesterol levels. Omitting cholesterol from the diet rapidly reduced plasma triglyceride and VLDL-cholesterol accumulation, associated with significantly lowered CD68 expression in liver together with other inflammatory genes [44]. 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 [45]. 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 the pro-inflammatory M1 macrophage marker TNF-alpha in liver. TNF-alpha is critically involved in the pathophysiology of liver steatosis, and this cytokine is primarily secreted by Kupffer cells and liver-infiltrating macrophages [46]. 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 [47-49]. ABCG1 has also been shown to be a reliable marker to assess Kupffer cell content in the liver, since ABCG1 is not expressed in hepatocytes [50, 51]. In the current study, a reduction in the hepatic TNF-alpha expression coincided with decreased CD68 and ABCG1 gene expression in liver, and also a reduced number of macrophages in liver, indicating an attenuated macrophage infiltration into the liver and/or an increased macrophage efflux/emigration from the liver and thus an overall 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 [6] 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.

Fig. 8 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 efflux/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.

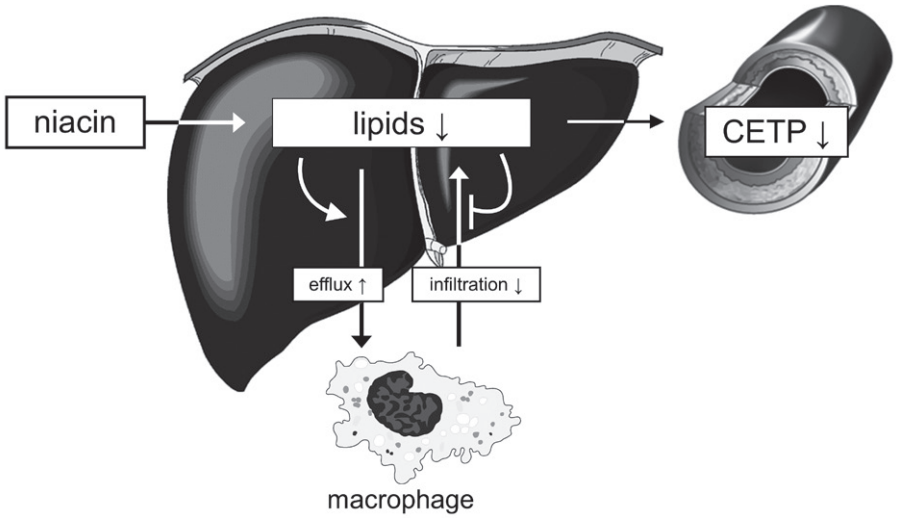


Figure 8. Proposed mechanism underlying the action of niacin on hepatic CETP expression and plasma CETP mass. We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated hepatic inflammation, and thus less macrophage infiltration 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. 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. These findings further substantiate our working hypothesis that CETP in plasma is primarily derived from bone marrow-derived cells, i.e. macrophages.

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