Nicotinic acid inhibits hepatic *APOA* gene expression: studies in humans and in transgenic mice[S]

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Abstract Elevated plasma lipoprotein(a) (LPA) levels are recognized as an independent risk factor for cardiovascular diseases. Our knowledge on LPA metabolism is incomplete, which makes it difficult to develop LPA-lowering medications. Nicotinic acid (NA) is the main drug recommended for the treatment of patients with increased plasma LPA concentrations. The mechanism of NA in lowering LPA is virtually unknown. To study this mechanism, we treated transgenic (tg) *APOA* mice with NA and measured plasma APOA and hepatic mRNA levels. In addition, mouse and human primary hepatocytes were incubated with NA, and the expression of APOA was followed. Feeding 1% NA reduced plasma APOA and hepatic expression of *APOA* in tg-*APOA* mice. Experiments with cultured human and mouse primary hepatocytes in addition to reporter assays performed in HepG2 cells revealed that NA suppresses APOA transcription. The region between −1446 and −857 of the human APOA promoter harboring several cAMP response element binding sites conferred the negative effect of NA. In accordance, cAMP stimulated APOA transcription, and NA reduced hepatic cAMP levels. It is suggested that cAMP signaling might be involved in reducing *APOA* transcription, which leads to the lowering of plasma LPA.—Chennamsetty, I., K. M. Kostner, T. Claudel, M. Vinod, S. Frank, T. S. Weiss, M. Trauner, and G. M. Kostner. **Nicotinic acid inhibits hepatic *APOA* gene expression: studies in humans and in transgenic mice. *J. Lipid Res.* 2012.** 53: **2405–2412.**

Supplementary key words primary human hepatocytes • reporter assay • apolipoprotein(a) • mRNA expression • atherosclerosis

There is mounting evidence that elevated plasma LPA levels contribute significantly to the incidence of cardiovascular disease (1). LPA belongs to the apoB-100-containing lipoproteins consisting of an LDL-like core particle linked to the glycoprotein apolipoprotein(a) (APOA) by a disulfide bridge. APOA is a large polymorphic glycoprotein with a molecular mass of 350–700 kDa that exhibits a striking structure homology to plasminogen (2). APOA has multiple kringle-4-like repeats that reflect its pronounced size heterogeneity (3). Plasma LPA concentrations range from <1 to >100 mg/dl and are to >90% genetically determined. The number of kringle-4 repeats correlates negatively with plasma LPA levels, accounting for approximately 50% of the inheritance (4). Despite intensive research, the physiological function of LPA remains elusive.

APOA is almost exclusively synthesized in the liver. Although LPA shares structural similarities with LDL, these two lipoproteins are differentially metabolized. Unlike LDL, LPA does not directly originate from VLDL but is likely assembled at the surface of hepatocytes or in circulating blood from APOA and LDL (5, 6). There are, however, studies supporting an intracellular assembly of LPA (7). Turnover studies in humans revealed that the rate of APOA biosynthesis greatly determines plasma LPA levels, whereas LPA catabolism appears to play only a minor role (8, 9).

Due to its high atherogenicity, numerous studies have been conducted to find drugs that lower plasma LPA. Unfortunately, most of the lipid-lowering drugs have little or inconsistent effects on LPA, and a specific LPA-lowering medication does not exist except for apheresis (reviewed in Ref. 10). Nicotinic acid (niacin) (NA) has been shown

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Abbreviations: FCS, fetal calf serum; LPA, lipoprotein(a); APOA, apolipoprotein(a); NA, nicotinic acid; cAMP, cyclic adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure.

to significantly reduce plasma LPA (11), but the mechanism of how it lowers LPA is unknown (12, 13). (NA) also has pleiotropic effects and reduces not only triglycerides and APOB-containing lipoproteins but also increases APOA-1 and HDL (14, 15). The molecular mechanisms of these effects on plasma lipids and HDL are beginning to unravel, yet the mode of action on LPA is unknown.

We have recently shown that a pathological increase of plasma bile acids strongly negatively correlates with plasma LPA and vice versa (16). Bile acids have a dual effect on APOA expression. One pathway is mediated by a HNF4α binding site at –826 to −814 of the APOA promoter that is competitively inhibited by activated FXR (16). The other mechanism is mediated by FXR stimulation of FGF-19 expression in the intestine that binds to FGFR4 on liver cells and in turn down-regulates APOA expression by MAPK-ERK1/2 signaling (17). In the present study, none of these pathways related to the action of NA on LPA. We provide evidence that NA reduces plasma concentrations of APOA by more than 50%in transgenic mice whose APOA expression is under the control of the genuine human promoter. In cultured primary hepatocytes from mice and humans, *APOA* mRNA was reduced by 30–40% by NA treatment. Luciferase reporter assays revealed that the observed effect of NA on *APOA* transcription is mediated through a segment between −1446 and −857 bp in the APOA promoter that contains binding sites for several regulatory elements involved in lipid metabolism.

MATERIALS AND METHODS

Chemicals

NA, 8-Br-cAMP, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (Vienna, Austria). cAMP assay kit was purchased from R&D systems (Vienna, Austria). Collagenase was from Worthington Corp. (Harrison, NJ).

Animal experiments

The research was conducted in conformity with the Public Health Service policy, and all animal experiments were performed after approval of the protocol by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments (Vienna, Austria). Transgenic *APOA* (tg-*APOA*) mice generated by Frazer et al. (18) carried a 110kb human *APOA* gene surrounded by more than 60 kb 5′- and 3′-flanking DNA in the YAC controlled by its native promoter and were the same as used in previous experiments (16, 17). Mice were hosted under a standard 12 h light/12 h dark cycle and fed standard rodent chow diet and water ad libitum. Female mice, between 10 and 12 weeks old, were used in all the experiments. For feeding studies, tg-*APOA* ($n = 10$) expressing the human APOA were divided into two groups (n = 6 per group). Animals were randomized based on plasma APOA levels. One group received a normal rodent chow diet (control), whereas the other group received the same diet supplemented with 10 g of NA per kilogram of chow for 2 weeks. Mice were fasted for 4 h before blood samples were collected. Liver samples were harvested for further analysis.

Cell cultures

Primary hepatocytes from tg-*APOA* mice were prepared and cultured as described previously (19), with minor modifications. The mouse liver was perfused with collagenase solution, and liver cells were collected. After filtration and centrifugation, the isolated hepatocytes were resuspended in DMEM (Invitrogen) supplemented with 20% (v/v) fetal calf serum (FCS) (Sigma-Aldrich), 100 units/ml penicillin, and 100 units/ml streptomycin and placed in 6-well, collagen-coated plates (BD Biosciences) at a density of 8×10^5 cells/well at 37°C in an atmosphere of 5% CO_2 for 4 h. Thereafter, cells were cultured in DMEM supplemented with 10% FCS and 100 units/ml penicillin/streptomycin for 24 h. Further experiments were performed in serum-free DMEM supplemented with various concentrations of NA (Sigma-Aldrich). The HepG2 cells were obtained from ATCC and were maintained in DMEM containing 10% FCS and 100 units/ml penicillin/ streptomycin.

PHH cultures

Non-neoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state-controlled foundation HTCR (Human Tissue and Cell Research), with the informed consent of patients. The experiments adhered to the principles of the Declaration of Helsinki as well as to Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects and were approved by the local ethical committee of the University of Regensburg. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (20). Viability of isolated hepatocytes was determined by trypan blue exclusion and cells with a viability > 85% were used for further work. Cells were plated on collagen-coated plates (BD Biosciences) at a density of 1.2×10^5 cells/cm^2 in an appropriate volume of culture media. The medium consisted of DMEM with 10% FCS, 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin, and supplements as follows: 125 mU/ml insulin, 7.3 ng/ml glucagon, and 0.8 µg/ml hydrocortisone. After 16 h of plating, medium was replaced by FCS-free DMEM without supplements. Cells were incubated at 37°C in a humidified incubator with 5% CO_2, and media were changed daily except otherwise stated. Viability of hepatocytes during the culture period was monitored by cell morphology (light microscopy, image analysis) and determination of enzyme release into culture medium (AST activity). Further experimental details are shown in the figures legends.

Cyclic AMP ELISA

cAMP levels were measured using the Cyclic AMP Assay kit from R&D Systems according to the manufacturer's protocol. Cells were treated for 15 min with 30 µM forskolin ± 1 mM IBMX before 1 h exposure to NA. The cAMP levels were calculated using a standard curve as per the protocol. Data represent mean ± SEM.

Plasma lipid parameters in mice

Blood was collected by retro-orbital bleeding, and EDTA plasma was harvested. Plasma concentrations of APOA were measured enzymatically by an in-house DELFIA method (21). Plasma triglyceride (DiaSys) and total cholesterol concentrations (Greiner Diagnostics AG) were determined enzymatically according to the manufacturer's protocols. Plasma samples from six mice of each group were pooled and used for lipoprotein separation by fast protein liquid chromatography (FPLC) using the Pharmacia P-500 FPLC System and a Superose 6 column (Amersham Biosciences, Piscataway, NJ).

RNA extraction, reverse transcription, and real-time PCR

Total RNA from cells and mouse tissues was isolated using TrIzol (Invitrogen) according to the manufacturer's protocol.

Two micrograms of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed on a Light Cycler 480 instrument (Roche Diagnostics), using the Quanti Fast SYBR Green PCR Kit (Qiagen). Primer sequences were identical to those published (16). The gene expression values were normalized to cyclophilin A (*Ppia*) as a housekeeping gene. The data were analyzed by the public domain program Relative Expression Software Tool (22). Values are presented as mean ± SEM.

Protein extraction and immunoblotting

Livers were homogenized, or cells were lysed in ice-cold RIPA buffer. The lysates were centrifuged (12,000 *g*) at 4°C for 10 min, and the supernatant was collected. Protein was quantitated using the Bradford protein assay (Bio-Rad). Equivalent amounts of protein homogenates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antibodies to human APOA (1:1,250) and a monoclonal anti-mouse β-actin (1:2,000) (Santa Cruz Biotechnology Inc.). The immunoblots were visualized by the Pierce ECL Chemiluminescence Detection System (Thermo Scientific).

Plasmids

Expression plasmids encoding the human *APOA* promoter construct (h*APOA* –1,952/+52) was obtained by PCR amplification using human genomic DNA as a template. The PCR product was cloned into the pGL3 basic vector (Promega) as a *Mlu*I/*Bgl*II fragment to generate human *APOA*-Luc. The primers used are as follows: for forward reaction, 5′-ACGCGTTCTGAGAGGGAGGTCAAAGTTTTC-3′; for reverse reaction, 5′-AGATCTCTTGAGAAAGCCAGCCCCAAAGGT-3′. The 5′ promoter deletion constructs were the same as described in detail previously (16). All constructs were verified by DNA sequencing (LGC Genomics).

Transient transfection and reporter gene assays

Reporter gene assays were performed in HepG2 cells. Cells at 60–70% confluency were transiently transfected with the indicated reporter or expression plasmids using FuGENE® 6 reagent as described previously (16).

Statistics

Statistical analyses of the experiments were performed with GraphPad Prism 5.0. Two-tailed, unpaired Student's *t*-test was applied to determine statistical significance (*** $P \leq 0.001$; ** $P \leq 0.01$; * $P < 0.05$).

RESULTS

NA reduces plasma APOA levels and mRNA expression in the liver in tg-APOA mice

To verify the validity of our mouse model for studying the NA-lowering effect on LPA, six tg-*APOA* mice were fed for 2 weeks with normal chow diet (control) or chow diet supplemented with 1% (wt/wt) NA. Supplementation with 1% NA resulted in significantly decreased plasma APOA levels by 43% (**Fig. 1A**) and hepatic APOA mRNA by 65% (Fig. 1B) as compared with controls. Western blot analysis of liver homogenates confirmed that this reduction also occurs at the protein level upon NA feeding in tg-*APOA* mice (Fig. 1C). No changes in body weight or food intake were observed between the control and treated groups (supplementary Fig. I). FPLC analysis revealed that the major reduction by NA was found in the LDL peak, whereas VLDL and HDL remained virtually the same (**Fig. 2A**). NA feeding significantly reduced plasma total cholesterol by 20 ± 6% and triglycerides by 24 ± 5% (Fig. 2B, C).

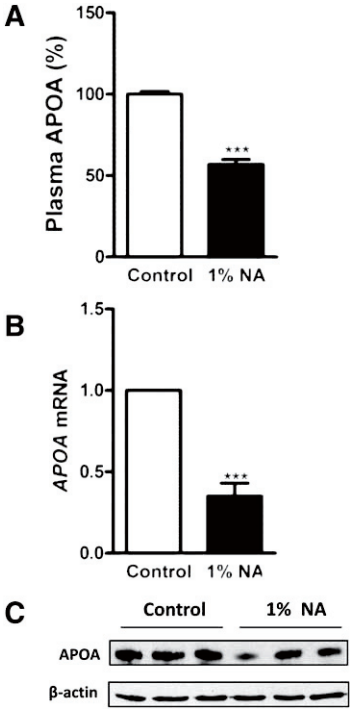

Fig. 1. NA decreases plasma levels and hepatic expression of *APOA* in mice. Tg-*APOA* mice were fed a diet containing 10 g of NA per kg of standard rodent chow (n = 6 per group) for 2 weeks. Control mice (n = 6 per group) received normal rodent chow. A**:** Plasma levels of APOA were measured by DELFIA and expressed as mean ± SD. B: Mouse liver *APOA* mRNA levels were analyzed by real-time quantitative PCR, normalized to cyclophilin, and expressed relative to control mice. Results represent the mean ± SEM (*** $P \leq 0.001$). C: Western blot analysis of APOA levels in the protein extracts from liver tissue of individual mice.

NA acts directly on hepatocytes reducing APOA mRNA abundance

Because NA primarily affects adipocytes and endothelial cells by binding to GPR109A followed by downstream signaling, it was important to address the question of whether the effect described in Fig. 1 might be direct or indirect. To study the effect of NA on human *APOA* gene expression, primary hepatocytes isolated from tg-*APOA* mice were incubated with increasing amounts of NA. Analysis by real-time quantitative PCR revealed a significant dose-dependent decrease of *APOA* mRNA levels (**Fig. 3A, 3B**). Western blot analysis confirmed that this NA-mediated repression also occurs at the protein level in cell lysates (Fig. 3C). Cell viability assessed by trypan blue exclusion test revealed that all concentrations of NA were well tolerated (data not shown).

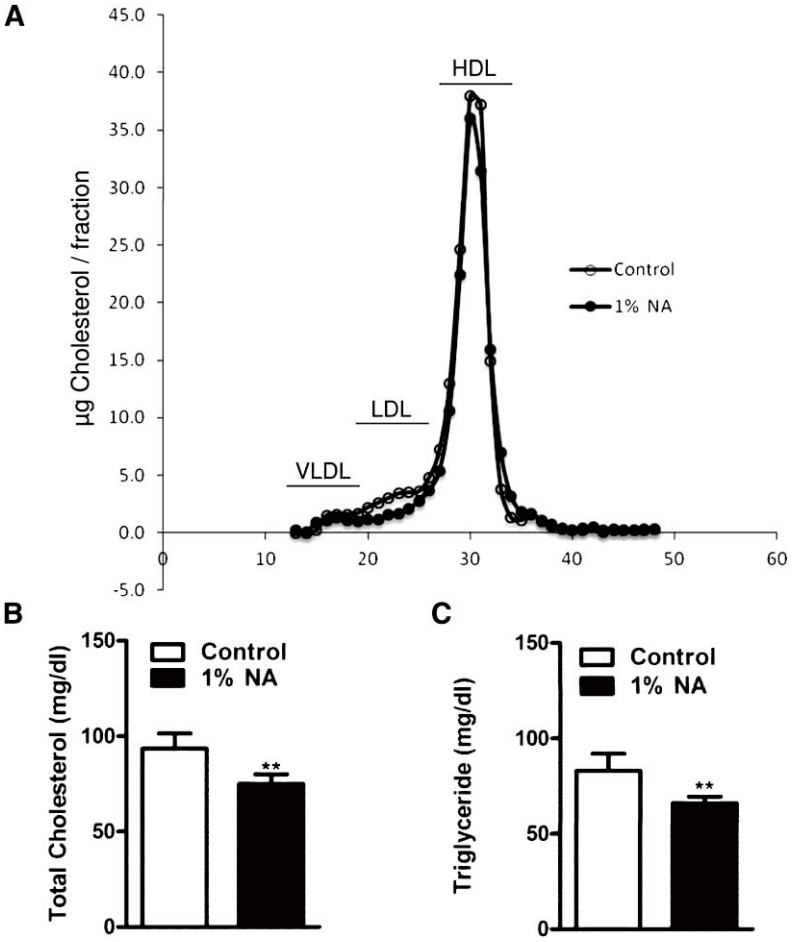

Fig. 2. Profile of plasma lipids and lipoproteins in control and 1% NA-treated mice. Tg-*APOA* mice were fed for 2 weeks a diet containing 10 g of NA per kg of standard rodent chow. A: Plasma from individual mice per group was pooled and subjected to FPLC, and the cholesterol content in each fraction was measured enzymatically. B and C: Total cholesterol and triglycerides levels were measured in plasma after a 4 h fasting period as described in Materials and Methods. Data are presented as mean ± SD (n = 6). ** $P \leq 0.01$ when compared with chow-fed control group.

NA reduces APOA mRNA in primary human hepatocytes

Because the expression of liver genes in mice might be quite different from that of humans, it was important to verify the results obtained in tg-*APOA* mice in the human system. Human hepatocytes demonstrate a greatly variable expression of APOA, dependent on the genotype of the donor that cannot be freely chosen for ethical reasons. The access to PHH is limited; therefore, only few control experiments could be performed in PHH. Most importantly, we observed a concentration-dependent reduction in *APOA* mRNA in PHH between 18% and 42% at NA concentrations ranging from 50 to 200 µM (**Fig. 4**).

Mapping the promoter region conferring the NA inhibition of APOA expression

To provide direct evidence for the NA-mediated inhibitory effect on *APOA* promoter and to further identify relevant promoter element(s), a 2 kb fragment of human *APOA* promoter (h*APOA* −1,952/+52) was cloned into a pGL3-luciferase reporter plasmid. In addition, a series of 5′ deletion constructs were generated (**Fig. 5A**).

HepG2 cells were transiently transfected with 5′ deletion constructs of the human *APOA* promoter in the absence or presence of NA (200 µM). Reduced promoter activities were noted for the −1952 and −1446 constructs by 49% and 44%, respectively (Fig. 5B). However, the repression was relieved for the −857, −757, −477, and −148 promoter constructs, indicating that the region between −1446 and −857 of the human *APOA* promoter contains a potential negative response element, which might be responsible for the observed NA effect.

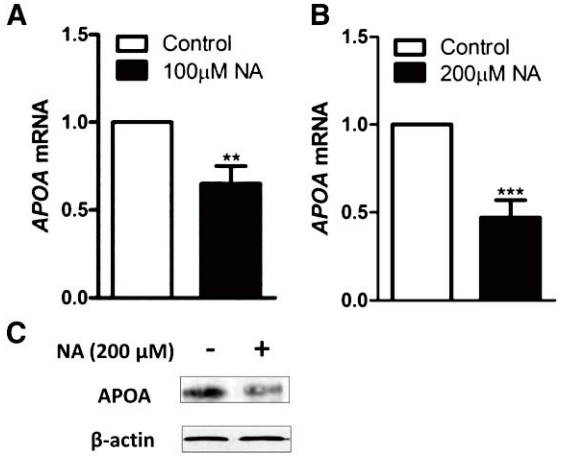

Fig. 3. NA represses *APOA* gene expression in a dose-dependent manner in primary mouse hepatocytes. A and B: Primary mouse hepatocytes from tg-*APOA* mice were incubated with increasing concentrations of NA (100 and 200 µM) or vehicle (control) for 24 h. mRNA levels of *APOA* were analyzed by real-time quantitative PCR. Results represent the mean ± SEM of three independent experiments (*** $P \leq 0.001$; ** $P \leq 0.01$). C: Western blotting analysis of APOA expression in whole cell lysates from hepatocytes treated for 24 h with NA.

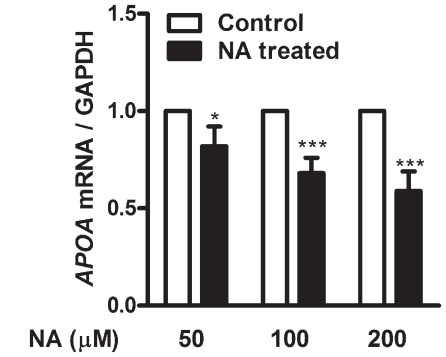

Fig. 4. NA down-regulates *APOA* gene expression in primary human hepatocytes. Human primary hepatocytes were treated with increasing concentrations of NA (50, 100, and 200 μM) or vehicle (control) for 24 h. mRNA levels of *APOA* were analyzed by real-time quantitative PCR. Results represent the mean ± SEM of three independent experiments (*** $P \leq 0.001$; * $P < 0.05$).

The APOA promoter at nt −1446 to −857 harbors four cAMP-responsive element binding sites

In silico Mat-Inspector promoter analysis using the Genomatix® database revealed 173 matches of possible high-affinity binding sites for transcriptional regulators (supplementary Table I). Among numerous potential response elements that are abundant also in other genes involved in lipid metabolism, we found several cAMP-responsive element binding sites in this promoter segment. This prompted us to study a possible cAMP effect on APOA expression in more detail.

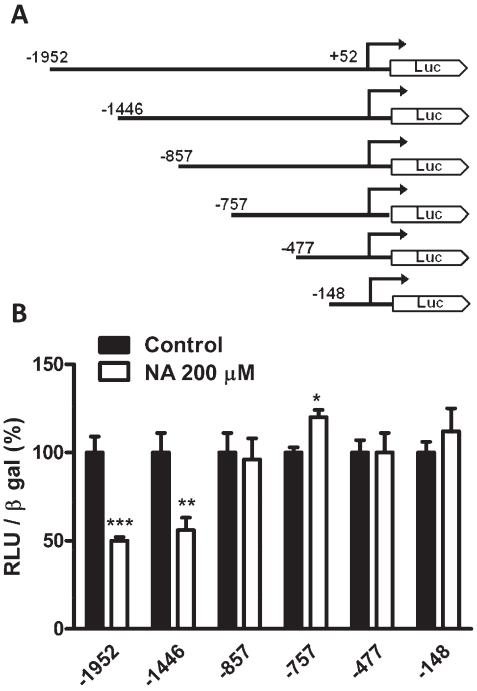

Fig. 5. NA down-regulates human *APOA* promoter activity in HepG2 cells. A: Scheme of the deletion constructs of the h*APOA* promoter used in the luciferase reporter assay. B: HepG2 cells were transfected with the indicated h*APOA* promoter reporter plasmids (150 ng). Cells were subsequently treated for 36 h with vehicle or with NA (200 μM) in serum-free DMEM. Transfections were performed in triplicates, and each experiment was repeated at least three times. Values are normalized to internal control β-galactosidase activity and expressed in percentage. Data are presented as mean ± SD (*** $P \leq 0.001$; ** $P \leq 0.01$). RLU, relative light units.

APOA transcription is influenced by cAMP

To examine the involvement of cAMP in the NA effect on APOA reduction, we treated primary mouse hepatocytes with the stable analog of cAMP, 8Br-cAMP, and followed the expression of APOA. 8Br-cAMP induced APOA expression in a dose-dependent manner (**Fig. 6A**). As a positive control, we measured the expression levels of Pepck. 8Br-cAMP strongly induced Pepck mRNA levels (Fig. 6B). The incubation of primary hepatocytes from tg-*APOA* mice with forskolin, a generally used inducer of adenylat cyclase together with IBMX, a phosphodiesterase inhibitor revealed a 3.7-fold elevation of *APOA* mRNA (Fig. 6C) when compared with vehicle-treated control cells.

To study the effect of cAMP on human *APOA* promoter activity, HepG2 cells were transfected with the full-length h*APOA* −1,952/+52 promoter reporter plasmid in the absence or presence of 8Br-cAMP. Incubation with 8Br-cAMP significantly increased the activity of the h*APOA* −1,952/+52 promoter (Fig. 6D).

NA reduces liver cAMP levels mainly by reducing cAMP production

To address the mode of action of NA on cAMP levels, primary hepatocytes from tg-*APOA* mice were incubated with forskolin with or without NA in the presence and absence of the phosphodiesterase inhibitor IBMX. Comparable effects of NA on cAMP levels were seen irrespective of the inhibition of cAMP degradation by IBMX (**Fig. 7**). From these experiments, we concluded that the main effect of NA on cAMP levels is due to interfering with cAMP production.

Taken together, our results demonstrate that cAMP significantly elevates *APOA* mRNA in hepatocytes from tg-*APOA* mice and that NA reduces intracellular cAMP levels in the liver, thereby possibly interfering with APOA transcription.

DISCUSSION

LPA has been suggested for a long time to be linked to cardiovascular diseases (23, 24), yet its role as a causal factor has been documented only in recent epidemiological trials (1, 25–28). There are very few drugs on the market that can be recommended for treatment of patients with elevated LPA, and the development of new medications is held back by the gaps in understanding its biosynthesis and catabolism. Many of the treatment regimes that have been recommended in the past show a low efficacy or are accompanied by adverse effects (10, 29). Optimal cardiovascular risk reduction in patients with coronary heart disease requires integrated pharmacotherapy to normalize all classes of lipoproteins, including LPA (30). NA or the derivatives thereof are considered to be good candidates to fulfill such a task because they not only lower plasma

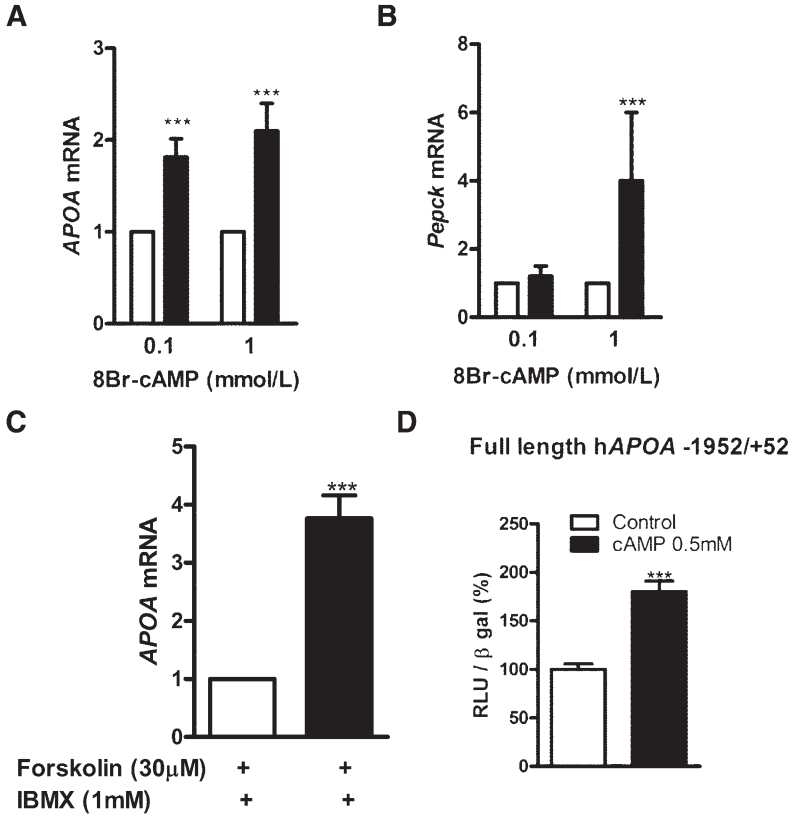

Fig. 6. 8Br-cAMP increases the expression of human *APOA*. Primary hepatocytes from tg-*APOA* mice were treated with 8Br-cAMP at the concentrations indicated for 24 h. mRNA levels of (A) *APOA* and (B) *Pepck* were analyzed by real-time quantitative PCR. C: Primary hepatocytes from tg-*APOA* mice were treated with forskolin (30 μM) and IBMX (1 mM) for 24 h. mRNA levels of *APOA* were analyzed by real-time quantitative PCR. Results represent the mean ± SEM of three independent experiments (*** $P \leq 0.001$; * $P < 0.05$). D: HepG2 cells were transfected with the full-length h*APOA* promoter reporter plasmid (150 ng). Cells were subsequently treated for 36 h with vehicle or with 8-Br-cAMP (0.5 mM) in serum-free DMEM. Data are presented as mean ± SD (*** $P \leq 0.001$). RLU, relative light units.

triglycerides and cholesterol but also are one of the most effective drugs in raising HDL. In addition, numerous trials have shown NA to significantly reduce plasma LPA (11–14, 29–32).

NA has pleiotropic effects far beyond its action on lipoproteins (33, 34). It affects the metabolism of numerous tissues, including endothelial cells, macrophages, adipose tissue, and liver, and in theory is suitable for the management of lipid abnormalities in type 2 diabetes mellitus and metabolic syndrome (35). With respect to its use as an antiatherogenic drug, NA is characterized by anti-inflammatory effects that are mediated by binding to its receptor GPR109A, which is expressed in adipocytes and some leukocytes (36). In a recent report, it has been also shown that NA induces the expression of heme oxigenase-1 by activating Nrf2 and the p38-MAPK signaling cascade (37). Heme oxigenase-1 catalyzes the formation of bilirubin, a very effective natural antioxidant. Nicotinic acid was shown to reduce lipoprotein associated phospholipase-A2 (38), an enzyme that has a high affinity for LPA (39).

The action of NA on plasma lipoproteins is highlighted in several review articles (30, 40, 41). Among the numerous suggested effects, NA reduces peripheral lipolysis of triglycerides, the flux of free fatty acids to the liver, and in turn the biosynthesis of VLDL. It activates the transcription of apoAI and the activity of lipoprotein lipase and reduces the biosynthesis of triglycerides in the liver. However, little is known about the mechanism causing the reduction of plasma LPA concentrations, and there are only speculations on the possible mode of action. One plausible mechanism that is not proven may relate to the interference of NA with the assembly of apoB-containing lipoproteins, as reported previously (40). In this study, we asked whether NA might directly interfere with APOA transcription.

Because such studies for ethical reasons are difficult to perform in humans in vivo, we first addressed the question whether NA might be active also in our tg-*APOA* animal model. Feeding tg-*APOA* mice with a 1% NA-containing diet for 1 week reduced APOA protein in plasma and mRNA in the liver by 53% and 59%, respectively (Fig.1). These mice do not form LPA lipoproteins because mouse apoB100 does not bind covalently to human APOA (42).

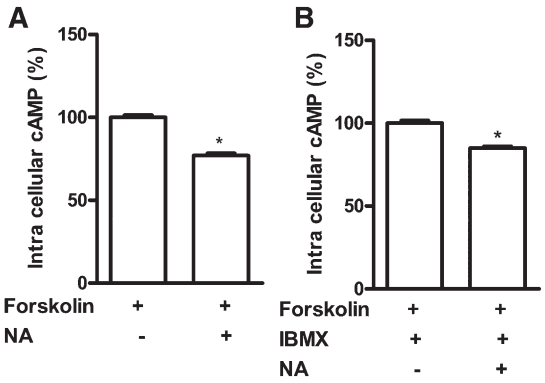

Fig. 7. Effect of nicotinic acid on cellular cAMP. Primary hepatocytes from tg-*APOA* mice were treated with 200 μM NA in presence or absence of forskolin (A) and forskolin with the phosphodiesterase inhibitor IBMX (B). The amount of cAMP present was calculated as described in Materials and Methods. Results represent the mean ± SEM of three independent experiments (*$P < 0.05$).

Accordingly, an interference of NA with APOA assembly as a major mechanism was ruled out. Next, in vitro experiments with cultured hepatocytes from tg-*APOA* mice and from human liver resections were performed, where reductions of *APOA* mRNA abundance up to 52% were found. This led us to conclude that NA acts directly on the liver and that the APOA-lowering effect might not be caused by secondary factors to a major extent. It was important to verify a direct effect of NA also on human liver because the expression particularly of liver specific genes in humans might deviate quite significantly from that of other mammalian species (43). It could not be ruled out that relevant regulatory sequences might be present outside the DNA fragment that had been inserted into the YAC clone for the production of the tg-*APOA* mouse (44). NA reduced APOA mRNA levels also in PHH in a concentration-dependent manner (Fig.4).

To get a closer look at the mechanism of NA action, luciferase reporter assays with constructs containing the APOA promoter were carried out. With the 2 kB reporter construct that had been used in previous studies (16, 17), we found a 50% reduction of activity. A comparable reduction was also demonstrated with the truncated reporter of 1446 nt but not with the constructs shorter than 857 nt. We therefore concluded that the responsible sequence for the NA effect is situated between −1446 to −857 nt upstream the transcription start side. In silico Mat-Inspector promoter analysis (45) using the Genomatix® database revealed 173 matches of potential high-affinity binding sites for transcriptional regulators. We observed response elements for RXR/FXR, several CCAAT/enhancer binding protein, HNF1, glucocorticoid receptor, SREBP-1 and -2, and four cAMP response elements in addition to others shown in supplementary Table I. Because it was impossible to test the role of all of them for the NA effect, we focused on the possible role of cAMP because it had been shown previously that NA reduces cAMP in the liver (46). cAMP stimulates HNF4α-phosphorylation, which has a lower DNA binding activity and may reduce transcriptional activation (47). We identified previously a DR-1 binding site for HNF4α (16), yet this DR-1 is situated at −830 to −816 in the APOA promoter, the sequence found not to be responsive to NA (Fig. 5). Incubation of primary mouse hepatocytes with 8Br-cAMP increased *APOA* mRNA abundance in a dose-dependent manner (Fig. 6A). When hepatocytes from tg-*APOA* mice were incubated with the cAMP inducer forskolin + IBMX, a 3.7-fold increase of APOA mRNA was observed (Fig. 6C). This was due to an elevated activity of the APOA promoter (Fig. 6D). Because the reducing effect of NA on cellular cAMP was comparable irrespective of the presence of IBMX (Fig. 7), we suggest that NA interferes with cAMP production but not with increasing cAMP degradation by PDE. Further studies are necessary to clarify the molecular mechanism of NA action in detail.

In summary, we show here for the first time that NA acts directly on liver cells and down-regulates *APOA* transcription. Mechanistically, the promoter segment –1,446 to –857 is involved, where, among 173 binding sites for transcriptional regulators, four cAMP response element binding sites have been found. Because cAMP strongly increases *APOA* mRNA in liver of tg-*APOA* mice and NA reduces the cellular cAMP content, we suggest that this mechanism might be responsible for the lowering effect of NA on LPA. Our results do not exclude the possibility that other mechanisms might act in parallel. Further work is necessary to identify the response element(s) involved and to clarify the molecular mechanism in detail. This may help to design medications for the treatment of patients with cardiovascular disease with elevated plasma LPA.

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