



Modulation of HDL metabolism by the niacin receptor GPR109A in mouse hepatocytes

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ABSTRACT

The niacin receptor GPR109A is a G_i-protein-coupled receptor which mediates the effects of niacin on inhibiting intracellular triglyceride lipolysis in adipocytes. However, the role of GPR109A in mediating the effects of niacin on high density lipoprotein (HDL) metabolism is unclear. We found niacin has no effect on HDL-C in GPR109A knockout mice. Furthermore, niacin lowered intracellular cAMP in primary hepatocytes mediated by GPR109A. We used an adeno-associated viral (AAV) serotype 8 vector encoding GPR109A under the control of the hepatic-specific thyroxine-binding globulin promoter to specifically overexpress GPR109A in mouse liver. Plasma HDL-C, hepatic ABCA1 and the HDL cholesterol production rate were significantly reduced in mice overexpressing GPR109A. Overexpression of GPR109A reduced primary hepatocyte free cholesterol efflux to apoA-I; conversely, GPR109A deficient hepatocytes had increased ABCA1-mediated cholesterol efflux. These data support the concept that the HDL-C lowering effect of niacin in wild-type mice is mediated through stimulation of GPR109A in hepatocytes; such an effect then leads to reduced hepatocyte ABCA1 expression and activity, decreased cholesterol efflux to nascent apoA-I, and reduced HDL-C levels. These results indicate that niacin-mediated activation of GPR109A in liver lowers ABCA1 expression leading to reduced hepatic cholesterol efflux to HDL.

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Plasma levels of high density lipoprotein (HDL) cholesterol (C) and its major apolipoprotein, apoA-I, show a strong inverse association with the risk of atherosclerotic cardiovascular disease [1,2]. HDL-C and apoA-I levels are reduced in acute and chronic inflammatory states [3,4]. Niacin (nicotinic acid) is the most effective pharmacologic approach currently available for raising HDL-C levels, but many questions remain regarding the molecular mechanisms responsible for such an effect [5–7]. Studies in cultured hepatocytes have suggested that niacin reduces the uptake of HDL [8] while kinetic studies in humans have mixed results, with reports that niacin reduces HDL clearance from plasma [9] or increases production [10] of the major HDL protein apoA-I.

Abbreviations: ABCA1, ATP binding cassette transporter A1; ApoA-I, apolipoprotein A-I; cAMP, cyclic adenosine monophosphate; CETP, cholesteryl ester transfer protein; GPCR, G-protein-coupled receptor; HDL-C, high density lipoprotein cholesterol; IBMX, 3-isobutyl-1-methylxanthine; LPS, lipopolysaccharide; TBG, thyroxine-binding globulin.

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GPR109A (also referred to as HM74A in humans and PUMA-G in mice) and GPR109B (also known as HM74 in human) are highly homologous G-protein-coupled receptors (GPCRs) and were identified as receptors for niacin [11–13] in 2003. Both GPR109A and GPR109B are highly expressed in adipocytes and activated immune cells [11–14]. GPR109B has only been found in humans, not in rodents [14–16]. β -Hydroxybutyrate, produced during fasting and starvation, was found to act as endogenous ligand of GPR109A [17]. Activation of GPR109A in adipose tissue leads to inhibition of adenylate cyclase, reduced cyclic adenosine monophosphate (cAMP), reduced activity of protein kinase A, reduced adipocyte triglyceride lipolysis, and reduced release of free fatty acids. Studies in GPR109A knockout mice confirmed that GPR109A was responsible for the antilipolytic (and cutaneous flushing) effects of niacin [18]. Reduced flux of free fatty acids from adipose to liver is believed to potentially underlie the triglyceride and LDL lowering effects of niacin.

Whether the HDL-raising effects of niacin are related to activation of GPR109A is unknown. Interestingly, niacin treatment of wild-type mice lacking cholesteryl ester transfer protein (CETP) consistently results in reduction of HDL-C, whereas in mice expressing CETP niacin has a modest HDL-raising effect [19–21]. The molecular mechanism(s) by which niacin reduces HDL-C in

wild-type mice lacking CETP and whether this involves activation of GPR109A in the liver are unknown. The aim of the present study is to establish whether GPR109A activation in liver influences HDL metabolism and the HDL response to niacin. By using GPR109A knockout mice, we demonstrate that GPR109A mediates niacin's effect of reducing HDL-C in the mouse. We established that GPR109A is expressed in primary murine hepatocytes and human-derived hepatocytes at low basal levels, and that expression is markedly inducible after exposure to inflammatory stimuli. Hepatic-specific overexpression of GPR109A in mice reduced plasma HDL-C levels accompanied by reduction in hepatic cAMP, ABCA1 protein, cholesterol efflux to apoA-I, and a reduced HDL cholesterol production rate. These data show that the reduction of HDL-C seen in mice in response to niacin is mediated by hepatic GPR109A.

1. Methods

1.1. Reagents and plasmid constructs

Forskolin, 3-isobutyl-1-methylxanthine (IBMX), niacin and puromycin were purchased from Sigma-Aldrich (St. Louis, MO,

USA). Ketamine was supplied by Fort Dodge Animal Health (Fort Dodge, Iowa, USA), xylazine was supplied by Vedco Inc. (Saint Joseph, MO, USA). The cAMP assay was purchased from R&D systems (Minneapolis, MN, USA). pCMV.SPORT6.1-hGPR109A plasmids were purchased from Invitrogen (Carlsbad, CA, USA). Polyclonal antibodies to HM74 and ABCA1 were from Novus Biologicals (Littleton, CO, USA); monoclonal antibodies to β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HRP-conjugated anti-mouse, anti-rabbit, anti-goat IgG and percoll were from GE Healthcare (Piscataway, NJ, USA); pQCXIP retroviral vectors were from BD-Clontech (Palo Alto, CA, USA). hGPR109A sequence from pCMV.SPORT6.1-hGPR109A was subcloned to pQCXIP vector. β -Hydroxybutyrate liquid assay kit was purchased from Stanbio Laboratory (Los Angeles, CA, USA). ^3H -cholesteryl ether was supplied by PerkinElmer Life Sciences Inc. (Boston, MA, USA).

1.2. Cell culture

HepG2 cells obtained from ATCC (Manassas, VA, USA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS).

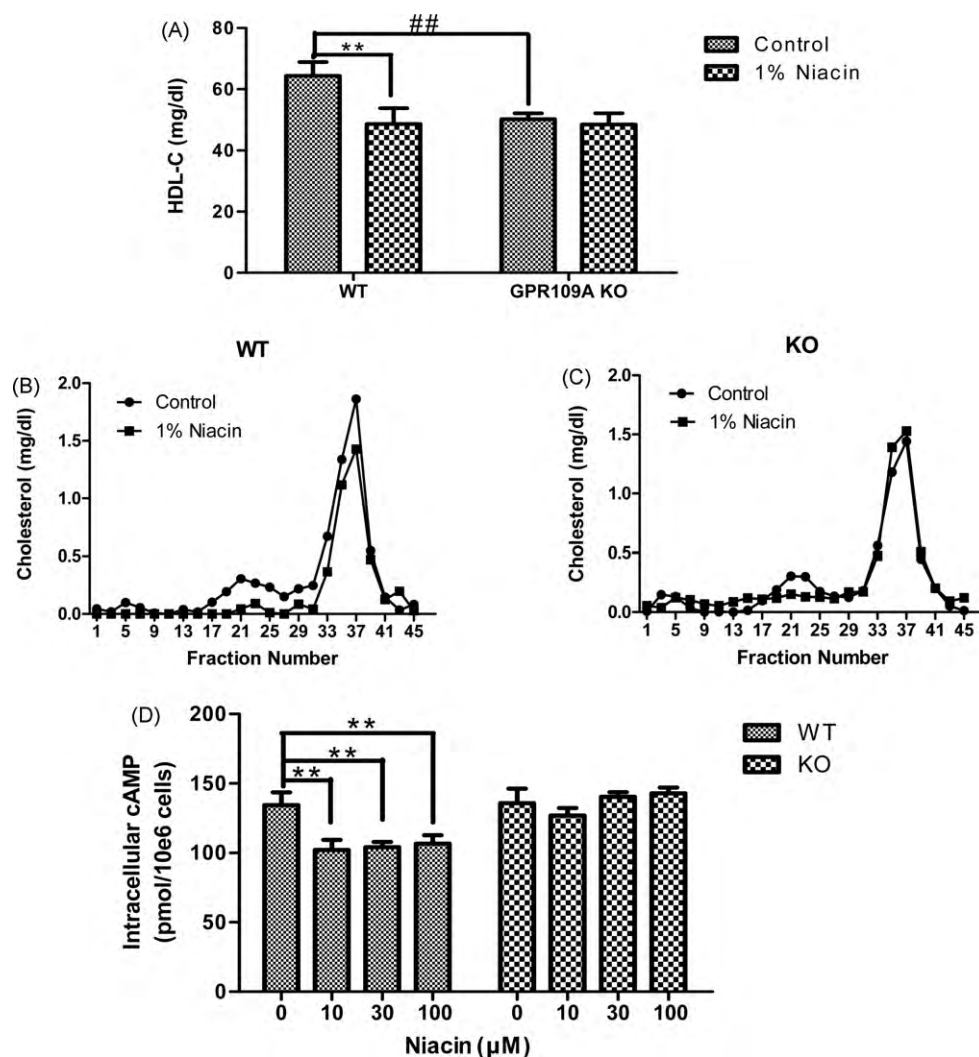


Fig. 1. Treatment of mice with a diet containing 1% niacin decreases plasma HDL-C in wild-type mice but not in GPR109A knockout mice. GPR109A knockout and wild-type control mice ($n = 8$) were fed a 1% niacin diet for 3 weeks. (A) Plasma HDL-C levels. Data are expressed as mean \pm SEM, $^{**}P < 0.01$, compared with control diet; $^{***}P < 0.01$ compared with wild type. Total cholesterol FPLC profile in GPR109A knockout (C) and wild-type mice (B). (D) Intracellular cAMP levels in the primary hepatocyte. Primary hepatocytes from GPR109A deficient or wild-type C57BL/6 mice were isolated and plated in 12-well plates. After pretreatment with or without 0, 10, 30 or 100 μM niacin for 1 hour, hepatocytes were treated with 1 mM IBMX and 30 μM forskolin for 15 min at 37 $^{\circ}$ C. The amount of cAMP present was calculated from a standard curve prepared using nonradioactive cAMP and was expressed as picomoles per 1×10^6 cells. Data are mean \pm SEM, $n = 3$, $^{***}P < 0.01$, compared with no niacin treatment.

For retroviral infections, viruses were produced from Phoenix ecotropic cells (Orbigen Inc., San Diego, CA, USA) by transient transfection of pQCXIP-human GPR109A, GPR109B or pQCXIP vector containing no insert (mock). HepG2 cells were subjected to one round of infection and selected 48 h after infection using 3 μ g/ml puromycin. HepG2-mock (stably expressing pQCXIP vector), HepG2-GPR109A (stably expressing human GPR109A) and HepG2-GPR109B (stably expressing human GPR109B) were grown in DMEM supplemented with 10% FBS and 1 μ g/ml puromycin.

Murine primary hepatocytes were isolated from adult female C57BL/6 mice. Using aseptic techniques, mice were anesthetized with ketamine/xylazine (70/7 mg/kg, respectively, IP) and subject to a mid-line laparotomy. Following a nonrecirculating *in situ* perfusion with Liver Perfusion Medium (Invitrogen, Carlsbad, CA, USA) through the portal vein, liver was digested with Liver Digestion Medium (Invitrogen) and minced. After washing with hepatocyte wash medium (Invitrogen), viable cells were purified by Percoll gradient centrifugation, and plated in collagen I-coated plates at 2×10^5 cells per well in a 12-well plate in Williams E medium supplemented with 10% FBS and antibiotics [22,23].

1.3. Animal studies

All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Breeder GPR109A knockout (KO) mice were kindly provided by Dr. Klaus Pfeffer (University of Düsseldorf, Düsseldorf, Germany) and then bred in our facility. GPR109A knockout mice have been backcrossed eight times to C57BL/6 to ensure a 98.3% similarity in genetic backgrounds with wild-type C57BL/6 mice. C57BL/6N mice were purchased from Taconic Company as wild-type (WT) control. Female C57BL/6J mice (6–8-week-old) were obtained from the Jackson Laboratory and maintained on a chow diet unless otherwise indicated. Diets were supplied by Research Diets, Inc. (New Brunswick, NJ). A simple purified ingredient diet, AIN-76A rodent diet, was used as the control diet and a 1% niacin diet consisted of the AIN-76A diet containing 10 g niacin per kilogram.

To overexpress hepatic GPR109A or GPR109B, C57BL/6 mice were injected with an AAV consisting of serotype 2/8 encoding human GPR109A (AAV2/8-TBG-hGPR109A) or GPR109B (AAV2/8-TBG-hGPR109B) under the control of the liver-specific, thyroxine-binding globulin (TBG) promoter, or with control null AAV2/8 (AAV2/8-TBG-Null) into the peritoneum (1×10^{12} particles/mouse) [24]. The AAV2/8-TBG-hGPR109A and AAV2/8-TBG-hGPR109B cis-plasmids were constructed by inserting human GPR109A or GPR109B cDNA followed by SV-40 poly A tail in front of liver-specific human TBG promoter flanked by AAV2-inverted terminal repeat sequence. The chimeric vector AAV2/8 with human GPR109A, GPR109B or null was then formed by fusing the capsid gene of serotype 8 with AAV2 rep gene by triple

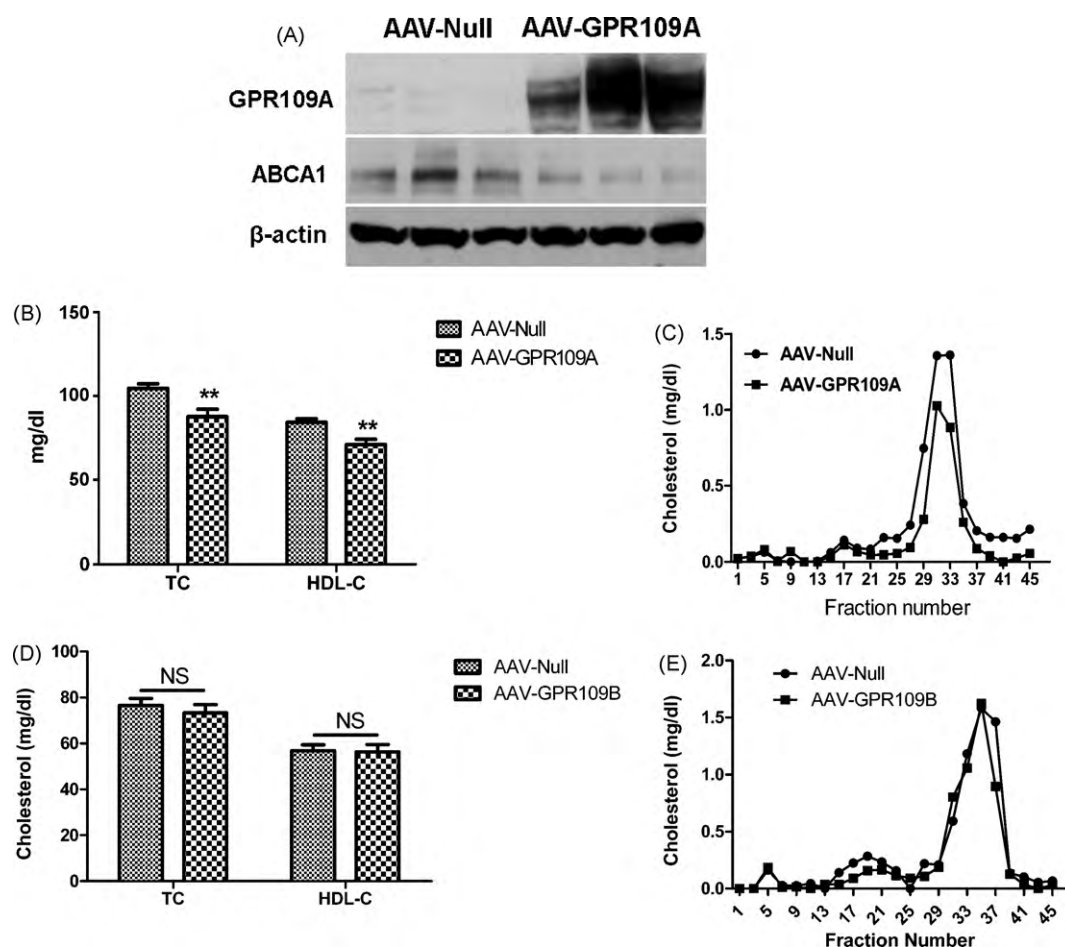


Fig. 2. Effects of liver-specific expression of GPR109A or GPR109B on plasma lipids *in vivo*. C57BL/6 mice ($n = 7$) were injected intraperitoneally with 1×10^{12} genome copies (GC) AAV2/8 GPR109A or control virus. (A) Western blot of GPR109A and ABCA1 in liver tissue from GPR109A overexpression and control mice. C57BL/6 mice were injected with 1×10^{12} GC AAV2/8 GPR109A or control virus IP, and 8 weeks later livers were harvested for total protein extraction. Experiments were repeated 3 times. (B) Plasma levels of total cholesterol and HDL-C after 8 weeks of transduction. Data are expressed as mean \pm SEM. $n = 7$, ** $P < 0.01$. (C) Total cholesterol FPLC Profile. C57BL/6 mice ($n = 8$) were injected intraperitoneally with 1×10^{12} GC AAV2/8 GPR109B or control virus. (D) Plasma levels of total cholesterol and HDL-C after 8 weeks of transduction. Data are expressed as mean \pm SEM. (E) Total cholesterol FPLC Profile.

transfection into 293 cells with adenovirus helper plasmid, cis-plasmid, and a chimeric packaging construct. Plasma lipid and lipoprotein analyses

Plasma total cholesterol and HDL cholesterol were measured on a Cobas Fara (Roche Diagnostics, Indianapolis, IN, USA) with the use of Sigma Diagnostic reagents. Fast protein liquid chromatography (FPLC) (Amersham Pharmacia Biotech, Uppsala, Sweden) analysis was performed on pooled samples. The cholesterol concentrations in the FPLC fractions were determined via an enzymatic assay (Wako Pure Chemical Industries Ltd, Richmond, VA, USA).

1.5. Kinetic study

The *in vivo* kinetic study was performed by co-injection of human HDL₃ labeled with ¹²⁵I-tyramine-cellobiose, which labels primarily apoA-I on HDL₃, and human HDL₃ exchange labeled with ³H-cholesteryl ether, a tracer of the cholesteryl ester core of HDL, as previously described [25]. Tyramine cellobiose was prepared and provided by William Cain, University of Delaware. Both tracers are non-hydrolyzable allowing tissue uptake to be accurately assessed. ¹²⁵I-Tyramine-cellobiose-HDL₃ and ³H-cholesteryl ether-HDL₃ were co-injected by the tail vein in each mouse, and blood samples were obtained by retro-orbital bleeding at 2 min and at 1, 3, 6, 9, 24 and 48 h. Forty-eight hours after dose injection, fasted animals were sacrificed, the vascular system was flushed with 15 ml ice-cold PBS, the liver and kidneys were harvested and radioactivity counted. The fractional catabolic rate (FCR) of apoA-I and HDL-cholesteryl ester was determined by fitting the corresponding tracer data to a single pool model containing an extravascular exchange using data normalized to the 2-min time point. Selective uptake was calculated as the difference between the HDL-cholesteryl ester and apoA-I FCRs. The production rate for HDL-cholesteryl ester was calculated by multiplying the FCR by the estimated HDL-cholesteryl ester pool size.

1.6. Statistics

Statistics were conducted using GraphPad Prism Software version 5.0. Results are presented as mean \pm SD or \pm SEM as

indicated. Differences were determined using 2-tailed Student's *t* test or a one-way ANOVA using a Newman-Keuls test to determine post-hoc differences. Differences were considered significant at a two-tailed *P* value of <0.05

2. Results

2.1. Niacin reduces plasma HDL-C in WT mice but not in GPR109A KO mice

It is well established that in wild-type mice, niacin treatment reduces plasma HDL-C levels [19–21]. To test whether this HDL-lowering effect of niacin is GPR109A-dependent, GPR109A knockout and wild-type control mice were treated with 1% niacin diet for 3 weeks. There were no differences in food consumption or weight gain between mice consuming the 1% niacin diet and control diet (data not shown). We found that niacin reduced HDL-C levels in wild-type mice as expected (Fig. 1A); interestingly, in GPR109A KO mice niacin had no effect on HDL-C levels (Fig. 1A). These results were confirmed by FPLC, showing that niacin reduced HDL in wild-type mice (Fig. 1B) but not in GPR109A knockout mice (Fig. 1C). This suggests that the HDL-lowering effect of niacin in mice is GPR109A dependent.

GPR109A is a G_i-protein-coupled receptor that when activated in adipocytes and GPR109A stably expressing cells reduces cAMP levels [11,12,14,26–29]. We hypothesized that niacin acts via GPR109A to modulate cAMP levels in hepatocytes. Primary hepatocytes from wild-type and GPR109A knockout mice were isolated and cAMP levels in response to niacin stimulation were measured. Niacin inhibited intracellular cAMP release in hepatocytes from wild-type mice whereas there was no effect of niacin on cAMP release from hepatocytes isolated from GPR109A knockout mice (Fig. 1D). These results suggest that primary hepatocytes respond to niacin via GPR109A despite low basal levels of receptor expression.

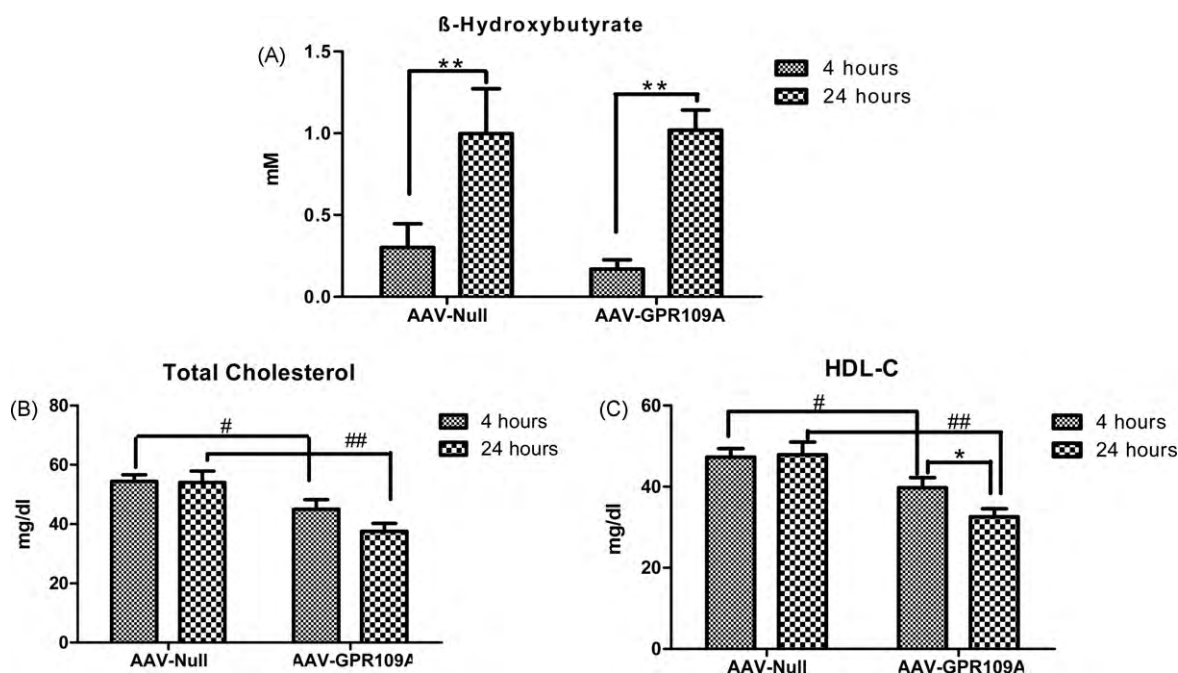


Fig. 3. Effects of 24 h fasting on both control and liver-specific expression of GPR109A. C57BL/6 mice were injected intraperitoneally with 1×10^{12} genome copies AAV2/8 GPR109A or control AAV2/8 Null. After 11 weeks of virus injection, chow-fed mice were subjected to 24 h fasting; plasma levels of β -hydroxybutyrate (A), total cholesterol (B) and HDL-C (C) were measured. Data are expressed as mean \pm SEM, $n = 7$. * $P < 0.05$ compared with 4 h fasting, # $P < 0.05$, ## $P < 0.01$ compared with AAV-Null.

2.2. LPS induces expression of GPR109A in murine hepatocytes *in vivo* and *ex vivo*

GPR109A is expressed in murine liver but the basal expression of GPR109A is low. However, after injection of very low dose endotoxin (LPS), expression of GPR109A was markedly upregulated in liver (Supplemental Fig. A). The maximal upregulation of GPR109A mRNA by LPS treatment occurred at 2 h (>80-fold) and returned to baseline by 24 h. The peak expression of GPR109A protein at 2 h was confirmed in the liver lysates by Western blotting (Supplemental Fig. B).

Macrophages are known to express GPR109A and therefore the increased GPR109A expression in liver after LPS stimulation could be due primarily to Kupffer cell expression. To determine whether LPS upregulates GPR109A expression specifically in parenchymal cells, C57BL/6 mice were injected intraperitoneally with LPS and hepatocytes were isolated from liver at baseline and 2 h after LPS injection. RNA was isolated and GPR109A mRNA determined by QRT-PCR. Hepatocyte GPR109A was markedly upregulated (>500-fold) by LPS injection (Supplemental Fig. C), establishing that

parenchymal cells respond to inflammation *in vivo* with pronounced upregulation of the receptor. Finally, mouse primary hepatocytes were isolated from C57BL/6 mice and were treated *ex vivo* with 100 ng/ml LPS for various time periods. GPR109A mRNA was increased by about 4-fold at 6 h compared with the baseline (Supplemental Fig. D). These studies establish that parenchymal hepatocytes express GPR109A at low levels under basal conditions and markedly upregulate its expression in response to inflammation.

2.3. Hepatocyte-specific overexpression of GPR109A in WT mice reduces HDL-C levels

Having established that GPR109A is expressed in mouse hepatocytes and is upregulated by inflammation, we wished to probe the effects of specific hepatocyte GPR109A overexpression on HDL metabolism. To test this, we utilized an AAV2/8-based vector encoding human GPR109A under the control of the liver-specific TBG promoter. GPR109B shows a high degree of sequence similarity to GPR109A; thus to test whether specific hepatic

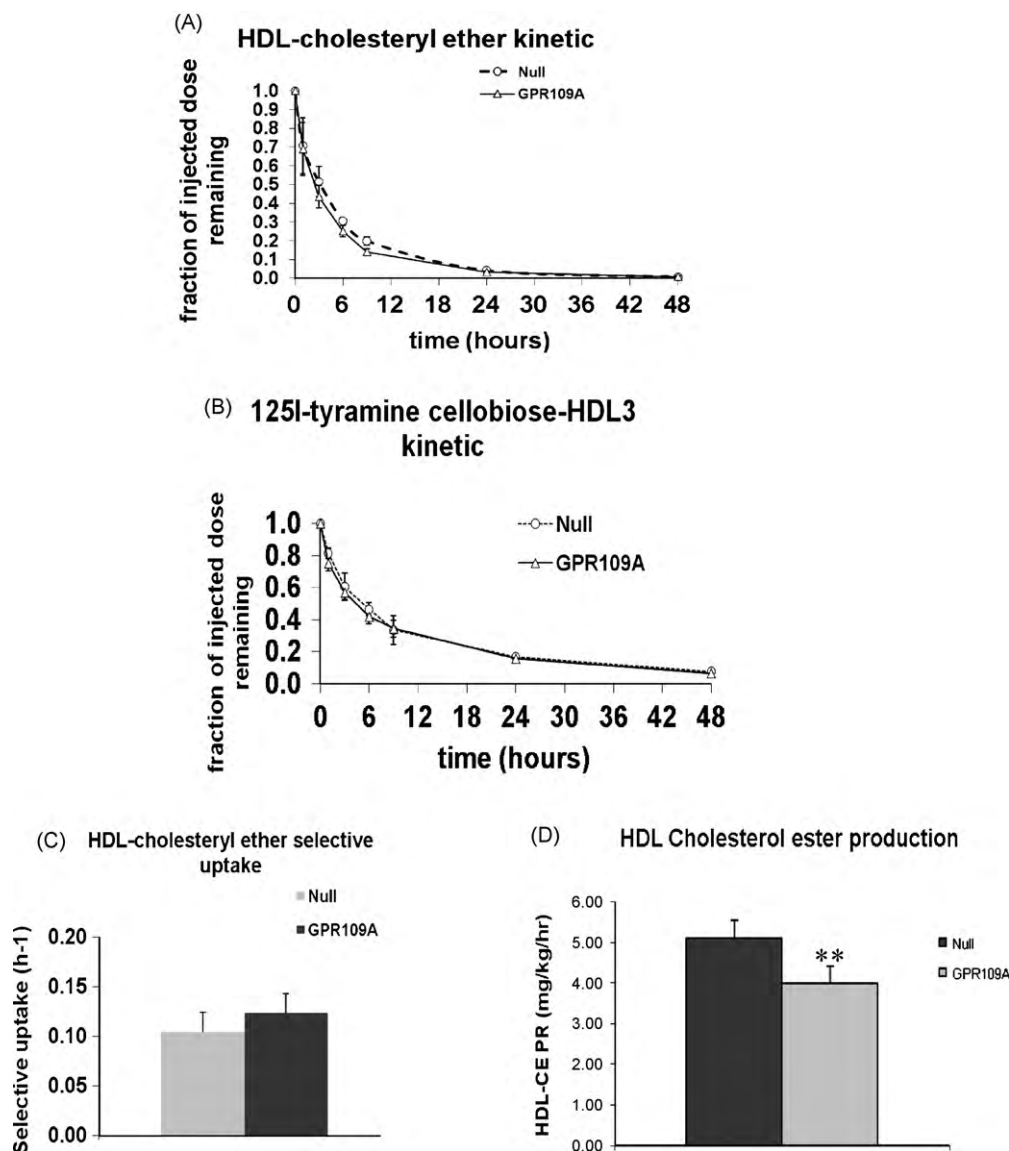


Fig. 4. Plasma kinetics of ^3H -cholesteryl ether and ^{125}I -labeled tyramine-cellobiose labeled human HDL₃. Blood samples were drawn at the time points indicated and analyzed for radioactivity. (A) ^3H -HDL-cholesteryl ether clearance from plasma. (B) ^{125}I -Tyramine-cellobiose HDL clearance from plasma. (C) Plasma HDL-cholesteryl ether selective uptake. Data are expressed as mean \pm SEM, $n = 6$. (D) Plasma HDL-cholesteryl ester transport rate. Data are expressed as mean \pm SEM, $n = 6$, $^{**}P < 0.01$.

GPR109B overexpression has a similar effect as GPR109A on HDL metabolism, the AAV2/8-TBG-GPR109B virus was made as a control. Injection of the AAV-GPR109A virus into wild-type mice resulted in detectable increase in the amount of GPR109A protein in liver (Fig. 2A). Eight weeks after injection, plasma total and HDL cholesterol (Fig. 2B) levels were significantly lower in GPR109A-overexpressing mice compared with mice injected with control vector. The decrease in HDL-C in mice overexpressing GPR109A in liver was confirmed by measurement of cholesterol in FPLC fractions (Fig. 2C). Furthermore, GPR109B overexpression had no effect on either total or HDL cholesterol (Fig. 2D and E).

2.4. Ligand activation of GPR109A in hepatocytes reduces HDL-C levels in vivo

β -Hydroxybutyrate is an endogenous ligand for GPR109A [17]. In order to test the role of hepatic GPR109A in HDL metabolism under endogenous ligand stimulated conditions *in vivo*, mice were subjected to 24 h fasting to stimulate production of β -hydroxybutyrate. Twenty four hours fasting significantly increased plasma β -hydroxybutyrate as expected (Fig. 3A). While the increase in β -hydroxybutyrate had no effect on total and HDL-C in control mice, fasting significantly reduced HDL-C in mice overexpressing GPR109A (Fig. 3B and C).

2.5. Hepatocyte-specific overexpression of GPR109A in WT mice reduces HDL-C production

To investigate the effect of hepatic overexpression of GPR109A on plasma HDL metabolism, 125 I-tyramine-cellobiose and 3 H-cholesteryl ether labeled HDL was IV injected into mice treated with AAV2/8-TBG-GPR109A or control vector (Fig. 4). There was no difference in the rate of HDL-cholesteryl ester (HDL-CE) (Fig. 4A) or protein clearance (Fig. 4B) between hepatic overexpressing GPR109A mice and control mice and there was no change in selective uptake

of HDL-CE (Fig. 4C). However, the HDL cholesterol production rate was significantly reduced in mice overexpressing GPR109A (Fig. 4D). These data indicate that the lowering of HDL-C in hepatic overexpressing GPR109A mice is not due to increased HDL cholesterol catabolism but due to reduced HDL cholesterol production.

2.6. Hepatocyte-specific overexpression of GPR109A in hepatocytes reduces cAMP, ABCA1 protein, and cholesterol efflux to apoA-I

In order to understand the mechanism of the reduced HDL-cholesteryl ester production, primary hepatocytes were isolated from GPR109A-expressing and control mice. Expression of GPR109A in hepatocytes dramatically reduced the forskolin-induced intracellular level of cAMP (Fig. 5A), indicating that overexpression of the receptor led to its activation, as has been described for other GPCRs [30,31]. Hepatic ABCA1 expression is a key determinant of HDL-C levels in mice through promoting cholesterol efflux from hepatocytes to apoA-I [32,33], and in some cells, such as macrophages, ABCA1 is upregulated at the protein level by cAMP [34,35]. We hypothesized that in hepatocytes the reduction of cAMP due to GPR109A activation would result in decreased ABCA1 protein abundance and function. We confirmed hepatic ABCA1 protein levels were substantially reduced in overexpressing GPR109A mice (Fig. 2A), consistent with the reduction in cAMP (Fig. 5A). GPR109A-overexpressing primary hepatocytes had reduced efflux of free cholesterol to apoA-I (a measure of ABCA1 activity) compared with control cells under basal (Fig. 5B) and forskolin-treated (Fig. 5C) conditions. In contrast, the GPR109A deficient liver had a higher ABCA1 protein expression compared with wild-type control mice liver (Fig. 5D). Cholesterol efflux data show that forskolin treatment evokes a higher free cholesterol efflux to apoA-I in GPR109A knockout hepatocytes (Fig. 5E).

To investigate whether the results observed in mouse hepatocytes were applicable to humans, we conducted experi-

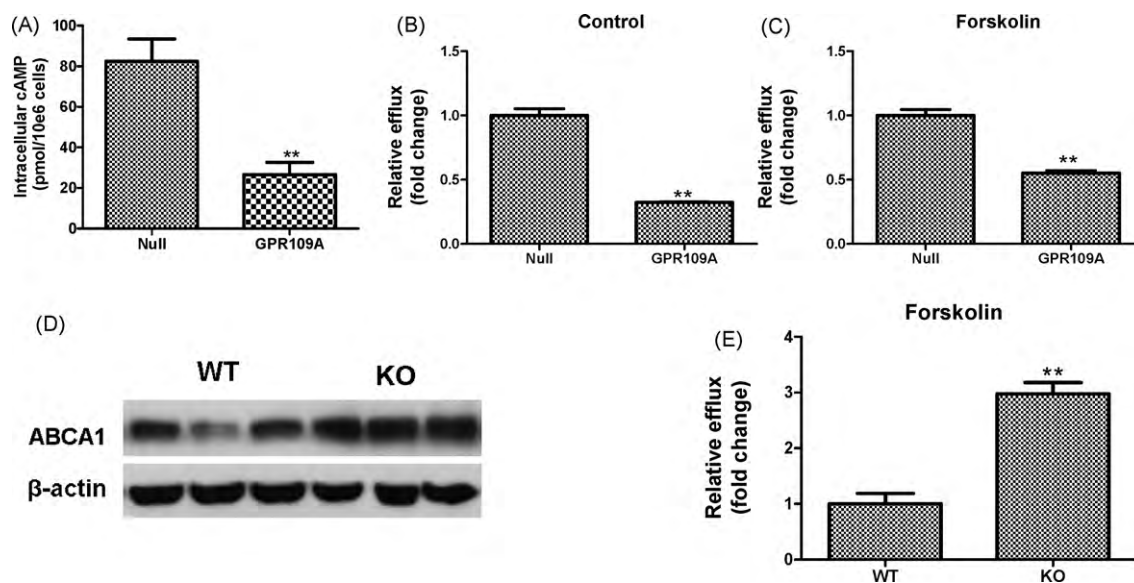


Fig. 5. GPR109A overexpression inversely related to free cholesterol efflux to apoA-I from hepatocytes. C57BL/6 mice were injected with 1×10^{12} GC AAV2/8 GPR109A or control virus IP, and 8 weeks later liver and hepatocytes were isolated. Primary hepatocytes from mice injected with either AAV-GPR109A or AAV-Null were cultured in 12-well plates. (A) GPR109A overexpression decreased intracellular cAMP release in response to forskolin. The amount of cAMP present was calculated from a standard curve prepared using nonradioactive cAMP and was expressed as picomoles per 1×10^6 cells. Data are mean \pm SEM, $n = 3$, $^{**}P < 0.01$, compared with AAV-Null. Primary hepatocytes were labeled with $2 \mu\text{Ci/ml}$ ^3H -cholesterol in medium for 24 h; cells were then equilibrated for 18 h at 37°C with DMEM/BSA (B) or containing the indicated reagents $30 \mu\text{M}$ forskolin plus 1 mM IBMX (C), separately. For cholesterol efflux, medium containing $10 \mu\text{g/ml}$ free apoA-I was added to cells. 9 h cholesterol efflux levels were measured. Values represent the average of triplicate determinations normalized to AAV-Null group (mean \pm SD), $^{**}P < 0.01$ compared with control vector. (D) Western blot of ABCA1 in liver tissue from GPR109A knockout and wild-type control mice. Experiments were repeated 3 times. (E) Cholesterol efflux to apoA-I from forskolin-treated GPR109A knockout and wild-type control primary hepatocytes. Primary hepatocytes from GPR109A deficient or wild type C57BL/6 mice were isolated, labeled, equilibrated with DMEM/BSA containing $30 \mu\text{M}$ forskolin plus 1 mM IBMX, then performed 9 h efflux study to human apoA-I. Values represent the average of triplicate determinations normalized to wild type group (mean \pm SD), $^{**}P < 0.01$ compared with wild-type.

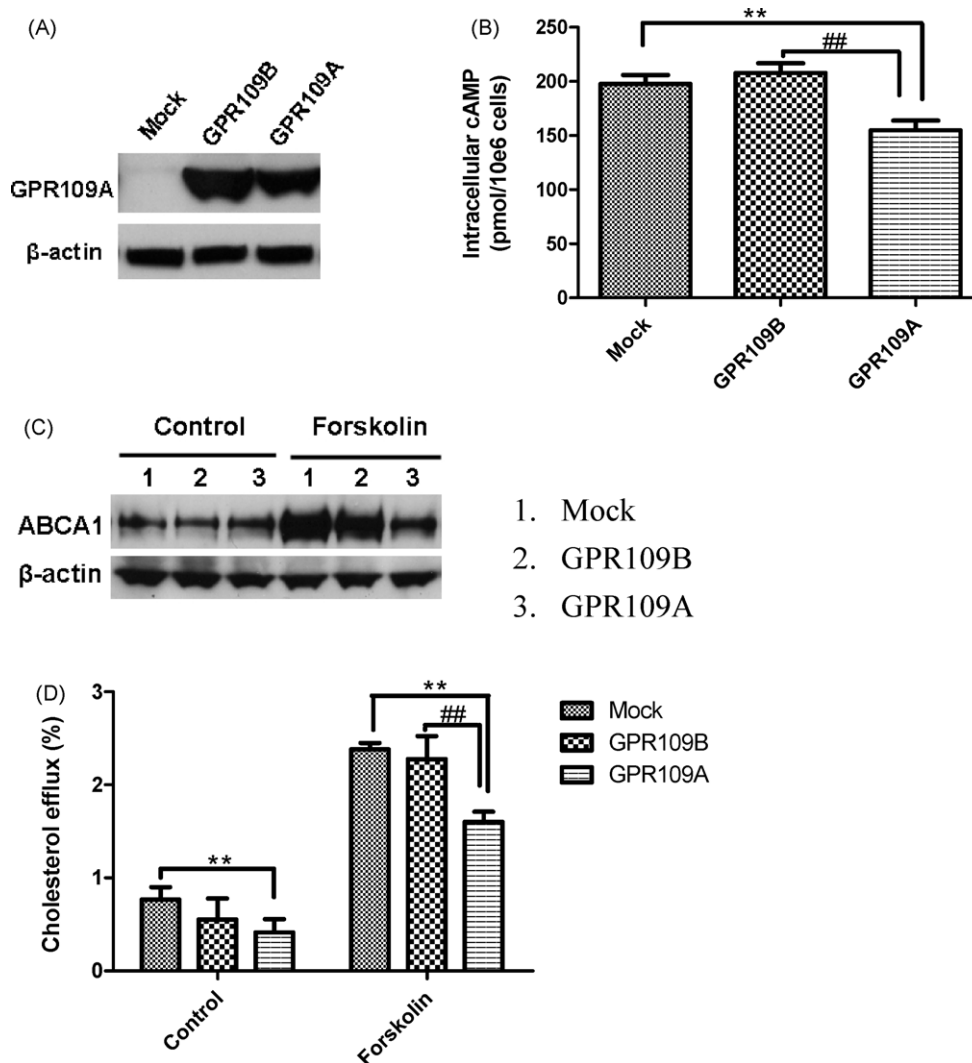


Fig. 6. Overexpression of GPR109A reduces ABCA1 expression and activity in HepG2 cells. (A) Western Blot of GPR109A in HepG2 cells. (B) Intracellular cAMP assay. HepG2 cells transfected with GPR109A or GPR109B or control HepG2 cells were treated with 30 μ M forskolin and 1 mM 3-isobutyl-1-methylxanthine for 15 min at 37 °C. Data from 3 independent experiments are expressed as mean \pm SD, $n = 6$, $^{**}P < 0.01$, compared with mock group, $^{##}P < 0.01$, compared with GPR109B group. (C) Western Blot of cell lysate of ABCA1. Experiments were repeated 3 times. (D) Cholesterol efflux to apoA-I from HepG2 cells. Efflux of free cholesterol into the media is expressed as percentage of the total radioactivity in the media and cells (^3H -cholesterol in the medium $\times 100$) / (^3H -cholesterol in the medium + ^3H -cholesterol in the cells). Values represent the average of triplicate determinations (mean \pm SD), $^{**}P < 0.01$ compared with control vector, $^{##}P < 0.01$, compared with GPR109B group.

ments using HepG2 cells stably transfected with GPR109A (HepG2-GPR109A) or GPR109B (HepG2-GPR109B). HepG2 cells transfected with a null retroviral vector were used as control cells (HepG2-mock). GPR109B differs from GPR109A in an extended C-terminal tail as well as in 16 amino acids [11,13]; despite this difference, the antibody we used recognized both GPR109A and GPR109B. Western blot analysis revealed a low level of GPR109A expression in the control cells and a substantially greater level of GPR109A or GPR109B protein in the stably transfected cells (Fig. 6A). As shown in Fig. 6B, overexpression of GPR109A reduced cellular cAMP levels in forskolin-treated cells, but not GPR109B. The GPR109A-expressing cells had a substantial reduction in ABCA1 protein levels in the presence of forskolin (Fig. 6C), accompanied by a 55% decrease in cholesterol efflux to apoA-I (Fig. 6D), while GPR109B expressing cells had a similar ABCA1 protein level (Fig. 6C) and cholesterol efflux capacity (Fig. 6D) with mock cells.

3. Discussion

The current study is the first to specifically investigate the role of hepatic GPR109A and GPR109B on HDL metabolism and

response to niacin therapy. While niacin raises HDL-C levels in humans, it has been reported by others [20,36] and confirmed by us here that niacin treatment reduced serum levels of HDL cholesterol in wild-type mice. This effect of reducing HDL-C was GPR109A dependent. GPR109A is a G_i -protein-coupled receptor that is known to reduce cAMP and lipase-mediated hydrolysis of triglyceride stores in adipose tissue. We found that niacin treatment also inhibited intracellular cAMP release response to forskolin stimulation in primary hepatocytes from wild-type mice. While hepatocyte expression of GPR109A in the basal state is low, it was markedly upregulated by very low dose endotoxin. GPR109A expression is upregulated in murine macrophages by inflammatory stimuli [37] but this phenomenon has not been reported in other cell types. While the basal expression of GPR109A in liver is low, we show that it is upregulated after LPS injection; furthermore, we show that this is due to upregulation in hepatocytes and not simply Kupffer cells. These studies establish that hepatocytes express the niacin receptor and upregulate its expression in response to inflammation.

In order to study the specific effects of increased hepatocyte expression of GPR109A on lipid, and particularly HDL, metabolism,

we made an AAV2/8-based vector encoding murine GPR109A driven by the hepatocyte-specific TBG promoter. Intraperitoneal injection of this vector into wild-type mice resulted in expression of human GPR109A at levels substantially greater than basal and in the range of what we observed after LPS injection. Several G-protein-coupled receptors have been shown to exist as homo- and hetero-oligomeric complexes in living cells and can exhibit ligand-independent activation, particularly when overexpressed [30,31]. We found that overexpression of GPR109A in hepatocytes resulted in activation as evidenced by marked suppression of forskolin-stimulated cAMP. This activation may be caused by dimerization of the GPR109A, oligomerization with another GPCR in the setting of overexpression, or simply increased receptor activation [38]. Although GPR109A and GPR109B are highly similar at the protein level and share a similar expression pattern, they are the products of different genes and clearly differ in their ability to be activated by niacin and β -hydroxybutyrate [16]. Our data establish that GPR109B expressed in hepatocytes does not have the same effects on cAMP and ABCA1 activity as does the expression of GPR109A.

Overexpression of GPR109A caused a reproducible and significant decrease in plasma HDL-C levels that we showed was not due to increased HDL catabolism but rather to reduced HDL cholesterol production. Hepatic ABCA1 plays a critical role in the biosynthesis of nascent HDL by promoting the efflux of cholesterol from hepatocytes to lipid-poor apoA-I [32]. Because activation of GPR109A is known to reduce cAMP in adipocytes, and ABCA1 protein and activity are known to be increased by cAMP in macrophages, we hypothesized that activation of GPR109A in hepatocytes would reduce cAMP and thus ABCA1 protein and activity. Our data support this model and provide a mechanistic basis for the decreased HDL-C levels with both niacin treatment and GPR109A overexpression in mice. In contrast, GPR109A deficiency was associated with significantly increased cholesterol efflux to apoA-I in primary hepatocytes, which is consistent with a role for GPR109A in modulating HDL-C metabolism in liver.

In summary, while basal expression of GPR109A in hepatocytes is low, it is substantially upregulated by inflammatory stimuli. Overexpression of GPR109A in mouse liver unexpectedly reduced levels of HDL cholesterol in plasma, apparently due to constitutive activation of the receptor with reduced cAMP production, leading to reduced ABCA1 protein and activity, reduced cholesterol efflux to apoA-I and reduced HDL-C levels in wild-type mice. The role of hepatocyte GPR109A expression in human physiology and the response to niacin in humans remains to be fully elucidated.

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Disclosure

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bcp.2010.07.023](https://doi.org/10.1016/j.bcp.2010.07.023).

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