

Niacin inhibits surface expression of ATP synthase β chain in HepG2 cells: implications for raising HDL

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Abstract Niacin is an effective agent for raising HDL, but its cellular target sites are largely unknown. We examined effects of niacin on the surface expression of ATP synthase β chain, a newly described HDL/apolipoprotein A-I (apoA-I) receptor for HDL endocytosis, in HepG2 cells. A significant amount of immunodetectable β chain was observed on the surface of HepG2 cells, which was competitively displaced by apoA-I. Niacin treatment reduced the surface expression of β chain in HepG2 cells by $\sim 27\%$, and decreased ^{125}I -labeled HDL uptake up to $\sim 35\%$. However, nicotinamide, a niacin metabolite that does not have clinical lipid effects, exhibited weaker inhibition on the β chain cell surface expression, and failed to show inhibitory action on ^{125}I -labeled HDL uptake. Furthermore, anti- β chain antibody significantly reduced ^{125}I -labeled HDL uptake and abolished the inhibitory effect of niacin. Niacin did not change β chain mRNA expression. These data suggest that niacin inhibits cell surface expression of the ATP synthase β chain, leading to reduced hepatic removal of HDL protein, thus implicating a potential cellular target for niacin action to raise HDL.—Zhang, L-H., V. S. Kamanna, M. C. Zhang, and M. L. Kashyap. Niacin inhibits surface expression of ATP synthase β chain in HepG2 cells: implications for raising HDL. *J. Lipid Res.* 2008. 49: 1195–1201.

Supplementary key words hepatocytes • HDL receptor • nicotinic acid • flow cytometry

HDL-cholesterol (HDL-C) levels are inversely correlated with risk of coronary artery disease (1). HDL plays a major role in reverse cholesterol transport, in which HDL acts as carrier in transferring cholesterol from peripheral tissues to the liver for elimination. HDL has also been shown to have the other cardio-protective properties of anti-inflammation, anti-oxidation, and anti-thrombosis (2). Niacin (nicotinic acid), a water-soluble vitamin and the most effective clinical agent in elevating plasma HDL-C and apolipoprotein A-I (apoA-I) levels, has been widely used

in the treatment of dyslipidemia (3–8). Either as monotherapy or in combination with other lipid-lowering agents, niacin significantly reduces cardiovascular risk. Furthermore, long-term follow-up of patients in the Coronary Drug Project with coronary arteriographic studies including the Cholesterol Lowering Atherosclerosis Studies, and the Familial Atherosclerosis Treatment Study, ARBITER 2 study, indicated that treatment with niacin significantly reduced total mortality and coronary events and retarded progression and even induced regression of coronary atherosclerosis (as reviewed in Refs. 3–6).

Although niacin has been commonly used to increase plasma HDL levels, the mechanism(s) by which niacin exerts its action is not clearly understood (4–10). Earlier kinetics studies showed that the rise in plasma apoA-I (major protein of HDL) in patients treated with niacin was a result of decreased fractional catabolic rates rather than alteration in its synthetic rates (7, 8). Consistent with in vivo observations, niacin does not change de novo apoA-I synthesis in cultured hepatoblastoma HepG2 cells (9). Interestingly, without altering cell uptake of HDL cholesteryl esters by HepG2 cells, niacin decreased ^{125}I -labeled HDL protein uptake, preferably LP-AI (a cardio-protective subfraction of HDL with only apoA-I but not apoA-II) (9, 10). These findings suggest that niacin does not act on the well-documented HDL receptor scavenger receptor class B type I (SR-BI) mechanism that is responsible for selective uptake of HDL cholesteryl ester (11, 12). Recently, using stably transfected cells with SR-BI cDNA, Nieland et al. (13) have shown that niacin did not affect cellular surface expression of SR-BI and the selective uptake of HDL protein or HDL-cholesteryl esters. Together, these studies suggest that niacin may act through an unknown pathway, but not SR-BI-mediated events, to reduce the hepatic uptake of HDL protein or HDL holoparticles (6, 12–14).

Recently, the mitochondrial β chain subunit of ATP synthase has been identified on the surface of hepatocytes as an apoA-I/HDL receptor (15). The β chain is one

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component of ATP synthase, composed of two linked multi-peptide complexes located in the inner membrane of mitochondria: F1 and F0. The water-soluble F1-ATP synthase consists of five subunits at the ratio of $\alpha\beta\beta_3\gamma\delta\epsilon$, forming a catalytic core with both forward ATP synthase activity and reverse ATPase activity. F0 is the detergent-soluble, membrane-spanning component, with three main subunits (a, b, c), comprising the proton channel (16, 17). The expression of cell surface α/β chains is cell specific. They were found to be present on hepatocytes, endothelial cells, lymphocytes, and some tumor cell lines but not on the CHO cell line (15, 18–20). The physiological significance of cell surface ATP synthase in these cells is not clear but may be involved in angiogenesis, hypertension, cell proliferation, and cytotoxicity in addition to HDL endocytosis (20–23).

The ectopic cell surface ATP synthase β chain mediates hepatic HDL holo-particle (protein + lipid) endocytosis (15). This HDL endocytosis is stimulated by ADP, which is generated by the ATPase activity of the β chain protein after its binding with apoA-I on the cell surface. Thus, expression of the β chain on the cell surface is required for HDL holo-particle endocytosis. The purpose of the present study was to examine the effects of niacin on the surface expression of the β chain in HepG2 cells, which is a key step for β chain-mediated HDL endocytosis. The results indicate that niacin inhibits β chain cell surface expression, leading to reduced cellular uptake of HDL protein.

MATERIALS AND METHODS

Cell culture

HepG2 cells, obtained from the American Type Culture Collection, were maintained in DMEM containing 10% FBS, supplemented with 100 U/ml penicillin G and 100 μ g/ml streptomycin sulfate at 37°C in a humidified atmosphere of 95% air, 5% CO₂. For serum starvation experiments, HepG2 cells were incubated in the same medium, but containing 0.2% FBS.

Confocal microscopy

HepG2 cells were washed twice in PBS before fixation with 3% paraformaldehyde at room temperature for 5 min. Cells were then incubated with the primary murine monoclonal antibodies against the human β chain of ATP synthase (clone 3D5; Molecular Probes, Eugene, OR) (5 μ g/ml) in PBS-1% BSA, followed by incubation with goat anti-mouse IgG conjugated to Alexa Fluor 488 (5 μ g/ml) (Molecular Probes). Samples stained with only the secondary antibodies, or stained with mouse isotypic IgG1 (Molecular Probes) in some experiments, were used as negative controls. After washing, cells were mounted on coverslips and analyzed using a Nikon Eclipse E800 microscope.

Flow cytometric analysis

HepG2 cells were washed three times with PBS, detached with 0.1 mM EDTA, and fixed in 3% paraformaldehyde at room temperature for 5 min. Cells were incubated with anti- β chain antibody (3D5; Molecular Probes) in PBS-1% BSA, 0.1% NaN₃, pH 7.4, at 20°C for 2 h. Isotypic mouse IgG was used as control. After a brief wash, cells were incubated with FITC-conjugated goat anti-mouse IgG 1 F(ab')₂ (Sigma, St. Louis, MO) at 20°C for

30 min. Cells were washed three times with PBS-1% bovine serum albumin, 0.1% NaN₃, pH 7.4, and then added to polystyrene flow cytometry tubes kept cold and in the dark whenever possible. The amount of staining was quantified using a FACScalibur (BD Biosciences, San Jose, CA) and CellQuest™ software for acquisition and analysis, with a total collection of 20,000 cell events.

HDL uptake assay

¹²⁵I-labeled HDL preparations and the uptake assay in HepG2 cells were performed according to the protocols described previously (9, 10). Briefly, HepG2 cells were preincubated in DMEM medium containing 0.2%, or 10% FBS in some experiments, with or without niacin at 37°C for 48 h. After washing with PBS, HepG2 cells were incubated with ¹²⁵I-labeled HDL (5–10 μ g/ml with specificity of 300–500 cpm/ng) at 37°C for 16 h in DMEM medium containing 5 mg/ml BSA free of fatty acids. The medium was removed after the incubation. The cells were washed two times with PBS-0.1 mg/ml BSA, and two times with PBS, and then dissolved in 250 μ l 1 N NaOH at room temperature for 2 h. Separate aliquots of cell lysate (100 μ l each) were used for γ -counting and for protein assay, with BSA as standard (BCA; Pierce, Rockford, IL). The data are expressed as counts per minute per microgram of cell protein. For the antibody blocking experiment, the monoclonal anti- β chain antibody or isotypic mouse IgG control (10 μ g/ml) was incubated for 30 min prior to the addition of ¹²⁵I-labeled HDL.

RT and real-time PCR

Total RNA extracted from cells was reverse transcribed with 100 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed by using the QuantiTect SYBG Green PCR reagents (Qiagen, Valencia, CA) in an iCycler with primer sets 5'-GAGACCAAGAAGGTCAAGATG and 5'-GAGAGGATTCGGCCCAATAATGCAG for β chain, and 5'-CTGACCCTGAAGTACCCCAT and 5'-TCTGCGCAAGTTAGGTTTTGT for an endogenous control gene, β -actin.

Statistical analysis

Data are expressed as means \pm SD. Student's *t*-test was used for statistical comparisons.

RESULTS

Initial studies showed that significant amounts of immunodetectable β chain protein were present on the surface of HepG2 cells (Fig. 1A). Preincubation of apoA-I substantially decreased the immunostained β chain protein, indicating the binding activity of the β chain for apoA-I/HDL (Fig. 1B). The ATP synthase β chain isolated from β chain cDNA-transfected cells was also able to co-precipitate with apoA-I (data not shown), suggesting that apoA-I directly binds to ATP synthase β chain. These observations are consistent with findings reported previously showing that the surface β chain is an apoA-I/HDL receptor (15, 18).

To study whether niacin affects the β chain, we first examined its overall expression in HepG2 cells treated with niacin. Real-time RT-PCR results showed that niacin did not change β chain mRNA levels (the ratios of β chain to β -actin at 0, 0.25, and 1 mM niacin were 1.824 ± 0.058 , 1.807 ± 0.116 , and 1.887 ± 0.263 , respectively).

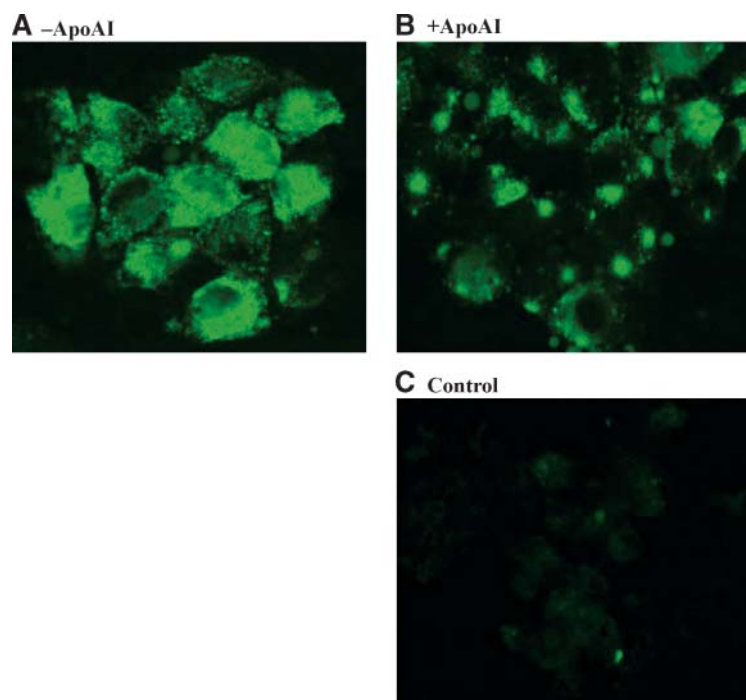


Fig. 1. Immunofluorescent confocal microscopic analysis of ATP synthase β chain expression in HepG2 cells. HepG2 cells grown on coverslips were preincubated without (A) or with (B) 10 $\mu\text{g}/\text{ml}$ apolipoprotein A-I, or with (C) control mouse IgG1 at 22°C for 30 min. After washing with PBS, the cells were immunostained with antibody against human ATP synthase β chain and Alexa Fluor 488-conjugated goat anti mouse IgG1, and analyzed by confocal microscopy.

The ATP synthase β chain is a mitochondrial protein. It was identified to be functioning as a hepatic apoA-I/HDL receptor when it presents on the cell surface. To enhance assay sensitivity, we assessed experimental conditions for overexpression of ATP synthase β chain on the cell surface, and HDL endocytosis. Overexpression of the β chain by cell transfection did not significantly increase its cell surface presentation (data not shown), suggesting that the surface β chain expression is not simply associated with changes in its total expression levels. Serum starvation (0.2% serum in the culture medium) has

been reported to increase cell surface expression of ATP synthase α chain in THP-1 cells and lymphocytes (21). We applied this condition to test β chain in HepG2 cells. Serum starvation did not significantly change overall β chain mRNA expression (data not shown). Flow cytometric analysis of immunofluorescence-stained HepG2 cells demonstrated that lowering serum concentrations from 10% to 0.2% in the culture medium dramatically increased cell surface expression of β chain by $\sim 77\%$ (mean fluorescence) (**Fig. 2A**). As a result, ^{125}I -labeled HDL uptake by HepG2 cells was also increased by $\sim 70\%$ (**Fig. 2B**).

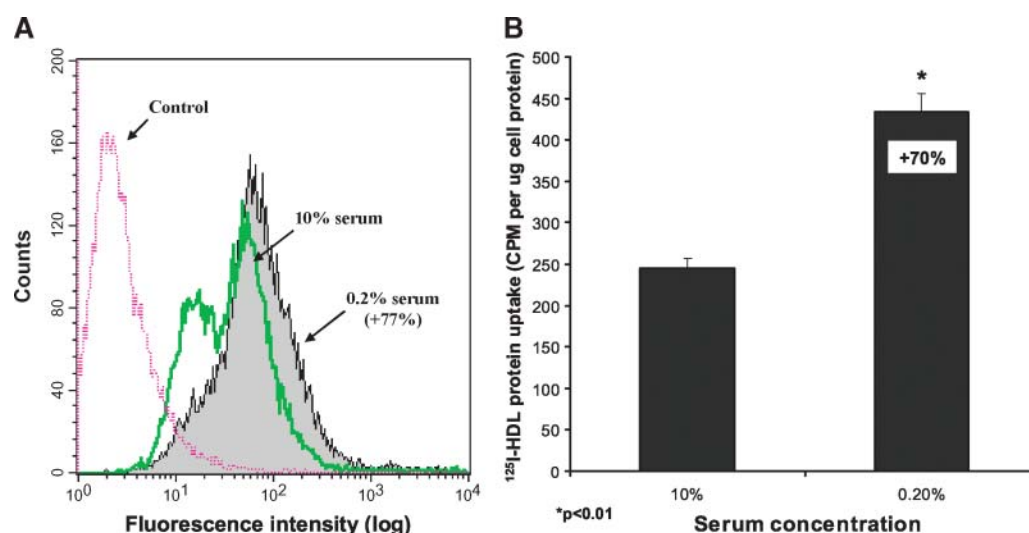


Fig. 2. Lowering serum concentration increased cell surface expression of ATP synthase β chain (A) and ^{125}I -labeled HDL uptake by HepG2 cells (B). HepG2 cells were incubated with DMEM medium containing 10% or 0.2% FBS at 37°C for 48 h, and then subjected to flow cytometric analysis after immunofluorescence staining of intact cells with anti- β chain antibody/FITC-conjugated goat anti-mouse IgG (A) (control: with isotypic IgG), and to ^{125}I -labeled HDL uptake assays (B). Data are expressed as mean \pm SD of triplicates.

Using regular cell culture conditions containing 10% FBS, initial studies indicated that niacin inhibited the surface β chain expression by $\sim 15\%$ (95.2 ± 24.9 vs. 80.4 ± 20.0 mean fluorescence, $P = 0.021$) and ^{125}I -labeled HDL uptake by $\sim 12\%$ (125.9 ± 4.2 vs. 110.6 ± 8.9 cpm/ μg cell protein, $P = 0.027$). We then further tested whether niacin inhibits β chain surface expression and HDL uptake in HepG2 cells grown in 0.2% serum culture medium. Under lower serum culture conditions, niacin treatment reduced cell surface expression of β chain by $\sim 27\%$ in a dose-dependent manner, as indicated by a shift in the distribution of the cells to lower fluorescence intensity and lower mean fluorescence (**Fig. 3A, B**). In some experiments, the reduced fluorescence resulted in the formation of a small second peak with left-shift, which may be from part of the HepG2 cells with the decreased cell surface β chain protein (not shown). Similarly, under the same conditions, niacin inhibited the uptake of ^{125}I -labeled HDL by HepG2 cells up to $\sim 35\%$ (**Fig. 4**). However, nicotinamide, a niacin metabolite, did not significantly reduce ^{125}I -labeled HDL uptake by HepG2 cells, because it has a much weaker inhibitory effect on the β chain surface expression at 0.25 and 0.5 mM concentrations, and only modestly decreased surface expression (-6%) at 1 mM (Figs. 3, 4).

To further test whether the β chain is involved in niacin action, we used the β chain antibody to block the

surface β chain prior to the HDL uptake assay (**Fig. 5**). As compared with mouse IgG control, the β chain antibody significantly reduced ^{125}I -labeled HDL uptake by HepG2 cells, and niacin did not show a further inhibitory effect (**Fig. 5**), suggesting that niacin inhibits hepatic HDL uptake through the surface β chain.

DISCUSSION

In this report, we show, for the first time, that niacin decreases the surface expression of the β chain of ATP synthase in cultured HepG2 cells. As indicated earlier, ATP synthase is a mitochondrial enzyme, and hepatic surface expression of its β chain is necessary for the holoparticle catabolism of HDL. A key element in this HDL catabolic process is the interaction of apoA-I with the β chain, resulting in a series of yet-to-be-defined steps culminating in HDL catabolism (15). This pathway is quite different from the SR-BI receptor, which mediates the selective removal of HDL-cholesteryl esters but not the HDL particle (11, 12). Recent studies have shown that niacin had no effect on the cellular surface expression of SR-BI and the SR-BI-mediated HDL uptake (13). These data, together with our studies, suggest that the effects of niacin on surface expression of β chain ATP synthase would be specific rather than generalized effects on surface

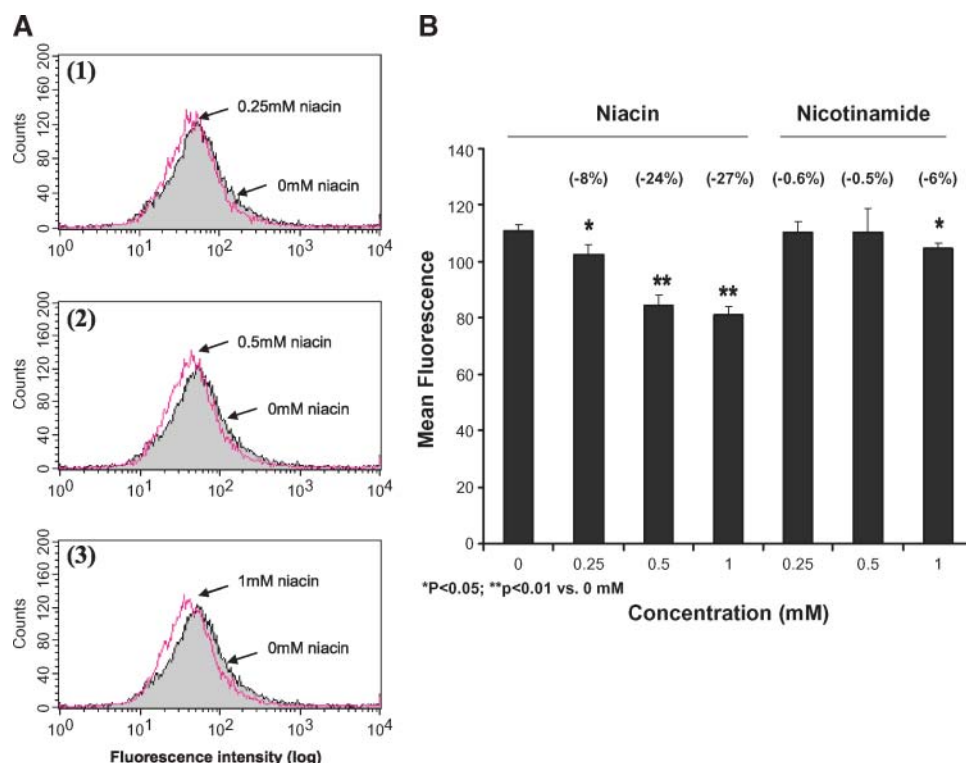


Fig. 3. Niacin decreased the surface expression of ATP synthase β chain in HepG2 cells. HepG2 cells were preincubated with or without niacin or nicotinamide in DMEM medium containing 0.2% FBS at 37°C for 48 h. β chain ATP synthase on the cell surface was immunostained with monoclonal antibody against human β chain/FITC-conjugated goat anti-mouse IgG F(ab')₂, and determined by flow cytometry. A: Representative flow cytometric histograms from three independent experiments. B: Mean fluorescence \pm SD from three experiments. Percent changes in parentheses are compared with 0 mM control.

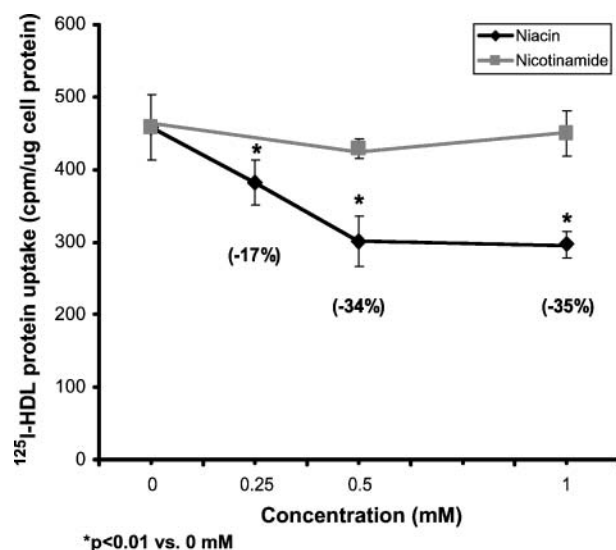


Fig. 4. Niacin but not nicotinamide inhibited ^{125}I -labeled HDL uptake by HepG2 cells. HepG2 cells were preincubated with or without niacin or nicotinamide in DMEM medium containing 0.2% FBS at 37°C for 48 h. After washing with PBS, HepG2 cell uptake assays were conducted in DMEM medium containing 5 mg/ml BSA free of fatty acid, and 10 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled HDL at 37°C for 16 h. Data are expressed as mean \pm SD of triplicates. Percent changes in parentheses are compared with 0 mM niacin.

proteins. Our key finding in this study suggests a potential molecular site of action of niacin, and significantly extends our previous reports that niacin decreases uptake of HDL protein in cultured HepG2 cells (9, 10).

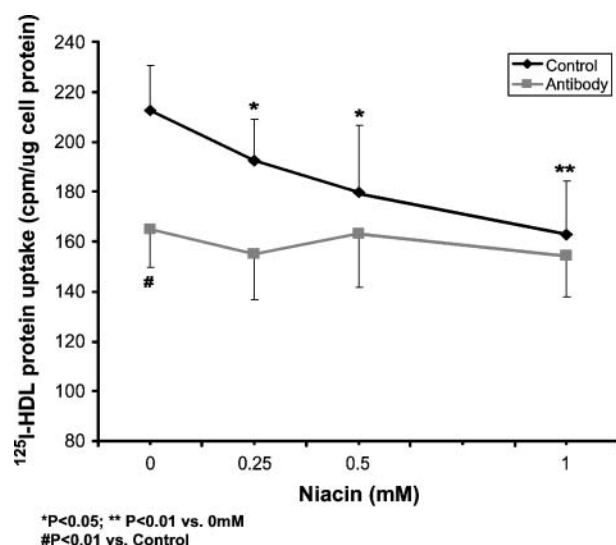


Fig. 5. Anti- β chain antibody reduced ^{125}I -labeled HDL uptake by HepG2 cells and eliminated the inhibitory effect of niacin. HepG2 cells were preincubated with niacin in DMEM medium containing 0.2% FBS at 37°C for 48 h. After washing with PBS, HepG2 cell uptake assays were conducted in DMEM medium containing 5 mg/ml BSA free of fatty acid, and prior to addition of ^{125}I -labeled HDL, incubated with 10 $\mu\text{g}/\text{ml}$ anti- β chain antibody or mouse IgG control at 37°C for 30 min. Data are expressed as mean \pm SD of triplicates.

In this investigation, we improved our previous methodology for quantifying HDL protein uptake by cultured HepG2 cells. On the basis of a report that serum starvation in cultured THP-1 and lymphocytes increases surface ATP synthase α chain presence (21), we found that compared with our previous assay, a reduction in serum concentration (0.2%) resulted in a significant increase in surface abundance of β chain by $\sim 77\%$ (Fig. 2A) and HDL-protein uptake by 70% (Fig. 2B), compared with 10% serum concentration (9, 10). We used this modified procedure in order to increase the sensitivity of our assay. Niacin also decreased both β chain surface expressions by up to 27% and HDL protein uptake by up to 35% in HepG2 cells. Thus, the modification greatly enhanced the sensitivity to detect effects of niacin on HDL protein uptake as reported previously (9, 10).

That niacin's effect was specifically targeted at the HepG2 cell surface expression of the ATP synthase β chain is supported by the results of several experimental approaches described in this communication. Using a commercially available specific β chain antibody, and an appropriate control isotypic antibody, we found that preincubation with apoA-I markedly diminished the binding of the antibody to β chain as assessed by confocal microscopy (Fig. 1). Using flow cytometry and a radio-labeled HDL protein assay, niacin significantly decreased both the surface abundance of β chain and HDL protein uptake, respectively (Figs. 3, 4). Importantly, nicotinamide, a niacin metabolite, which has no effect on HDL levels clinically, had no appreciable effect on β chain surface expression or HDL protein uptake. Preincubation with the specific ATP synthase β chain antibody abolished the effect of niacin on HDL protein uptake (Fig. 5), suggesting strongly that niacin's action was dependent on the availability of surface β chain in HepG2 cells.

The exact mechanism for niacin's action in reducing the surface presence of ATP synthase β chain is unclear at present and requires further investigation. Of preliminary significance is our observation that niacin had no effect on mRNA levels of ATP synthase β chain. Overexpression of ATP synthase by cell transfection did not result in increased surface presence of β chain. These data suggest that niacin may affect some step(s) in the translocation of β chain between mitochondria and cell surface. This hypothesis merits further research, because it would more specifically define the locus of its action. Not much is known about the translocation pathway and the steps involved in this important process (20–23).

Recent reports have indicated the presence of a specific niacin receptor, GPR109A, which may mediate some of the actions of niacin. This receptor is found in adipose tissue and in epidermal Langerhans cells, where it may mediate decreased lipolysis and prostaglandin D_2 production (resulting in flushing), respectively (as reviewed in Ref. 6). In the current study, it was found that GPR109A is not expressed in HepG2 cells (data not shown). Thus, it is unlikely that the niacin receptor may have a role in

niacin's effect on β chain expression in our HepG2 cell culture system.

When taken in the context of previous research on niacin's mechanism on HDL, the following proposed scheme is offered to explain how niacin's unique mechanism of action makes it a potent agent for atherosclerosis treatment. We propose that niacin downregulates the surface expression of the β chain of ATP synthase, which results in a decrease in the holo-particle removal of HDL, leading to higher plasma levels of largely apoA-I-rich particles. Increased accumulation of such particles results in a higher capacity and efficiency not only for effluxing cholesterol from peripheral tissues (e.g., arterial endothelium) but also for delivering cholesterol to the liver via the SR-BI pathway, which importantly, is unaffected by niacin (13). In addition, the cholesterol in such HDL is removed via the LDL-receptor pathway through the cholesteryl ester transfer protein (CETP)-mediated cholesterol transfer between the HDL and LDL. By this mechanism, niacin administration ultimately results in an accelerated reverse cholesterol transport.

Because the β chain of ATP synthase interacts primarily with apoA-I, its downregulation and the ensuing increase in plasma apoA-I concentration result in significant improvements in many of the beneficial properties of this HDL protein beyond the reverse cholesterol transport. These properties include anti-oxidant, anti-inflammatory, anti-thrombotic, and anti-endothelial dysfunction actions, all of which ameliorate atherosclerosis and its complications (2, 6).

Recent developments in atherosclerosis treatment have identified raising HDL as an important therapeutic target. However, HDL is a very complex class of molecules that are affected by many mechanisms, including the ATP binding cassette transporter proteins (e.g., ABCA1, ABCG1, etc.), enzymes (e.g., lipoprotein lipases, LCAT, ACAT, etc.), lipid transfer proteins (e.g., cholesteryl ester transfer protein, phospholipid transfer protein), nuclear receptors [e.g., peroxisome proliferator-activated receptors (PPARs), liver X receptor, etc.), and membrane receptors (e.g., SR-BI). Except for PPAR agents (fibrates), other pharmacologic HDL agents have not been found to be successful in human clinical trials for atherosclerosis, except for apoA-I Milano, in a small study (24). An important recent trial using torcetrapib, a CETP inhibitor, had to be prematurely terminated because of increased cardiovascular morbidity (25). It is unclear whether this toxicity was a result of depressing reverse cholesterol transport or related to the pharmacologic agent. Because niacin is a proven drug for atherosclerosis treatment, the unique mechanism by which it raises HDL may also be significant in the future development of HDL-based therapies.

In summary, the data presented in this communication strongly point to the β chain of ATP synthase as a molecular target site for action of niacin to raise apoA-I (and HDL) by decreasing its catabolism. The findings add to our expanding knowledge of the mechanism of niacin action on its wide range of clinical effects, includ-

ing apoA-I- and apoB-containing lipoproteins, and anti-inflammatory and anti-thrombotic and flushing effects.

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