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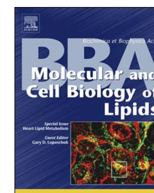

Activation of hepatic Nogo-B receptor expression—A new anti-liver steatosis mechanism of statins

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A B S T R A C T

Deficiency of hepatic Nogo-B receptor (NgBR) expression activates liver X receptor α (LXRα) in an adenosine monophosphate-activated protein kinase α (AMPKα)-dependent manner, thereby inducing severe hepatic lipid accumulation and hypertriglyceridemia. Statins have been demonstrated non-cholesterol lowering effects including anti-nonalcoholic fatty liver disease (NAFLD). Herein, we investigated if the anti-NAFLD function of statins depends on activation of NgBR expression. *In vivo*, atorvastatin protected apoE deficient or NgBR floxed, but not hepatic NgBR deficient mice, against Western diet (WD)-increased triglyceride levels in liver and serum. *In vitro*, statins reduced lipid accumulation in nonsilencing small hairpin RNA-transfected (shNSi), but not in NgBR small hairpin RNA-transfected (shNgBRi) HepG2 cells. Inhibition of cellular lipid accumulation by atorvastatin is related to activation of AMPKα, and inactivation of LXRα and lipogenic genes. Statin also inhibited expression of oxysterol producing enzymes. Associated with changes of hepatic lipid levels by WD or atorvastatin, NgBR expression was inversely regulated. At cellular levels, statins increased NgBR mRNA and protein expression, and NgBR protein stability. In contrast to reduced cellular cholesterol levels by statin or β-cyclodextrin, increased cellular cholesterol levels decreased NgBR expression suggesting cholesterol or its synthesis intermediates inhibit NgBR expression. Indeed, mevalonate, geranylgeraniol or geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate or farnesol, blocked atorvastatin-induced NgBR expression. Furthermore, we determined that induction of hepatic NgBR expression by atorvastatin mainly depended on inactivation of extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (Akt). Taken together, our study demonstrates that statins inhibit NAFLD mainly through activation of NgBR expression.

1. Introduction

In general, the nonalcoholic fatty liver disease (NAFLD) is characterized by accumulation of excessive triglycerides (TG) in hepatocytes of the patients without alcohol intake, chronic viral hepatitis or other liver diseases. NAFLD is one of the most common chronic liver diseases worldwide. The spectrum of NAFLD covers from the simple steatosis, a benign and non-progressive condition, to nonalcoholic steatohepatitis (NASH) which is characterized by hepatocellular necroinflammation and ballooning, and may further progress to liver fibrosis, cirrhosis and in some cases hepatocellular carcinoma [1]. Although about 20–30% of the general population may have NAFLD, a much higher prevalence of NAFLD (> 75%) can be found in patients with insulin resistance [2]. NAFLD is also considered as an independent

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risk factor for cardiovascular diseases [3]. The excessive lipid accumulation in the liver of NAFLD patients can be attributed to multiple factors, such as the increased uptake of plasma non-esterified fatty acids and *de novo* lipogenesis [1]. Statins, a class of medicines used for treatment of patients with hypercholesterolemia, can reduce cardiovascular diseases by 30–40%. Statins reduce serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels by, 1) inhibiting activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), the rate-limiting enzyme which catalyzes the conversion of HMG−CoA into mevalonate, thereby reducing *de novo* cholesterol synthesis; 2) activating hepatic LDL receptor (LDLR) expression which enhances cholesterol catabolism and excretion [4]. In plasma, TG is mainly presented in different lipoproteins, particularly in very low-density lipoprotein (VLDL) and LDL. In addition to cholesterol-lowering effects, statins can decrease TG levels in hypertriglyceridemic patients, even with a greater effect than that on LDL-C levels [5]. For instance, atorvastatin does not affect TG distribution but consistently reduces it in all lipoprotein fractions in hypertriglyceridemic patients. The epidemiological studies have demonstrated that statins can reduce NAFLD in overweight individuals. The clinical observational studies have shown positive outcomes of statin treatment on NAFLD patients [6–10]. However, the mechanisms by which statins protect patients against NAFLD have not been fully elucidated.

Nogo-B, also known as Reticulon 4B, is a member of the family of reticulon proteins. High expression of Nogo-B can be determined in caveolin-1 enriched microdomains or/and lipid rafts of endothelial cells (ECs) and vascular smooth muscle cells [11]. NgBR is a receptor specific for Nogo-B [12]. So far, several biological functions of NgBR have been identified. NgBR expression is necessary for Nogo-B stimulated chemotaxis and morphogenesis of ECs [12]. In zebrafish, NgBR expression is essential for angiogenesis [13]. Niemann-Pick type C2 (NPC2) is a protein for cholesterol trafficking between cellular membranes. The interaction between NgBR and NPC2 increases stability of NPC2 protein, thereby enhancing NPC2-mediated intracellular cholesterol trafficking. Thus, NgBR can play an important role in lipid metabolism [14]. We recently reported that deficiency of hepatic NgBR expression activates liver X receptor α (LXRα) through an adenosine monophosphate-activated protein kinase α (AMPKα)-dependent pathway, thereby resulting in liver steatosis which is due to TG accumulation [15]. Because of the anti-liver steatosis properties of statins and the importance of NgBR in hepatic lipogenesis, we hypothesized that inhibition of NAFLD by statins is completed, at least in part, by activating hepatic NgBR expression.

2. Materials and methods

2.1. Materials

Rabbit anti-NgBR antibody was generated as described [13]. Rabbit anti- extracellular signal-regulated kinases 1/2 (ERK1/2), phosphorylated ERK1/2 (pi-ERK1/2), protein kinase B (Akt) and phosphorylated Akt (pi-Akt) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-GAPDH, fatty acid synthase (FASN), AMPKα, phosphorylated AMPKα (pi-AMPKα), sterol-regulatory element binding protein 2 (SREBP2) and HMGCR antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-LXRα and Lamin A polyclonal antibodies were purchased from Proteintech Group, Inc. (Rosemont, IL). LDL was purchased from Athens Research & Technology, Inc. (Athens, Georgia). Manumycin A and GGTI-286 were purchased from Merck Millipore (Nottingham, UK). PD98059, U0126, LY294002, SP600125 and SB203580 were purchased from Selleck Chemicals (Houston, TX). Atorvastatin was purchased from AK Scientific Inc. (Palo Alto, CA). Farnesyl pyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), geranylgeraniol (GGOH), farnesol (FOH) and water soluble-cholesterol (Chol/CD) were purchased from Sigma-Aldrich (St Louis, MO).

PTEN expression vector (C2-PTEN) was prepared as follows: the cDNA encoding mouse PTEN was generated by reverse transcription with total cellular RNA isolated from differentiated 3 T3-L1 adipocytes and an oligo(dT)$_{18}$ primer, followed by PCR with the forward primer (5′-GGGAATTCATGACAGCCATCATCAAAGAGATCG-3′) and the backward primer (5′-CGGGATCCTCAGACTTTTGTAATTTGTGAATGC-3′). After the sequence was confirmed, the PCR product was digested with *Eco*RI and *Bam*HI followed by ligation into the pEGFP-C2 expression vector. Mature SREBP2, MKK1* and Akt1* expression vectors (They are also ligated into the pEGFP-C2 vector) were purchased from Addgene (Cambridge, MA).

2.2. In vivo studies

The protocols for animal study were approved by the Ethics Committees from Nankai University and Medical College of Wisconsin, respectively, and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH), USA.

Liver specific NgBR deficient (NgBR^{LKO}) and the littermate control (NgBR floxed, NgBR$^{fl/fl}$) mice were generated as described [15]. ApoE deficient (apoE$^{-/-}$) mice were purchased from the Animal Center of Nanjing University (Nanjing, China). ApoE$^{-/-}$, NgBR^{LKO} and NgBR$^{fl/fl}$ mice (males, ~8-week old) were randomly divided into 3 groups (6 mice/group), and fed normal chow, Western diet (WD, 21% fat and 0.5% cholesterol) or WD containing atorvastatin [15 mg/day/kg bodyweight (mpk)] for 2 weeks, respectively. During the treatment, we routinely checked the food intake, body weight gain and water drinking, and did not observe difference between control mice and mice receiving atorvastatin treatment, indicating the high safety with atorvastatin at this dose. At the end of experiment, all the mice were anesthetized and euthanized by i.p injection of 2,2,2-tribromoethanol (640 mg/kg bodyweight) followed by collection of liver and serum samples. Serum TG and TC levels were analyzed by enzymatic methods with an automatic biochemical analyzer (Model 7020; Hitachi, Tokyo, Japan).

2.3. Cell culture

HepG2 cells, a human hepatic cell line (ATCC, Manassas, VA), were cultured in MEM medium containing 10% fetal calf serum, 50 μg/ml penicillin/streptomycin and 2 mM glutamine. Both nonsilencing small hairpin RNA-transfected (shNSi) cells and NgBR small hairpin RNA-transfected (shNgBRi) cells were established as described [15]. Cells received treatment at ~85% confluence. HepG2 cells lacking AMPKα or LXRα expression were established using the CRISPR-Cas9 technology, respectively. The selection of mutated clonal cell lines was completed using the standard protocol, and the knockout of target gene expression was confirmed by Western blot. The cells lacking AMPKα or LXRα expression were named as Cas9-AMPKα or Cas9-LXRα cells, and the corresponding control cells as Cas9-NS cells.

Mouse primary hepatocytes were isolated from C57BL/6 mice (males, ~8-week old, also purchased from the Animal Center of Nanjing University) by a collagenase perfusion method. Briefly, after anesthetized the midline laparotomy was performed, and the inferior vena cava was cannulated with an angiocatheter. The liver was then perfused with 1 ml heparin (320 U/ml), 40 ml solution I (Kreb's solution containing 0.1 mM EGTA) and 30 ml solution II (Kreb's solution containing 2.74 mM $CaCl_2$ and 0.05% collagenase I) at 37 °C, sequentially. The perfused liver was then passed through a 400 μm screening size filter by flushing with the cold DMEM medium. The isolated hepatocytes were collected after centrifuge for 5 min at 50 g, re-suspended with DMEM medium and plated in 6-well plates (the cell density is ~1 × 10^6 cells/well). The viability of the isolated hepatocytes was ~90% which was determined by the method of trypan blue exclusion.

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