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Nordihydroguaiaretic acid protects against high-fat diet-induced fatty liver by activating AMP-activated protein kinase in obese mice

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ABSTRACT

Nonalcoholic fatty liver disease, one of the most common causes of chronic liver disease, is strongly associated with metabolic syndrome. Nordihydroguaiaretic acid (NDGA) has been reported to inhibit lipoprotein lipase; however, the effect of NDGA on hepatic lipid metabolism remains unclear. We evaluated body weight, adiposity, liver histology, and hepatic triglyceride content in high-fat diet (HFD)-fed C57BL/6J mice treated with NDGA. In addition, we characterized the underlying mechanism of NDGA's effects in HepG2 hepatocytes by Western blot and RT-PCR analysis. NDGA (100 or 200 mg/kg/day) reduced weight gain, fat pad mass, and hepatic triglyceride accumulation, and improved serum lipid parameters in mice fed a HFD for 8 weeks. NDGA significantly increased AMP-activated protein kinase (AMPK) phosphorylation in the liver and in HepG2 hepatocytes. NDGA downregulated the level of mature SREBP-1 and its target genes (acetyl-CoA carboxylase and fatty acid synthase), but, it upregulated expression of genes involved in fatty acid oxidation, such as peroxisome proliferator-activated receptor (PPAR)α, PPARγ coactivator-1, carnitine palmitoyl transferase-1, and uncoupling protein-2. The specific AMPK inhibitor compound C attenuated the effects of NDGA on expression of lipid metabolism-related proteins in HepG2 hepatocytes. The beneficial effects of NDGA on HFD-induced hepatic triglyceride accumulation are mediated through AMPK signaling pathways, suggesting a potential target for preventing NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), a major cause of liver disease, is strongly associated with obesity, insulin resistance, and hypertension [1]. Adaptation of the western lifestyle has rapidly increased the prevalence of NAFLD, which is estimated to occur in approximately 75–100% of the obese population [2]. The pathogenesis of NAFLD is not completely understood, but recent research has revealed that excess fat accumulation in the liver plays a direct role in its initiation and development. Treating NAFLD requires the prevention of hepatic fat accumulation and a better understanding of the molecular mechanisms involved in lipid metabolism [3]. Mounting evidence suggests that AMP-activated protein kinase (AMPK) and sterol regulatory element binding protein (SREBP) are critical regulators of hepatic lipid metabolism [4,5].

AMPK functions as a cellular energy regulator [6]. During energy depletion, AMPK inhibits de novo fatty acid synthesis by inactivating acetyl-CoA carboxylase (ACC) and stimulates fatty acid oxidation by upregulating gene expression of carnitine palmitoyltransferase-1 (CPT-1), peroxisome proliferator-activated receptor (PPAR)α, and uncoupling protein (UCP) [7]. SREBPs are transcription factors that regulate expression of lipogenic enzymes, such as ACC, fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) [8]. Considerable studies have reported that increased SREBP expression is strongly associated with fatty liver in two mouse models of diabetes mellitus [9,10]. Recently, it has been reported that AMPK inactivates SREBP-1 and inhibits hepatic steatosis in high-fat diet-induced animal models [11]. Therefore, AMPK and SREBP have emerged as particularly promising therapeutic targets to prevent fatty liver disease.

Nordihydroguaiaretic acid (NDGA) is a plant lignan derived from the *Larrea divaricata*. NDGA, widely known as lipoxygenase inhibitor, possesses antioxidant and anti-cancer properties [12,13] and inhibits lipoprotein lipase, an enzyme responsible for maintaining the level of circulating fatty acids [14]; however, the metabolic effects of NDGA are unclear. In the present study, we determined the effect of NDGA on body fat accumulation and fatty liver disease in high-fat diet (HFD)-fed mice. In addition, we characterized the molecular mechanism underlying the NDGA effect in HepG2 hepatocytes.

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2. Materials and methods

2.1. Animal studies

Thirty-two male C57BL/6J mice (DooYeol Biotech, Seoul, Korea), 4 weeks of age, were housed in a controlled environment (25 ± 2 °C, 55 ± 5% relative humidity with a 12 h light–dark cycle). Throughout the experiment, the mice were allowed free access to food and tap water. After acclimatization for 1 week, eight mice received the AIN93G (DooYeol Biotech) normal diet (Normal), which provided 64.7% of energy as carbohydrates, 18.7% as protein, and 7% as fat. The remaining 24 mice were fed with a high-fat diet (Rodent diet D1245; Research Diet, New Brunswick, NJ), providing 45% of energy as fat, 35% as carbohydrates, and 20% as protein. After 7 weeks of dietary manipulation to induce obesity, 24 animals in the high-fat diet group were divided into three experimental groups (each group, $n = 8$): Group 1 received a high-fat diet (HFD; 45% of energy as fat); Group 2 received the HFD with low-dose NDGA (100 mg/kg/day); Group 3 received the HFD with high-dose NDGA (200 mg/kg/day). NDGA was dissolved in water and animals were orally administrated with NDGA at doses of 100 or 200 mg/kg/day by oral zonde for 8 weeks. Oral administration volume was approximately 200 μl per mouse. Mice in the Normal group and the HFD group were given an equal volume of water. Water, food intake and body weight were measured twice per week throughout the experiment.

At the end of the 8-week oral administration period, all mice were sacrificed with diethyl ether after an overnight fast. Their fat pads and liver were removed, weighed, and frozen in liquid nitrogen. Micro-computed tomography (micro-CT) experiments were performed with an animal positron emission tomography (PET)/CT/single photon emission computed tomography (SPECT) system (INVEON, Siemens, USA) at the Korea Basic Science Institute in Ochang. This study adhered to the Guide for the Care and Use of Laboratory Animals developed by the Institute of Laboratory Animal Resources of the National Research Council, and was approved by the Institutional Animal Care and Use Committee of Yonsei University in Seoul, Korea.

2.2. Chemicals and reagents

NDGA (a minimum 90% purity) and insulin was purchased from Sigma Chemicals (St. Louis, MO). NDGA was further purified 99% or more using preparative HPLC (column: GS-310, 20.0 mm ID × 500 mm L, Japan Analytical Industry Co., Ltd., Tokyo, Japan) eluted with 100% methanol. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Welgene (Daegu, Korea). Antibodies against ACC, phosphorylated ACC (Ser79), AMPK, and phosphorylated AMPK (Thr172) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against SREBP-1; active amino terminal fragment of SREBP-1, FAS, PPARα, PPARγ coactivator-1 (PGC-1), CPT-1 liver form (CPT-1L), UCP2, UCP3, and α-tubulin, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Compound C were purchased from Calbiochem (San Diego, CA) and electrochemiluminescence solution for Western blot analysis was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

2.3. Blood analysis

Blood was collected by heart puncture from all mice and held at room temperature for 1 h; serum was then prepared by centrifugation at 4000 rpm for 15 min and stored at −70 °C until analysis. Serum lipid profiles were determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN, USA).

2.4. Hepatic histology and triglyceride analysis

Liver tissues obtained from all mice were embedded in tissue-freezing medium (Leica, Wetzlar, Germany) and fixed as previously described [15]. After fixation, they were stained with hematoxylin and eosin (H&E) and analyzed for hepatic lipid accumulation and adipocyte size with an Eclipse TE2000U Inverted Microscope with twin CCD cameras (magnification, ×200; Nikon, Tokyo, Japan). To determine hepatic triglyceride content, liver tissue homogenates were mixed with chloroform–methanol solution (chloroform–methanol–water 8:4:3). The mixture was shaken for 1 h and then centrifuged at 12,000 rpm for 15 min. The bottom layer was collected and resuspended for analysis of hepatic lipids. Total lipids were measured with the enzymatic hydrolysis method.

2.5. Cell culture

HepG2 human hepatoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM supplemented with antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. To investigate the hypolipidemic effects of NDGA, we pretreated HepG2 hepatocytes with insulin (1 μM) for 12 h, and then HepG2 cells were treated with or without NDGA (5–50 μM) for another 12 h. After NDGA treatment, hepatic triglyceride content was measured by oil red O staining.

2.6. Western blot analysis

Homogenized tissues were lysed with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% deoxycholate, 1 mM EDTA, and 1 mM PMSF) and incubated on ice for 10 min. The mixture was then centrifuged, and the supernatant was used to determine protein concentration. After treatment of NDGA (5–50 μM for 48 h), HepG2 hepatocytes were lysed and whole cell lysates were collected to evaluate the effects of NDGA on expression of lipid metabolism-related proteins. To measure phosphorylation of AMPK and ACC by NDGA, we treated HepG2 cells with NDGA (5–25 μM) for 30 min and whole cell lysates were collected. To evaluate whether an AMPK inhibitor, compound C, inhibits the effects of NDGA on expression of lipid metabolism-related proteins, HepG2 cells were preincubated for 30 min with or without compound C (10–20 μM). After treatment of NDGA (5–50 μM for 30 min) with the cells, HepG2 cells were lysed and proteins were extracted to evaluate the effect of NDGA on AMPK activation. Total protein (30 μg) was subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blotted membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies for 16 h at 4 °C. After three washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 2 h. Proteins were detected with the chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) and visualized with a LuminoImager (LAS-3000 Bio Imaging Analysis System; Fuji Film Co., Toktheyo, Japan).

2.7. Reverse-transcription PCR

After treatment of NDGA (5–50 μM for 24 h) with HepG2 cells, total RNA from the cells was isolated with Trizol reagent (Invitrogen, Madison, WI) and converted to cDNA with reverse transcriptase