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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

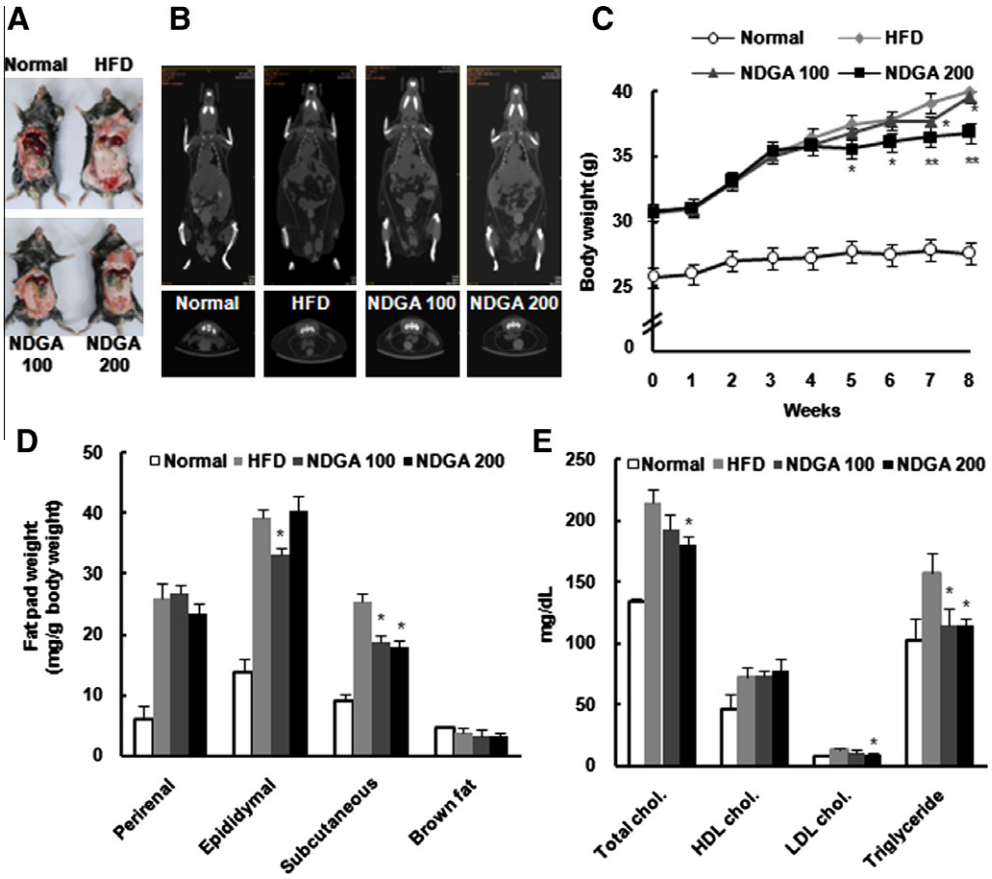

Fig. 1. Nordihydroguaiaretic acid attenuates high-fat diet-induced adiposity and weight gain. (A) Gross appearance and (B) Micro-computed tomography (CT) images of whole body and abdomen of mice fed a normal diet (Normal), high-fat diet (HFD), HFD with 100 mg/kg/day nordihydroguaiaretic acid (NDGA 100), or HFD with 200 mg/kg/day NDGA (NDGA 200). (C) Body weight changes in mice fed a normal diet, HFD, or HFD with NDGA (100 or 200 mg/kg/day). (D) Fat pad mass in mice fed a normal diet, HFD, or HFD with NDGA (100 or 200 mg/kg/day). (F) The serum levels of total cholesterol (Total chol.), low density lipoprotein cholesterol (LDL chol.), high density lipoprotein cholesterol (HDL chol.), and triglyceride, were evaluated. Data are expressed as mean ± SD, ($n = 8$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with HFD control (each treatment group, $n = 8$).

(Invitrogen, Carlsbad, CA). To quantify mRNA expression, the cDNA was amplified by PCR with gene-specific primers and AccessQuick™ RT-PCR System Master Mix (2×) (Promega, Madison, WI). The following primer sequences were used: human PGC-1α, 5′-CAGA CCTGACACAACACGG-3′ (sense) and 5′-CTTGAAA-AATTGCTTGCG TC-3′ (antisense); human PPARα, 5′-CGTCCTGGCCTTCT-AAACG TAG-3′ (sense) and 5′-CCTGTAGATCTCCTGCAGTAGCG-3′ (antisense); human CPT-1L 5′-ATGGCAG-AGGCTCACCAAGCTGTG-3′ (sense) and 5′-CCTCTGTGGTACACAACAATGTGC-3′ (antisense); human UCP2, 5′-ATGGTTGGACTGAAGCCTTCAG-3′ (sense) and 5′-TCA AAACGGTGATTCCCGTAAC-3′ (antisense); human SREBP-1, 5′-TCA ACAA-CCAAGACAGTGACTTCCCTGGCC-3′ (sense) and 5′-GTTCTC CTGCTTGAGC-TTCTGGTTGCTGTG-3′ (antisense); human FAS, 5′-T CGTGGGCTACAGCATGGT-3′ (sense) and 5′-GCCCTCTGAAGTCGA AGAAGAA-3′ (antisense); human ACC, 5′-CTGTAGAAACCCGGAC AG-TAGAAC-3′ (sense) and 5′-GGTCAGCATACATCT-CCATGTG-3′ (antisense); human β-actin, 5′-TGTGATGGTGGGAATGGGTCAG-3′ (sense) and 5′-TTTGATGTCACGCACGATTTCC-3′ (antisense).

2.8. Statistics

Results are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 9.0. Significance was assessed by analysis of various followed by a Duncan's test. Statistical significance was set at $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Effects of NDGA on high-fat diet-induced body weight gain and adiposity

Adiposity was considerably higher in the HFD control group than in mice fed a normal diet or those treated with NDGA, as shown by gross appearance (Fig. 1A) and micro-CT analysis (Fig. 1B). Further, mice receiving low-dose or high-dose NDGA gained less weight during the experimental period (Fig. 1C). At the end of the experiment, mice treated with low-dose NDGA gained 11% less weight ($p < 0.05$) and those treated with high-dose NDGA gained 39% less weight ($p < 0.01$) than mice in the HFD control group. Food intake did not significantly differ among the HFD groups (data not shown). These results suggested that NDGA reduces diet-induced body weight gain which is not attributed to the amount of food intake.

Next, the effects of NDGA on fat pads were evaluated. As shown in Fig. 1D, mice treated with low-dose or high-dose NDGA had less subcutaneous and epididymal fat compared with the HFD control group; however, this effect was not dose-dependent. In contrast, no significant difference in perirenal fat or brown fat was observed among treatment groups. In addition, the serum levels of total cholesterol, low density lipoprotein (LDL) cholesterol, and triglyceride were significantly decreased in mice treated with high-dose NDGA;

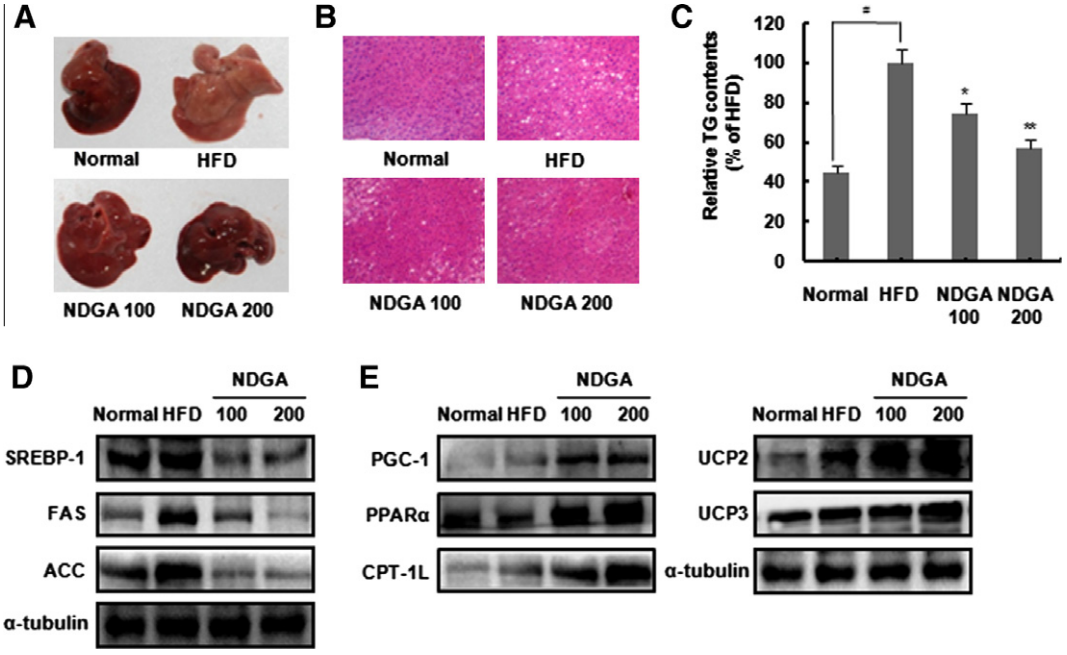

Fig. 2. Nordihydroguaiaretic acid prevents high-fat diet-induced liver steatosis in C57BL/6 J mice. (A) Gross appearance of the liver from mice fed a normal diet, HFD, or HFD with NDGA (100 or 200 mg/kg/day). (B) Histological analysis of liver of mice fed a normal diet, HFD, or HFD with NDGA (magnification,×200). (C) HFD-induced liver triglyceride levels in C57BL/6 J mice (each treatment group, $n = 8$). $^{\#}p < 0.01$ compared with Normal control. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with HFD control. Data are expressed as mean ± SD, ($n = 8$). (D) Western blot analysis of SREBP-1 and its target genes FAS and ACC in the liver. (E) Western blot analysis of PGC-1, PPARα, CPT-1L, UCP2, and UCP3 expression in the liver. α-Tubulin used as a protein loading control. Western blots shown represent the tendency of 8 mice in groups.

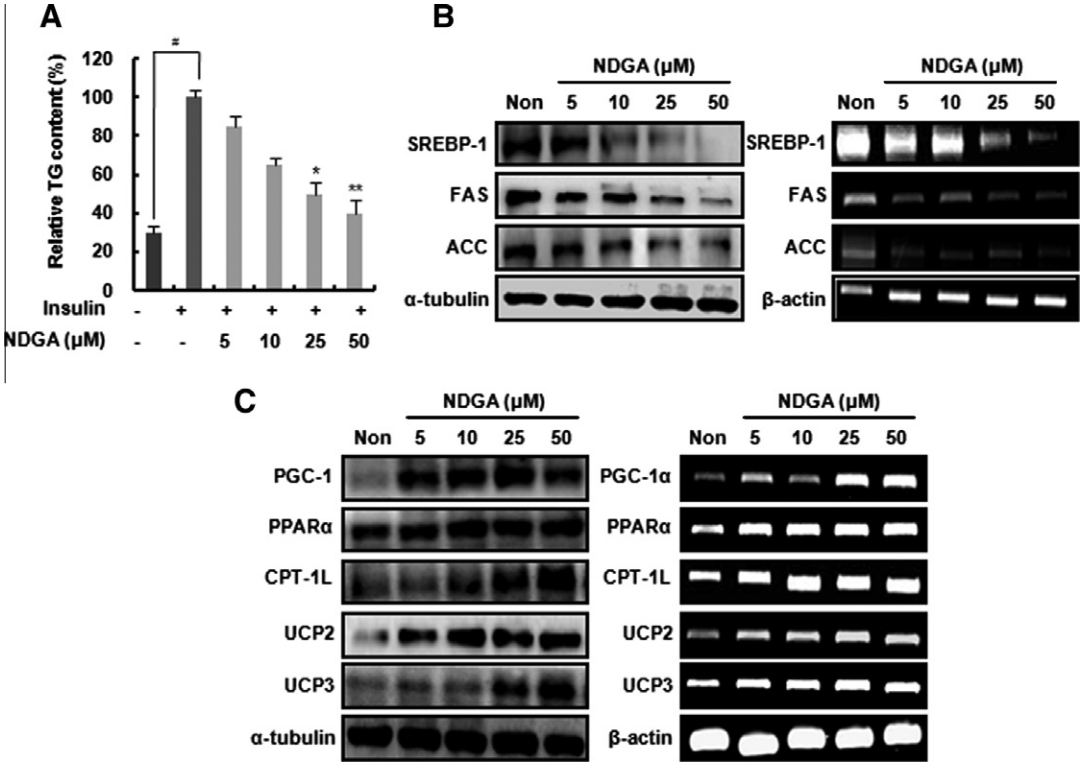

Fig. 3. Nordihydroguaiaretic acid reduces insulin-induced triglyceride accumulation in HepG2 cells. (A) HepG2 cells were preincubated with insulin for 12 h, and then were incubated with NDGA for another 12 h. All data are expressed as mean ± standard deviation from 3 independent experiments. $^{\#}p < 0.01$ compared with untreated control, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with insulin-treated cells. (B) Western blot analysis and RT-PCR analysis of SREBP-1 and its target genes FAS and ACC in HepG2 hepatocytes. (C) Western blot analysis and RT-PCR analysis of PGC-1, PPARα, CPT-1L, UCP2, and UCP3 protein expression in HepG2 hepatocytes treated with NDGA. α-Tubulin used as a protein loading control. Western blots shown represent three independent experiments.

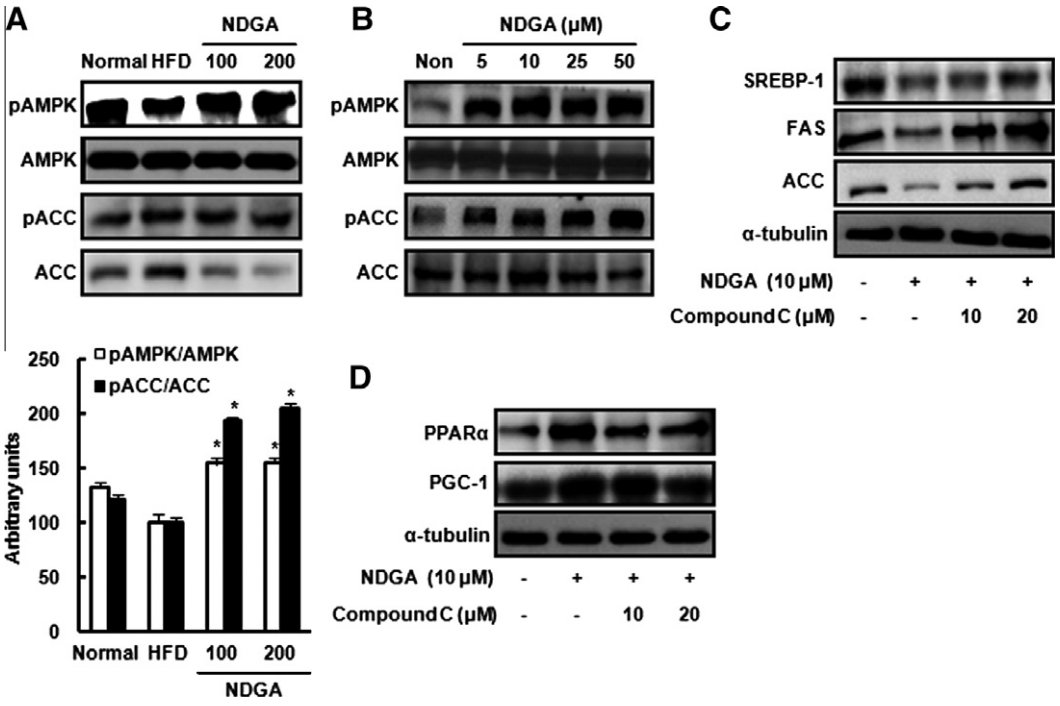

Fig. 4. Nordihydroguaiaretic acid activates AMPK *in vivo* and *in vitro*. (A) Western blot analysis of phosphorylated AMPK (pAMPK) and ACC (pACC) in the liver of mice fed a normal diet, HFD, or HFD with NDGA. The values are the means SD from 7–8 animals, and significance from HFD (*$p < 0.05$) was assessed by analysis of various followed by a Duncan's test. (B) Western blot analysis of pAMPK and pACC in HepG2 cells treated with NDGA (5–50 Mm). AMPK and ACC were used as a protein loading control of pAMPK and pACC, respectively. (C) Western blot analysis also shows that NDGA-induced expression of SREBP-1 and its target genes FAS and ACC was attenuated by a pretreatment with specific AMPK inhibitor, compound C, for 30 min. (D) Western blot analysis shows that NDGA-induced expression of PPARα was attenuated by a pretreatment with compound C, in HepG2 cells for 30 min. α-Tubulin used as a protein loading control. Western blots shown represent three independent experiments.

however, high density lipoprotein (HDL) cholesterol was not altered between the HFD group and NDGA-treated groups (Fig. 1F). These results suggest that NDGA administration might improve high-fat diet-induced obesity.

3.2. Effects of NDGA on hepatic triglyceride accumulation in the animal model

The gross appearance and H&E analysis of the liver revealed fatty infiltration in the HFD control group compared with mice fed the normal diet; however, fatty infiltration was not observed in livers of the NDGA-treated mice (Fig. 2A and B). As shown in Fig. 2C, NDGA treatment decreased HFD-induced hepatic triglyceride levels: low-dose NDGA reduced hepatic fat content by 21%, and high-dose NDGA by 40%.

To determine the molecular mechanism by which NDGA exerts hypolipidemic effects, Western blot analysis was performed to examine expression of proteins involved in lipid metabolism. As shown in Fig. 2D, NDGA decreased the level of mature SREBP-1 in the liver. Further, expression of SREBP-1 target genes FAS and ACC was reduced by NDGA. However, the level of lipolytic proteins, such as PPARα, PGC-1, CPT-1L, UCP2, and UCP3, was increased by NDGA treatment (Fig. 2E). These results suggest that NDGA regulates hepatic lipid metabolism through regulating expression of proteins related to lipid metabolism, eventually leading to both the suppression of lipogenesis and the fat degradation.

3.3. Effects of NDGA on insulin-induced triglyceride accumulation in HepG2 hepatocytes

To characterize hypolipidemic effects of NDGA *in vitro*, we treated HepG2 cells with insulin (1 μM) to induce hepatic lipid accumulation [16] and various concentration of NDGA. Consistent with *in vivo* data, NDGA dose-dependently reduced intracellular triglyceride accumulation; lipids were reduced by 60% at 50 μM NDGA (Fig. 3A) without cytotoxicity (data not shown). Western blot analysis revealed that the lipogenic mRNA and protein levels of ACC, FAS, and SREBP-1 were reduced in NDGA-treated HepG2 cells and the lipolytic mRNA and protein levels of PPARα, PGC-1, CPT-1L, UCP2, and UCP3 were increased (Fig. 3B and C). These results indicate that NDGA treatment decreases hepatic triglyceride accumulation in HepG2 cells, suggesting that reduced triglyceride accumulation were mechanisms by which NDGA reduced fat accumulation in the liver of the animal model.

3.4. Effects of NDGA on hepatic AMPK activation in vivo and in vitro

AMPK plays an important role in regulating fatty acid oxidation and lipogenesis in metabolic tissues [17]. To determine whether the hypolipidemic effect of NDGA could be mediated by AMPK, we examined AMPK phosphorylation, which indicates AMPK activation. NDGA supplementation dose-dependently increased AMPK and ACC phosphorylation compared to the HFD control group (Fig. 4A). These data suggest a role for AMPK in the NDGA effects on fat accumulation *in vivo*.

To investigate whether NDGA directly activates hepatic AMPK in the cell model, HepG2 cells were incubated with NDGA. As shown in Fig. 4B, NDGA stimulates AMPK phosphorylation in a dose-dependent manner. To confirm the role of AMPK in the regulation of metabolic gene expression, HepG2 hepatocytes were pre-incubated for 1 h with or without the selective AMPK inhibitor compound C. Compound C pretreatment also attenuated inhibitory effects of NDGA on SREBP-1, FAS, and ACC expression (Fig. 4C). NDGA-induced PPARα expression was blocked by compound C; however, PGC-1 expression was unchanged (Fig. 4D). These results support the role of AMPK as a mediator of NDGA's effects on

PPARα, SREBP-1, and SREBP-1-target gene expression. These results support the role of AMPK as a mediator of NDGA effects on PPARα, SREBP-1, and SREBP-1-target gene expression.

4. Discussion

In the present study, we examined the effects of NDGA on HFD-induced fat accumulation in white adipose tissues and the liver in C57BL/6J mice. Body weight gain, the serum levels of cholesterol and triglyceride, and hepatic fat accumulation were significantly lowered in NDGA-treated mice compared to the HFD control group. Typically, reductions in fat accumulation are accomplished by decreasing food consumption. However, food consumption did not differ significantly among the HFD-fed groups. These results suggest that NDGA attenuates fat accumulation by downregulating fatty acid synthesis and upregulating fatty acid oxidation.

The hypolipidemic effects of NDGA might be caused by altered hepatic lipid metabolism. We therefore assessed whether NDGA decreased hepatic lipogenesis through reducing the mature SREBP-1 level in mouse livers and HepG2 hepatocytes. In the present study, expression of mature SREBP-1 and its target lipogenic genes was markedly downregulated by NDGA. In addition, we examined effects of NDGA on expression of fatty acid oxidation gene in mouse liver and HepG2 hepatocytes. We found that NDGA significantly increased expression of genes involved in fatty acid transport, β-oxidation, and thermogenesis in both liver and HepG2 hepatocytes. These results imply that NDGA decreases hepatic fat accumulation without reducing food intake by inducing fatty acid oxidation as well as decreasing hepatic lipogenesis.

AMPK belongs to a family of serine/threonine kinases that regulates energy homeostasis. After activation by energy depletion (e.g., exercise), AMPK exerts multiple effects on genes involved in both glucose and fatty acid metabolism [18]. We hypothesized that AMPK may play an important role in the hypolipidemic effects of NDGA. We observed phosphorylation of AMPK and its direct substrate ACC, indicating that NDGA activated AMPK. Consistent with these results, pretreatment with the specific AMPK inhibitor compound C attenuated the effects of NDGA on lipid accumulation and expression of PPARα and SREBP-1 proteins. These observations suggest that the hypolipidemic effects of NDGA require the AMPK signaling pathway. Furthermore, we are the first to report that NDGA may attenuate hepatic fat accumulation by targeting AMPK.

The present study demonstrates that NDGA administration decreases hepatic triglyceride accumulation through AMPK-mediated modulation of fatty acid metabolism. Furthermore, we report novel hypolipidemic properties of NDGA for the first time and the molecular mechanism underlying its beneficial effects. Taken together, our findings demonstrate that NDGA may improve diet-induced fatty liver disease though regulating hepatic lipid metabolism.

Acknowledgments

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