Effects of exogenous DLK1 administration on the hepatic steatosis and fibrosis in two different murine models

Hyun Min Kim

Department of Medicine

The Graduate School, Yonsei University

Effects of exogenous DLK1 administration on the hepatic steatosis and fibrosis in two different murine models

Directed by Professor Bong Soo Cha

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Hyun Min Kim

June 2016

This certifies that the Doctoral Dissertation of Hyun Min Kim is approved.

Thesis Supervisor: Bong Soo Cha

Thesis Committee Member#1: Sahng Wook Park

Thesis Committee Member#2: Jae-woo Kim

Thesis Committee Member#3: Eun-Jung Rhee

Thesis Committee Member#4: Yong-ho Lee

The Graduate School
Yonsei University

June 2016

ACKNOWLEDGEMENTS

During my doctor's course, I have desperately felt I am not good enough to do it. Thanks to many people's helps and supports, I can complete it.

Most of all, I sincerely appreciate my academic advisor, Professor Bong Soo Cha. He led me from beginning to end of this research, encouraged me, endured me who is not good enough and gave me opportunities.

I would like to say a word of gratitude to the degree committee, Professor Sahng Wook Park, Jae-woo Kim, Eun-Jung Rhee, and Yong-ho Lee, too. Their practical advices and helps led me complete this study well. I appreciate Mi-Ra Yun and Eugene Shin who gave a great help in the experiment process. Also, especially, I thank Professor Jaetaek Kim from Chung-Ang University.

Thank God and lastly I would like to thank all my loving family. I sincerely appreciate my parents and parents in law who always pray for me and give me great helps and loves. Thank you and love you my dear husband, Heetae Yu, and my dearest Hyun Kyu and Hyun Ji.

June 2016
Hyun Min Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Effects of exogenous DLK1 administration on the hepatic steatosis and fibrosis in two different murine models

Hyun Min Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Bong Soo Cha)

Notch signaling activation is involved in development and progression of lipogenesis and fibrosis in liver, and results in various spectrums of non-alcoholic fatty liver disease (NAFLD). One of the endogenous inhibitors of Notch signaling, Delta-like 1 homolog (DLK1), is widely expressed in developing tissues. Recent finding showed the effect of DLK1 on modulating adipogenesis and muscle development. Therefore, we investigated the effect of exogenous DLK1 in vitro and in vivo on the development or progression of NAFLD.

A soluble DLK1 peptide was generated with fusion between a human Fc fragment and extracellular domain of DLK1. DLK1 were treated in the *db/db* mice and methionine and choline deficient (MCD) diet–fed mice for 4 weeks. HepG2 and LX-2 cells were used for in vitro experiments.

After exogenous DLK1 administration, hepatic triglyceride content and lipid droplets in liver tissues, as well as serum levels of liver enzymes, were markedly decreased in *db/db* mice. DLK1 treatment induced phosphorylation of AMP-activated protein kinase (AMPK) in hepatocytes and increasing fatty acid oxidation in liver. Furthermore,

DLK1-treated MCD diet-fed mice showed significantly lower levels steatosis and fibrosis, with decreased hepatocyte apoptosis compared with the vehicle-treated group. In activated LX-2 cells, DLK1 treatment increased AMPK activation, reduced TGF-β/Smad2/3 phosphorylation, and decreased fibrogenic gene expression. Furthermore, DLK1 treatment prevented hepatocyte apoptosis by AMPK activation in HepG2 cells.

In conclusion, this study demonstrated that exogenous administration of DLK1 reduced hepatic steatosis, fibrosis, and apoptosis by AMPK activation. These results suggest that DLK1 may be a novel therapeutic approach for treating NAFLD.

Key words: Delta-like 1 homolog, non-alcoholic fatty liver disease, hepatic fibrosis, AMP-activated protein kinase

Effects of exogenous DLK1 administration on the hepatic steatosis and fibrosis in two different murine models

Hyun Min Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Bong Soo Cha)

I. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is currently a significant health concern globally[1,2]. A disease spectrum of NAFLD ranges from simple steatosis to steatosis with liver inflammation and fibrosis, referred to as non-alcoholic steatohepatitis (NASH)[3]. Though simple hepatic steatosis is considered benign, NAFLD with the presence of histologic changes consistent with NASH significantly affects the life expectancy. About ten to twenty percent of NAFLD patients have NASH, and about ten percent of NASH patients are known to progresses into liver cirrhosis. Patients with liver cirrhosis have higher risk for the development of hepatocellular carcinoma[1,4]. Furthermore, obesity and type 2 diabetes are closely linked to the progression of NAFLD[5]. However, despite the development of several classes of therapeutic agents for treating type 2 diabetes or obesity, there are no clinically available drugs to treat or prevent hepatic steatosis, NASH, or progression to liver cirrhosis.

Notch pathway has an essential role in many fundamental processes during early development and mature tissue homeostasis by regulating cell fate determination, proliferation, differentiation and death[6,7]. Recently, Notch

signaling has been recognized to have a crucial role in the development of NAFLD and diabetes[8,9]. Notch activation is known to enhance lipogenesis and gluconeogenesis in hepatocytes, resulting in increased insulin resistance[8,9]. In humans, Notch signaling is activated in patients with obesity-related liver disease such as NAFLD, and diabetes, and may represent a therapeutic target for these patients[10]. Also, Notch pathway interacts to control the fate of hepatic satellite cells (HSC) involved in adult liver repair by modulating epithelial-to-mesenchymal–like/mesenchymal-to- epithelial–like transitions[11]. The canonical Notch signaling pathway is activated by direct interaction between one of four Notch receptors (Notch1–4) and transmembrane Notch ligands of the Jagged or Delta-like families on a neighboring cell, resulting in a series of proteolytic cleavages that induce the transcription of Notch targets[7,12]. Delta-like 1 homolog (DLK1) is known as a transmembrane protein belonging to the epidermal growth factor-like repeat-containing family, which also includes Notch receptors and their ligands[13]. Various evidences shows that DLK1 interacts with Notch1 and functions as an inhibitory regulator of Notch signaling[14,15]. Furthermore, the soluble extracellular domain of DLK1 produced by a protease of tumor necrosis factor-α converting enzyme suppressed adipogenesis in vitro and in vivo[16]. Based on these previous studies, DLK1 may be a promising preventive or therapeutic target for hepatic steatosis and fibrosis due to metabolic dysfunction by inhibiting Notch signaling.

Therefore we investigated the therapeutic effect of the recombinant DLK1 protein in two different murine models of hepatic steatosis and fibrosis.

II. MATERIALS AND METHODS

1. Soluble DLK1 protein

To produce a soluble form of DLK1 protein, the extracellular domain of DLK1 (Glu25 to Gly302) was fused to a human Fc fragment. Recombinant plasmids, pYK602-sDLK1, which contains a signal sequence for secretion and the CMV promoter, were generated. Expression was performed as described previously[17]. Purification was then conducted with using protein A-Sepharose (GE Healthcare, Uppsala, Sweden). Soluble DLK1 protein was kindly given by Dr. Y.W. Park from Korea Research Institute of Bioscience and Biotechnology.

2. Animal procedure

Seven-week-old *db/db* male mice and C57BL/6J mice were purchased from Orient Bio (Sungnam, Korea). Mice were housed in an animal facility maintained at a temperature of 23 ± 2 °C and a humidity of 55 ± 5%. The mice were exposed to a 12-hr light, 12-hr dark cycle and fed an unrestricted diet with monitoring for food intake and body weight twice a week. In *db/db* mice, the DLK1-treated group (n = 12) received soluble DLK1 protein intraperitoneally (25 mg/kg) twice a week, and the vehicle-treated group (n = 12) were given the same volume of PBS instead of DLK1. C57BL/6J mice were divided into three groups. The mice in the first group (n = 6) were fed a standard chow diet and treated with vehicle as control, the second group (n = 6) were fed a diet completely devoid of methionine and choline (MCD) and treated with vehicle. The third group (n = 6) of mice was fed a MCD diet and administered soluble DLK1 protein intraperitoneally (15 mg/kg) twice a week. The mice were sacrificed after 4 weeks of treatment. While eating the experimental formulas, all animals had free access to drinking water. Animals were killed after fasting for 6 hr. All of the procedures were approved by the Institutional Animal Care and Use Committee at the Yonsei University College of Medicine.

3. Biochemical analysis

Blood samples were obtained with heparinized syringes by puncture into the inferior vena cava and immediately centrifuged at 5000 *g* for 15 min. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by ELISA (BioAssay Systems, Hayward, CA, USA).

4. Cell culture and treatment

HepG2 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; GE Healthcare Hyclone, Sungnam, Korea) supplemented with 25 mM D-glucose, 10% fetal bovine serum, 100 U penicillin, and 100 μg streptomycin. Hepatic stellate cells (HSCs), LX-2 cells were kindly obtained from Dr. Friedman SL (Mount Sinai School of Medicine, NY, USA) and maintained in DMEM, high glucose (25 mM D-glucose), GlutaMAX media (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 1% fetal bovine serum, 100 U penicillin, and 100 μg streptomycin. To stimulate HSCs activation, 1 ng/mL of Kdo2-lipid A (KLA) (Avanti Polar Lipids, AL, USA) or 5 ng/mL of transforming growth factor beta 1 (TGF-β1, R&D Systems, Minneapolis, MN, USA) was treated in LX-2 cells for 24 hrs. KLA is an active component of lipopolysaccharide with an analogous response. To induce apoptosis in hepatocyte, 200 μM of palmitate and 10 ng/mL TGF-β1 or 10 ng/mL activin A (R&D Systems, Minneapolis, MN, USA) were treated in HepG2 for 24 hrs. Compound C (Sigma-Aldrich, St Louis, MO, USA) was used to block AMP-activated protein kinase (AMPK) activation, and WY14643 (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control.

5. Protein extraction and immunoblotting

Mouse livers, HepG2 cells, and LX-2 cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA), and the protein content was measured using the Bradford assay (Bio-Rad, 162-0115, Hercules, CA, USA).

Nuclear and cytosolic proteins were extracted using the NE-PER kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein (30 μg) were heat-denatured in 4 × sample buffer (2% sodium dodecyl sulfate, 62.5 mM Tris (pH 6.8), 0.01% bromophenol blue, 1.43 mM β-mercaptoethanol and 0.1% glycerol), separated on 10 or 12% sodium dodecyl sulfate-polyacrylamide gels and electro-transferred onto nitrocellulose membrane (Bio-Rad). Membranes were subsequently blotted with antibodies against the following proteins: AMPK (cat#2603, Cell Signaling Technology), pAMPK (cat#2535, Cell Signaling Technology), Smad2 (cat#5339S, Cell Signaling Technology), Smad3 (cat#9523S, Cell Signaling Technology), pSmad2 (Ser465/467) (cat#3101S, Cell Signaling Technology), p-Smad3 (Ser423/425) (cat#9520S, Cell Signaling Technology), and GAPDH (cat#sc-25778, Santa Cruz).

6. RNA isolation and real-time PCR

Total RNA was extracted using Tirol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions and subjected to reverse transcription with the high capacity complementary DNA transcription kit (Applied Biosystems, Foster City, CA, USA) followed by quantitative real-time PCR using the ABI 7500 sequence detection system (Applied Biosystems). PCR was conducted using the following primers (for SYBR Green): acyl-Coenzyme A dehydrogenase (ACADM), forward (5′-TGA CGG AGC AGC CAA TGA-3′) and reverse (5′-TCG TCA CCC TTC TTC TCT GCT T-3′); carnitine palmitoyltransferase 1 (CPT1), forward (5′-GGG AGG ACA GAG ACT GTA CGC TC-3′) and reverse (R 5′-TGT AGG AAA CAC CAT AGC CGT CAT-3′); ACOX, forward (5′-GGG TGG TAT GCT GTC GTA C-3′) and reverse (5′-CAA AGA CCT TAA CGG TCA CGT AGT G-3′); AMPK, forward (5′-TGA CGG AGC AGC CAA TGA-3′) and reverse (5′-TCG TCA CCC TTC TTC TCT GCT T-3′); alpha-smooth muscle actin (α-SMA), forward (5′-ACT GGG ACG ACA

TGG AAA AG-3′) and reverse (5′-CAT CTC CAG AGT CCA GCA -3′); collagen, forward (5′-TGC CGT GAC CTC AAG ATG TG-3′) and reverse (5′-CAC AAG CGT GCT GTA GGT GA-3′); TGF-β, forward (5′-CGG CAG CTG TAC ATT GAC TT-3′); and reverse (5′-TCA GCT GCA CTT GCA GGA GC-3′); and GAPDH, forward (5′-AAC TTT GGC ATT GTG GAA GG-3′) and reverse (5′-TGT TCC TAC CCC CAA TGT GT-3′). Quantitative analyses were performed using the ΔΔcycle threshold method and StepOne Software version 2.2.2 (Grand Island, NY, USA).

7. Hepatic triglyceride measurement and Oil Red O staining

After homogenization, triglyceride content in liver tissues was measured with the Triglycerides Quantification Kit (K622, Biovision, Milipitas, CA, USA) according to the manufacturer's instructions. Lipid droplets in HepG2 cells were visualized and subsequently quantified by Oil Red O staining after treatment with palmitate and DLK1. To measure the quantification of lipid accumulation, Oil Red O was eluted by adding 100% isopropanol and the optical density was measured by spectrophotometry at 520 nm.

8. Hepatic hydroxyproline content

For quantification of fibrosis, hepatic hydroxyproline content was measured using a hydroxyproline assay kit (BioVision, Mountain View, CA, USA). Briefly, frozen liver tissue from mice were weighed and homogenized in distilled H_2O and hydrolyzed. Ten L of hydrolysate was transferred to a 96-well plate and evaporated to dryness under vacuum; a standard curve was applied following the kit protocol. Total hydroxyproline content was analyzed and data presented per gram of wet weight liver tissue.

9. Histological analysis

The mice livers were fixed using 10% neutral-buffered formalin for 48 hr,

paraffin embedded and sectioned into 4-mm thick slices for hematoxylin and eosin (H-E) stain or Masson's trichrome stain. After sealing the slides containing the tissue slices with neutral gum, the stained tissue slices were microscopically examined at 200× magnification. Histological images were analyzed using ImageJ software (NIH Image, Bethesda, MA, USA).

10. In situ apoptosis detection

The terminal deoxynucleotidyl transferase (TdT) mediated digoxigenin-dUTP nick end labeling (TUNEL) method was carried out using a commercially available kit (Trevigen, Gaithersburg, MD, USA). Paraffin sections (4 μm) were dewaxed, rehydrated, treated with protease K, and blocked with H_2O_2. After labeling with TdT and biotin-labeled dNTP, the sections were incubated with peroxidase conjugated streptavidin, followed by counter-staining with hematoxylin. Labeled cells were then visualized under a light microscope. Cells which stained brown by the TUNEL assay (TUNEL positive) were judged apoptotic; non-apoptotic cells remained blue.

11. Cell viability assays

WST-8 Cell Counting Kit-8 (#CK04; Dojindo Laboratories, Kumamoto, Japan) was used to evaluate the viability of HepG2 cells. Cells were grown in 96-well plates at 5,000 cells in 200 mL culture medium per well. Cells were labeled with the Iron oxide-PLL complex overnight at 37℃ in a 5% CO_2 atmosphere. Unlabeled cells which were kept under identical conditions served as control cells. After overnight incubation, 20 mL of WST-8 solution per well was added to the growing cells and were incubated for an additional 1-4 hr. The absorbance of the formazan product was then measured at a wavelength of 450 nm subtracting absorbance at 600 nm or above according to manufacturer's instructions.

12. Statistical analysis

All statistical analyses were performed with SPSS software (version 18.0; SPSS, Chicago, IL, USA) and graphs were plotted using GraphPad Prism (Version 5.0, GraphPad, San Diego, CA, USA). Results are expressed as a mean ± SD. Statistical significance was calculated using Student's t test to assess the differences between the groups. Data with a p value < 0.05 were considered significant.

III. RESULTS

1. DLK1 ameliorated hepatic steatosis in *db/db* mice.

To investigate the metabolic effect of DLK1 in the animal model of fatty liver and diabetes, soluble DLK1 peptide consisting of the N-terminal extracellular domain of DLK1 fused to a human Fc fragment was generated (Fig. 1). This recombinant protein contains six epidermal growth factor-like domains and the juxtamembrane portion, which corresponds to residues 25–302 of DLK1 (P80370, UniProt Knowledgebase).

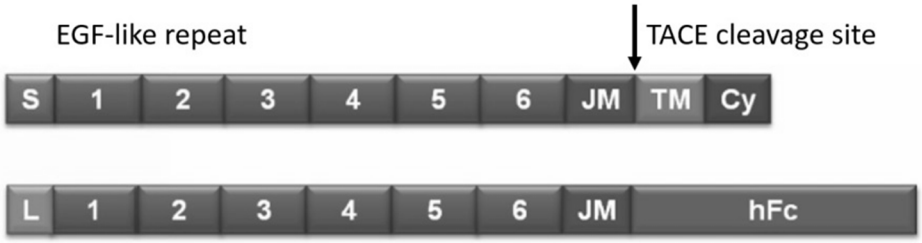

Fig. 1. Schematic of the structure of DLK1. Schematic of the structure of endogenous DLK1 (upper) and DLK1-human Fc fragment fusion protein (lower). Tumor necrosis factor alpha converting enzyme (TACE) cleavage site is marked. S, signal sequence; JM, juxtamembrane domain; TM, transmembrane domain; Cy, cytoplasmic region; hFc, human Fc fragment.

Vehicle- and DLK1-treated *db/db* mice exhibited comparable body weights and food intakes for 4 weeks (Fig. 2). The fasting glucose level was lower in DLK1-treated mice than vehicle-treated mice after 2 weeks of treatment. In histologic analysis, however, the lipid droplets markedly decreased in DLK1-treated mice compared to control. Serum levels of AST and ALT, and hepatic triglyceride content were also significantly decreased in mice administered with DLK1 (Fig. 3).

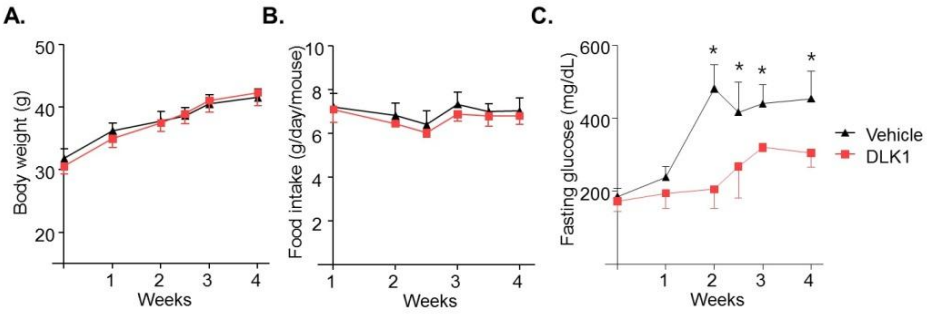

Fig. 2. DLK1 did not affect body weight and food intake in *db/db* mice. (A) Body weight, food intake (B), and oral glucose tolerance test (C) were performed after a 4-week treatment of DLK1 or vehicle. Data are presented as the mean ± SD (n = 6). *$p < 0.05$ compared with the vehicle group.

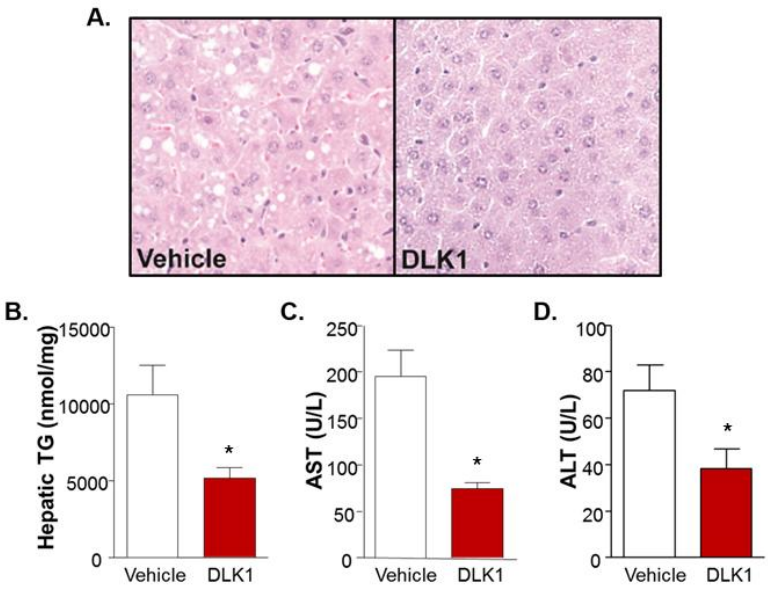

Fig. 3. DLK1 ameliorated hepatic steatosis in *db/db* mice. (A) Liver histology (Magnification, × 200), (B) hepatic TG content, (c) serum AST, (D) and serum ALT were evaluated after a 4-week treatment of DLK1 or vehicle. Data are presented as the mean ± SD (n = 6). *$p < 0.05$ compared with the vehicle group.

2. DLK1 reduced hepatic lipid accumulation via AMPK activation in *db/db* mice and HepG2 cells.

Next, we investigated the underlying mechanisms for the anti-steatotic effects of DLK1. One of antidiabetic agents such as metformin showed some possibility in treatment for NAFLD targeting AMPK, leading to increased fatty acid oxidation and suppression of lipid accumulation in hepatocytes[18,19]. Similarly, we observed that DLK1 treatment in *db/db* mice significantly enhanced Thr172 phosphorylation on AMPK-α (Fig. 4A and B). Phosphorylation of ACC, a downstream target of AMPK, was also induced in the liver of *db/db* mice treated with DLK1. Among the genes involved in fatty acid oxidation, our data demonstrate that hepatic expression of acyl-Coenzyme A dehydrogenase (ACADM) was significantly elevated in DLK1-treated mice (Fig. 4C).

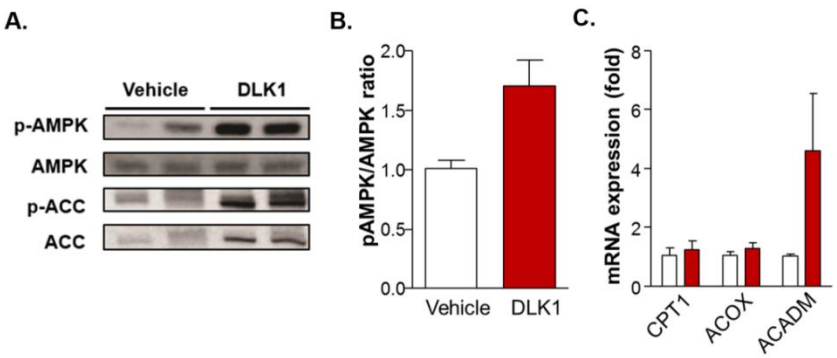

Fig. 4. DLK1 stimulated AMPK activation followed by induction of genes related to fatty acid oxidation in *db/db* mice. (A) The levels of phosphorylated and total AMPK, as well as phosphorylated and total ACC, were determined in hepatocytes by immunoblotting. (B) The graph shows densitometric analysis of the optical density-based data of the phosphorylated AMPK/total AMPK ratio from the immunoblots shown on the left. (C) Hepatic mRNA expression of CPT1, ACOX, and ACADM were measured by real-time PCR. Data are representative of four specimens. *$p < 0.05$.

We also confirmed that the activation of AMPK by DLK1 in HepG2 cells in vitro (Fig. 5A and B) was consistent with in vivo studies. These results suggest that DLK1 induces fatty acid oxidation in hepatocytes by activating AMPK

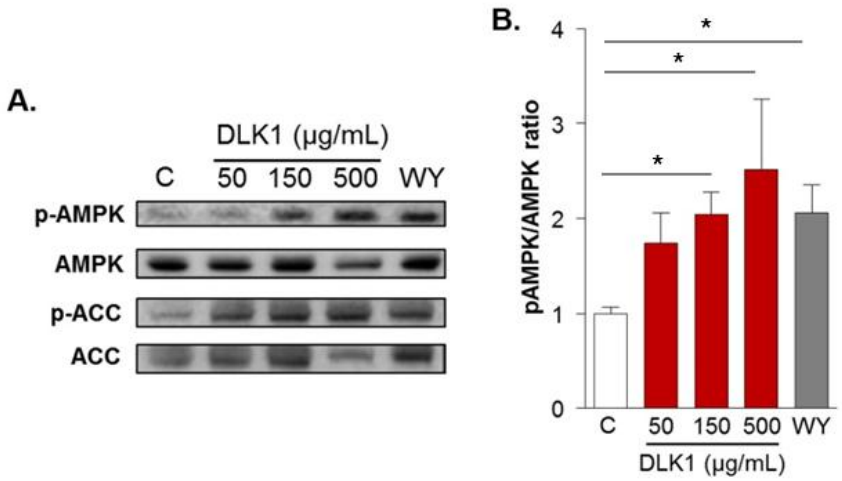

Fig. 5. DLK1 stimulated AMPK activation in a dose-dependent manner in HepG2 cells. (A) Level of phosphorylated and total AMPK, as well as phosphorylated and total ACC in HepG2 cells treated with DLK1 or WY14643 (as a positive control) were analyzed by immunoblotting. (B) The graph shows densitometric analysis of the optical density-based data of the phosphorylated AMPK/total AMPK ratio from the immunoblots. Data are representative of four specimens. $*p < 0.05$.

To assess whether DLK1 treatment ameliorates lipid accumulation in hepatocytes via AMPK activation in vitro, we stained lipid droplets with Oil Red O and quantified them by spectrophotometry. As shown in Fig. 6A and B, DLK1 significantly reduced intracellular lipid accumulation in the presence of palmitate compared with control. However, lipid accumulation was not affected by DLK1 administration after pretreatment with compound C (Fig. 6B).

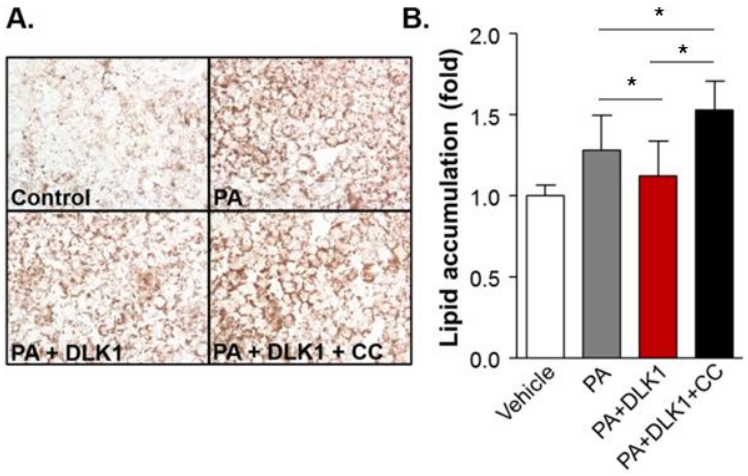

Fig. 6. DLK1 inhibited lipid accumulation by activation of AMPK. (A) Oil Red O staining was performed to assess intracellular lipid accumulation in HepG2 cells treated with palmitate (0.3 mM) and DLK1 (150 μg/ml). (B) Lipid accumulation was quantified by spectrophotometry. Data are presented as the mean ± SD (n = 6). *$p < 0.05$.

3. DLK1 ameliorated hepatic steatosis, fibrosis, and apoptosis in MCD diet-fed mice.

To investigate the effects of in advanced NAFLD such as fibrosis and apoptosis, we repeated similar experiment in another animal model, MCD diet-fed mice. After maintaining for 4 weeks with MCD diet, mice exhibited markedly severe steatohepatitis assessed by increased lipid droplet in H-E stain, increased hepatic triglyceride contents, and elevation in serum ALT level (Fig. 7A, D, E, and F). Besides, in MCD diet-fed mice, the fibrosis assessed by Masson's trichrome stain, fibrogenic gene expression, and quantification of hepatic hydroxyproline content were ameliorated by DLK1 administration (Fig. 7B, G, and H). Ultimately, MCD diet-induced hepatocyte apoptosis significantly decreased with DLK1 treatment (Fig. 7C).

4. DLK1 ameliorated HSC activation and hepatic fibrosis by AMPK activation.

In the setting of chronic liver disease including NAFLD, HSCs are the primary target of fibrogenic stimuli in the diseased liver. TGF-β signals through the sequential phosphorylation of Smad2 or Smad3 are considered one of the major underlying mechanisms in the process of HSCs activation. So we investigate these pathways in MCD-diet fed mice. To stimulate HSCs, we used KLA induced LX-2 cells. KLA increased mRNA expression of TGF-β in a dose dependent manner (Fig. 8A). Then we tested the anti-fibrotic effect of DLK1 treatment. After a dose of 1 ng/mL of KLA treatment for 24 hours, TGF-β, the marker of HSCs activation, and fibrogenic gene expression such as α-SMA and collagen were much increased. DLK1 pretreatment recovered this change (Fig. 8B-D).

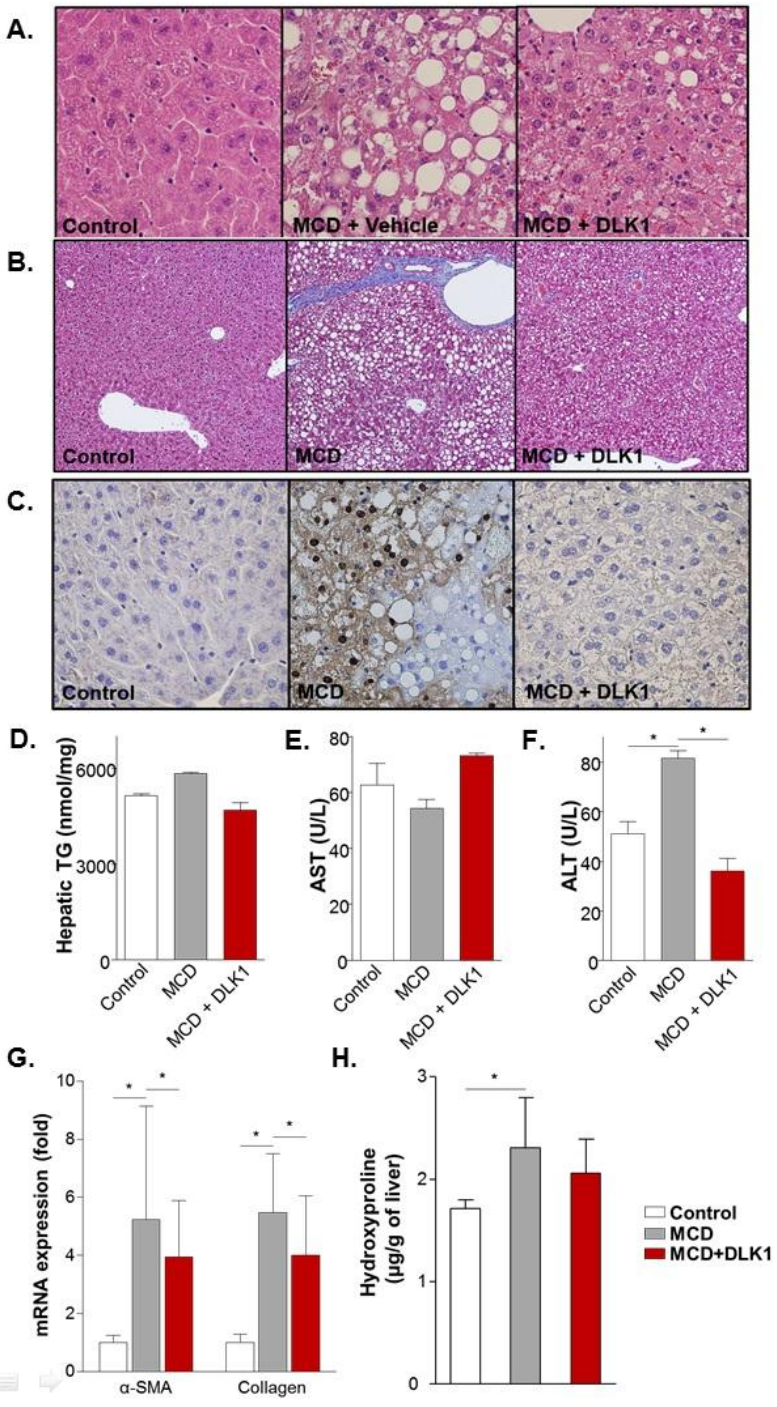

Fig 7. DLK1 ameliorated hepatic steatosis, fibrosis, and apoptosis in MCD diet-fed mice. (A) Liver histology (Magnification, × 200), (B) Masson's trichrome stain (Magnification, × 200), and (C) TUNEL stain were performed in MCD diet-fed mice with DLK1 treatment for 4 weeks. (D) Hepatic TG content, (E) serum AST, (F) and serum ALT were evaluated. (G) Hepatic mRNA expression of α-SMA and collagen were measured by real-time PCR. (H) Hydroxyproline content was quantified. Data are representative of four specimens. *$p < 0.05$.

To investigate the underlying mechanism with DLK1 treatment, we stimulated LX-2 cells with 5 ng/mL of TGF-β. Consistent with the previous reports, TGF-β treatment increased phosphorylated form of Smad2/3 in LX-2 cells, however, DLK1 treatment reduced pSmad2/3 expression. Furthermore, 10 μM of compound C treatment blocked this effect of DLK1 on TGF-β/Smad signaling activation (Fig. 9).

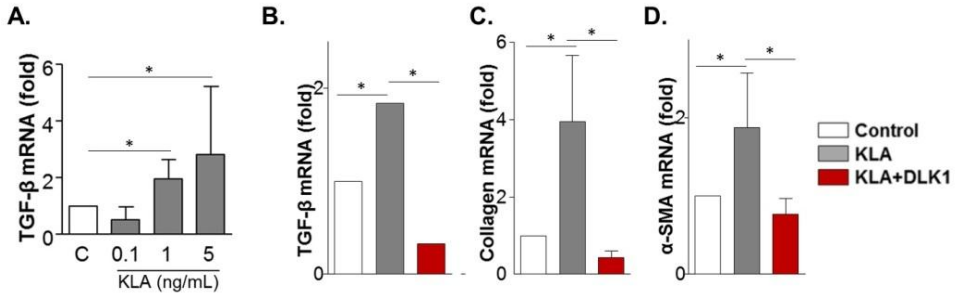

Fig. 8. DLK1 decreased HSCs activation and fibrosis in KLA-induced LX-2 cells. (A) KLA treatment activated LX-2 cells in a dose-dependent manner assessed by increased TGF-β expression. (B) Hepatic mRNA expression of TGF-β, Collagen, and α-SMA were measured by real-time PCR in LX-2 cells treated with TGF-β and DLK1. Data are representative of four specimens. *p < 0.05.

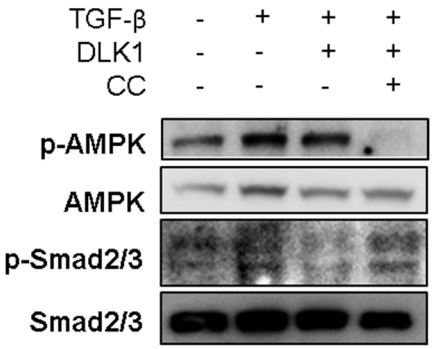

Fig. 9. DLK1 inhibited TGF-β-induced Smad2/3 phosphorylation in LX-2 cells via AMPK activation. Level of phosphorylated and total AMPK, as well as phosphorylated and total Smad2/3 were analyzed by immunoblotting in LX-2 cells treated with TGF-β and DLK1.

5. DLK1 reduced activin A-induced cell death in steatotic hepatocyte by AMPK activation.

Lastly, we tested whether DLK1 reduces activin A-induced apoptosis in steatotic hepatocyte and whether the mechanism is attained by AMPK activation. Activin A, as well as TGF-β, is thought to be involved in apoptosis during the progression of NAFLD. As expected, treatment with palmitate and activin A decreased AMPK phosphorylation, increased Smad2 phosphorylation, and increased apoptosis assessed by cleaved caspase 3 expression. Soluble DLK1 pretreatment increased AMPK activation and decreased apoptosis, furthermore, compound C treatment interrupted this anti-apoptotic effect (Fig. 10A). We confirmed this effect using MTT assay (Fig. 10B).

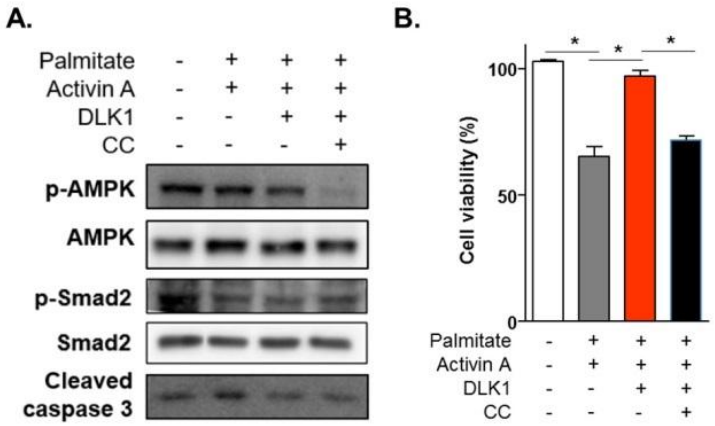

Fig. 10. DLK1 inhibited activin A-induced apoptosis in steatotic hepatocyte by AMPK activation in HepG2 cells. (A) Level of phosphorylated and total AMPK, phosphorylated and total Smad2, and cleaved caspase 3 were analyzed by immunoblotting in HepG2 cells treated with palmitate and DLK1. (B) Cell viability was assessed by MTT assay. Data are representative of four specimens. *$p < 0.05$.

IV. DISCUSSION

The present study reports that exogenous DLK1 administration ameliorated hepatic steatosis and fibrosis in two different murine models. By generating a fusion protein containing the extracellular domain of DLK1 and the human Fc region, which increases its stability, we found the unexpected action of DLK1 on prevention of steatosis by increased hepatic fatty acid oxidation via AMPK activation in *db/db* mice, one of the typical animal models for obesity and hepatic steatosis. This animal model, however, has a limitation that it represents only hepatic lipid accumulation, not severe inflammation and cirrhosis as shown in advanced form of NAFLD in human. So, we tried to investigate the effect of exogenous DLK1 administration on the development and progression of NAFLD to NASH in another model. In mice fed MCD diet, exogenous DLK1 administration significantly decreased hepatic steatosis, fibrosis, and finally hepatocyte apoptosis.

NAFLD has become an important worldwide health problem due to its high prevalence and close association with various metabolic diseases, including obesity, type 2 diabetes, metabolic syndrome, and cardiovascular disease. The major cause of the clinical significance of NAFLD is that it could progress from simple steatosis to steatohepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma. Actually, NAFLD is projected to replace hepatitis C as the leading cause for liver transplantation by 2020 in USA.[20]. In spite of this current situation, however, it is still unclear what triggers the progression of NAFLD to NASH accompanied by inflammation and fibrosis. A "multiple-hit" hypothesis for the pathogenesis of NAFLD based on an animal model has been proposed, however, the exact mechanism remains incomplete.

DLK1 was first known from a 3T3-L1 preadipocyte complementary DNA library and has been linked to the inhibition of adipogenic differentiation[21]. Some researches using DLK1-null mice and transgenic mice overexpressing

DLK1 in adipose tissue, demonstrated that DLK1 has an important role as a negative regulator of adipogenesis. DLK1-null mice exhibited accelerated body weight gain with increased mass of adipose tissues [22]. These conflicting findings may be due to the differences in the dosing of DLK1 and the timing of action. This explanation is supported by a recent study showing that tightly regulated dosage control of DLK1 is important for its regulatory function in neurogenesis[23]. Furthermore, DLK1 has been identified as a dosage-critical gene during development and growth[24]. So the effect of exogenous DLK1 administered in adult animal models may be different from that of previous genetic animal models in which DLK1 expression during early life has been manipulated. In contrast to previous findings from genetically engineered mice, we observed that exogenous administration of soluble DLK1 did not affect the development of adipose tissue.

AMPK acts as a metabolic master regulator by regulating cellular ATP concentration and energy homeostasis[25]. Therefore AMPK activation could be the treatment of diabetes, obesity, and NAFLD. In our study, DLK1 treatment protected the mice or cell from hepatic steatosis in *db/db* mice by AMPK activation. Activated AMPK has been known to be related with suppressing biosynthetic pathways like fatty acid synthesis, and stimulating catabolic processes such as fatty acid oxidation in the liver[26].

Recently, Charalambous *et al.* demonstrated that DLK1 overexpression ameliorated hepatic steatosis with reduced body weight and improved insulin sensitivity in obese mice[27] This might be consistent with our data on positive metabolic effect and protection from hepatic steatosis. However, the mechanisms of the metabolic effect are different, because the body weight, adipose tissue mass as well as the food intake did not decrease with DLK1 treatment in the present study. So it is not a secondary effect with decreased energy intake or body weight. Further study to clarify the direct function of DLK1 administration on liver metabolism would be warranted.

We confirmed the anti-fibrotic and anti-apoptotic effect of exogenous DLK1 treatment in another animal model. These interesting effects were also attained by activation of AMPK, which leads to inhibition of Smad signaling pathway. There have been several reports of protective effect of AMPK activation from hepatic fibrosis, however, the exact mechanisms remained unknown. *Lim et al.* showed the inhibition of TGF-β-induced fibrosis in LX-2 cells by AMPK agonist such as metformin and AICAR. AMPK activation, however, did not affect TGF-β-stimulated phosphorylation of Smad2 or Smad3. AICAR or metformin decreased Smad3 interaction with transcriptional coactivator p300[28]. In contrast, another study with adiponectin-based short peptide, another AMPK activator, demonstrated the association between AMPK activation and TGF-β/Smad2 signaling in thioacetamide-induced hepatic fibrosis cell model[29]. In addition, AMPK activation could inhibit hepatic fibrosis by targeting Smad pathway by itself[28].

Furthermore, our study suggests the ant-apoptotic role of DLK1 in progression from NAFLD to NASH induced by activin signaling. Activins, one of TGF-β superfamily members, have been known to be related with hepatic fibrosis and apoptosis. It has been discovered that activin A plays as important roles in liver regeneration and fibrosis among activins[30]. In this process, HSCs were identified as the major source of activin A, and expression of activin A was rapidly induced upon culturing of these cells[31]. Activin A acts in an autocrine and paracrine manner on hepatocytes and activated HSCs[30]. In hepatic fibrosis, activin A pathway involves Smad2 and Smad3 signaling through direct serine phosphorylation after receptor binding[32]. Also, in hepatocytes with activin A stimulation, Smad proteins are known to be involved in the apoptotic process[33,34]. In our study, treatment with palmitate and activin A in HepG2 cells reproduced the condition with apoptotic signal in steatotic hepatocyte in our experiment. Co-treatment with palmitate and activin A markedly increased hepatocyte lipoapoptosis, however, DLK1 pretreatment ameliorated the

apoptosis. When treated with compound C, an AMPK inhibitor, the anti-apoptotic effect of DLK1 disappeared.

This study has some limitations. Further research will be required to better determine the underlying molecular mechanism of DLK1 in AMPK activation, and the direct relationship between AMPK activation and Smad2/3 pathway inhibition in the process of hepatic fibrosis and apoptosis. In addition, the effective dosage of soluble DLK1 protein to activate AMPK is high, at least 150 μg/mL in our study with HepG2 cells. Because there is few studies using soluble DLK1 protein in animal study, we could not compare the dosage directly. However, delicate pharmacokinetic and pharmacodynamics studies should also be conducted to define the optimal therapeutic concentration of DLK1 in the blood to avoid causing adverse effects. In spite of these, this study is still meaningful. The strength of this study is it demonstrates that DLK1 administration showed beneficial effects on hepatic steatosis, fibrosis, and apoptosis in murine models. These results provide novel evidence that DLK1 may be a good therapeutic option for treating various spectrums of obesity-related chronic liver disease.

V. CONCLUSION

In conclusion, the present study demonstrated that exogenous administration of DLK1 markedly reduced hepatic steatosis by activation of fatty acid oxidation through increased AMPK phosphorylation. Furthermore, DLK1 significantly protected the progression of hepatic fibrosis and apoptosis in MCD diet-fed mice by inhibition of Smad signaling pathway following AMPK activation. Taken together, these findings suggest that DLK1 might be a novel therapeutic strategy for the treatment of various stages of NAFLD from steatosis to fibrosis.

REFERENCES

1. Day CP. Non-alcoholic fatty liver disease: a massive problem. Clin Med (Lond) 2011;11:176-8.
2. Sanyal AJ, Brunt EM, Kleiner DE, Kowdley KV, Chalasani N, Lavine JE, et al. Endpoints and clinical trial design for nonalcoholic steatohepatitis. Hepatology 2011;54:344-53.
3. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annu Rev Pathol 2010;5:145-71.
4. Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol 2010;53:372-84.
5. Loomba R, Abraham M, Unalp A, Wilson L, Lavine J, Doo E, et al. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. Hepatology 2012;56:943-51.
6. Bolos V, Grego-Bessa J, de la Pompa JL. Notch signaling in development and cancer. Endocr Rev 2007;28:339-63.
7. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009;137:216-33.
8. Pajvani UB, Shawber CJ, Samuel VT, Birkenfeld AL, Shulman GI, Kitajewski J, et al. Inhibition of Notch signaling ameliorates insulin resistance in a FoxO1-dependent manner. Nat Med 2011;17:961-7.
9. Pajvani UB, Qiang L, Kangsamaksin T, Kitajewski J, Ginsberg HN, Accili D. Inhibition of Notch uncouples Akt activation from hepatic lipid accumulation by decreasing mTorc1 stability. Nat Med 2013;19:1054-60.
10. Valenti L, Mendoza RM, Rametta R, Maggioni M, Kitajewski C, Shawber CJ, et al. Hepatic notch signaling correlates with insulin resistance and nonalcoholic fatty liver disease. Diabetes

2013;62:4052-62.

11. Xie G, Karaca G, Swiderska-Syn M, Michelotti GA, Kruger L, Chen Y, et al. Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. Hepatology 2013;58:1801-13.
12. Dufraine J, Funahashi Y, Kitajewski J. Notch signaling regulates tumor angiogenesis by diverse mechanisms. Oncogene 2008;27:5132-7.
13. Lai EC. Notch signaling: control of cell communication and cell fate. Development 2004;131:965-73.
14. Baladron V, Ruiz-Hidalgo MJ, Nueda ML, Diaz-Guerra MJ, Garcia-Ramirez JJ, Bonvini E, et al. dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. Exp Cell Res 2005;303:343-59.
15. Nueda ML, Baladron V, Sanchez-Solana B, Ballesteros MA, Laborda J. The EGF-like protein dlk1 inhibits notch signaling and potentiates adipogenesis of mesenchymal cells. J Mol Biol 2007;367:1281-93.
16. Wang Y, Sul HS. Ectodomain shedding of preadipocyte factor 1 (Pref-1) by tumor necrosis factor alpha converting enzyme (TACE) and inhibition of adipocyte differentiation. Mol Cell Biol 2006;26:5421-35.
17. Backliwal G, Hildinger M, Chenuet S, Wulhfard S, De Jesus M, Wurm FM. Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. Nucleic Acids Res 2008;36:e96.
18. Pernicova I, Korbonits M. Metformin--mode of action and clinical implications for diabetes and cancer. Nat Rev Endocrinol 2014;10:143-56.
19. Zhang BB, Zhou G, Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab 2009;9:407-16.
20. Kneeman JM, Misdraji J, Corey KE. Secondary causes of nonalcoholic

fatty liver disease. Therap Adv Gastroenterol 2012;5:199-207.

21. Smas CM, Sul HS. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 1993;73:725-34.
22. Moon YS, Smas CM, Lee K, Villena JA, Kim KH, Yun EJ, et al. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol Cell Biol 2002;22:5585-92.
23. Ferron SR, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, et al. Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. Nature 2011;475:381-5.
24. da Rocha ST, Charalambous M, Lin SP, Gutteridge I, Ito Y, Gray D, et al. Gene dosage effects of the imprinted delta-like homologue 1 (dlk1/pref1) in development: implications for the evolution of imprinting. PLoS Genet 2009;5:e1000392.
25. Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 2004;117:5479-87.
26. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 2012;13:251-62.
27. Charalambous M, Da Rocha ST, Radford EJ, Medina-Gomez G, Curran S, Pinnock SB, et al. DLK1/PREF1 regulates nutrient metabolism and protects from steatosis. Proc Natl Acad Sci U S A 2014;111:16088-93.
28. Lim JY, Oh MA, Kim WH, Sohn HY, Park SI. AMP-activated protein kinase inhibits TGF-beta-induced fibrogenic responses of hepatic stellate cells by targeting transcriptional coactivator p300. J Cell Physiol 2012;227:1081-9.
29. Wang H, Zhang H, Zhang Z, Huang B, Cheng X, Wang D, et al. Adiponectin-derived active peptide ADP355 exerts anti-inflammatory and anti-fibrotic activities in thioacetamide-induced liver injury. Sci Rep 2016;6:19445.

30. Werner S, Alzheimer C. Roles of activin in tissue repair, fibrosis, and inflammatory disease. Cytokine Growth Factor Rev 2006;17:157-71.
31. De Bleser PJ, Niki T, Xu G, Rogiers V, Geerts A. Localization and cellular sources of activins in normal and fibrotic rat liver. Hepatology 1997;26:905-12.
32. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 2003;113:685-700.
33. Chen W, Woodruff TK, Mayo KE. Activin A-induced HepG2 liver cell apoptosis: involvement of activin receptors and smad proteins. Endocrinology 2000;141:1263-72.
34. Yang L, Roh YS, Song J, Zhang B, Liu C, Loomba R, et al. Transforming growth factor beta signaling in hepatocytes participates in steatohepatitis through regulation of cell death and lipid metabolism in mice. Hepatology 2014;59:483-95.

ABSTRACT(IN KOREAN)

DLK1의 투여가 지방간의 발생 및 섬유화에 미치는 효과

<지도교수 차 봉 수>

연세대학교 대학원 의학과

김 현 민

Notch 신호전달체계의 활성화가 지방간의 다양한 단계에서 작용한다는 사실이 알려져 있다. Delta-like 1 homolog (DLK1) 는 Notch 신호전달체계의 내인적인 억제제인데, 최근 보고에 따르면 DLK1 이 지방조직으로의 분화나 골격근의 형성 뿐 아니라 간의 재생 과정에도 관여한다고 한다. 따라서 본 연구에서는 DLK1의 투여가 지방간의 발생이나 지방에 어떠한 역할을 하는지 알아보고자 하였다.

융합 DLK1 단백질은, DLK1의 세포외도메인과 인간 면역글로불린의 Fc 분절을 융합하여 만들었다. 동물 실험을 위해서는 *db/db* 마우스 동물 모델과 메티오닌-콜린 결핍 식이를 유지한 C57BL/6 마우스 모델을 사용하였다. DLK1 은 4주동안 투여하였다. HepG2 세포주와 인간 간성상세포인 LX-2 세포주를 이용하여 세포 실험을 진행하였다.

융합 DLK1 단백질의 투여는 *db/db* 마우스 동물 모델에서 간조직내 중성지방 함량을 줄이고 간효소수치를 호전시키는 등 지방간의 진행을 현저히 감소시켰다. 또한 DLK1의 투여는 간조직

에서 AMP-activated protein kinase (AMPK)의 인산화를 촉진하였고, 지방산의 산화를 담당하는 유전자의 발현을 높였다. HepG2 세포에서도 마찬가지 결과를 보였다. 메티오닌-콜린 결핍 식이 마우스 모델에서는 DLK1의 투여가 지방간의 진행 뿐 아니라 간의 섬유화, 간세포의 세포자멸사를 감소시켰다. LX-2 세포에서 DLK1의 투여는 간성상세포의 활성화를 억제하였고, 섬유화를 일으키는 유전자 발현을 감소시켰는데, 이는 AMPK의 활성화를 통하여 TGF-β/Smad 신호전달체계를 억제함으로써 나타났다. 또한 DLK1 의 투여는 간세포 지방 축적 자멸사를 줄이는 결과를 보였다.

결론적으로, DLK1의 투여는 지방간의 발생과 진행을 다양한 단계에서 억제하며, 이는 간세포와 간성상세포에서 AMPK의 활성화를 통하여 작용한다. 이는 DLK1이 지방간의 예방 및 치료에 있어 새로운 약물의 타겟이 될 가능성을 제시하였다.

핵심되는 말: Delta-like 1 homolog, 비알코올성지방간, 간 섬유화, AMP-activated protein kinase

PUBLICATION LIST

Lee YH, Yun MR, Kim HM, Jeon BH, Park BC, Lee BW, et al. Exogenous administration of DLK1 ameliorates hepatic steatosis and regulates gluconeogenesis via activation of AMPK. Int J Obes (Lond) 2016;40:356-65.