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약학석사 학위논문

Effects of Docosahexaenoic Acid on SREBP1-Mediated Lipogenic Enzyme Expression in Hepatocytes;

Role of Long Chain Fatty Acid Sensing GPCRs

간 세포 내 G단백질 연결 수용체에 작용하는 DHA의

SREBP-1을 통한 지질 생성 억제 효과

2018년 8월

서울대학교 대학원

약학대학 약물학 전공

온 승 태

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Role of Long Chain Fatty Acid Sensing GPCRs

지도교수 강 건 욱

이 논문을 약학석사 학위논문으로 제출함
2018년 8월

서울대학교 대학원
약학과 약물학 전공
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온승태의 약학석사 학위논문을 인준함
2018년 8월

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Abstract

Effects of Docosahexaenoic Acid on SREBP1-Mediated Lipogenic Enzyme Expression in Hepatocytes;
Role of Long Chain Fatty Acid Sensing GPCRs

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Non-Alcoholic Fatty Liver Disease (NAFLD) is the most copious liver malady and if proper treatment is not applied, it may progress to cirrhosis, the end stage liver disease. Considering the disease's prevailing and vicious outcome around the world, treatment options are limited and further research is warranted. Failure to resolve inflammatory stimuli evoked by fatty acids accumulation in NAFLD can result in the dysfunction of hepatocytes and tissue damage. Thus, it is important to seek potential pharmacological target(s) to ameliorate impairments prompted by fatty acids. Omega-3 fatty acids are clinically proven to lower serum triglyceride (TG) levels. Furthermore, these omega-3 fatty acids such as long chain free fatty acids are known to activate G-protein coupled receptor 40 (GPR40) and G-protein coupled receptor 120 (GPR120), playing a pivotal role in myriad

physiological homeostatic mechanisms. We found that exposure of murine primary hepatocytes to 300 μM docosahexaenoic acid (DHA) for 12 hr increased mRNA levels of GPR40 and GPR120. Moreover, DHA lowered protein or mRNA expression levels of lipogenic enzymes such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and stearoyl-CoA desaturase-1 (SCD1) in primary hepatocytes incubated with liver X receptor (LXR) agonist or a high glucose with insulin milieu. DHA also inhibited protein expression of both nuclear and total sterol response-element binding protein (SREBP)-1, a key lipogenesis transcription factor. These anti-lipogenic effects of were also confirmed by visualization of lipid droplets (LDs). In primary hepatocytes exposed to DHA, LD size greater than 10 μm^2 representing pathologic lipid accumulation disappeared and total area of LD per cell decreased. To ascertain whether DHA's anti-lipogenic effects were due to activity of GPR120 or GPR40, GPR120 knock-out (KO) or Compound A, a GPR120 specific agonist, were used. Interestingly, with CpdA treatment, SREBP-1 levels remain elevated with either an LXR agonist or a high glucose/insulin. Moreover, DHA's anti-lipogenic effects were sustained in the GPR120 KO mouse. In contrast, AMG-1638, a GPR40 specific agonist, lowered lipogenic enzyme levels as DHA did. Additionally,

GW1100, a GPR40 antagonist, reversed the anti-lipogenic effects of DHA. Collectively, our findings demonstrate that the lipid sensor, GPR40, has a key role in sensing DHA in hepatocytes and, therefore, in the control of expression of lipogenic enzymes and hepatic steatosis.

Keywords: GPR40, GPR120, DHA, Omega-3 fatty acid, SREBP-1, Hepatocytes

Student ID number: 2016-25825

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
AST	Aspartate transaminase
CpdA	Compound A
DHA	Docosahexaenoic acid
FAS	Fatty acid synthase
FFA	Free fatty acid
GPR120	G-protein coupled receptor 120
GPR40	G-protein coupled receptor 40
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene Ontology
LDs	Lipid droplets
LXR	Liver X receptor
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ND	Not detected

SCD1	Stearoyl-CoA desaturase-1
SREBP-1	Sterol regulatory element binding protein-1
TG	Triglyceride
T090	T0901317

Introduction

Non-alcoholic fatty liver disease (NAFLD) is rapidly increasing and affecting an estimated 80 to 100 million people in the U.S. alone[1]. In The States, most patients who visited a primary care provider for a routine checkup found their alanine aminotransferase (ALT) and aspartate aminotransferase (AST), markers for liver damage, elevated[2]. Out of all these people who have increased serum levels of ALT and AST, 33 percent of them eventually are diagnosed with NAFLD[2]. This occurrence is not only limited to the western world. In Asia, 25 percent of individuals above 18 years old are diagnosed with NAFLD[3]. Even though this widespread disease has no specific signs and symptoms, it can progress to non-alcoholic steatohepatitis (NASH) and eventually lead to cirrhosis, terminal end stage liver disease[4]. Though treatment options for NAFLD are limited, diet, exercise, thiazolidinedione and Vitamin E are the only options for NAFLD proven effective[5]. Even with these limited options, unfortunately, medication treatments offer a finite benefit compared to diet and exercise[5]. Therefore, in order to attenuate the worldwide burden caused by NAFLD, it is necessary to excavate a specific receptor for possible pharmaceutical remedies to lower fatty acid accumulation in the liver. Omega-3

fatty acids could provide a crucial energy source as nutrients, but also act as a panacea for serum hyperlipidemia. As of now, commercially available fish oil supplements are the most sought after over the counter medications for managing elevated serum triglyceride (TG); in the U.S alone, around 18.8 million adults are taking fish oil supplements[6]. Moreover, omega-3-acid ethyl esters such as Omacor® and Lovaza® are used as prescription medications for dyslipidemia[7]. However, their mechanism of action in lowering lipids is not clear. These omega-3 fatty acids are recognized as ligands for G-protein coupled receptor 40 (GPR40) and G-protein coupled receptor 120 (GPR120). Nevertheless, the proposed direct effects of omega-3 fatty acids on G protein-coupled receptor (GPCR) in hepatocytes are largely uncharacterized and controversial. In the present study, we assessed the effect of docosahexaenoic acid (DHA) on hepatic lipogenesis and attempted to clarify the roles of GPR40 and GPR120 in anti-lipogenesis effect of DHA in primary hepatocytes.

Materials and Methods

Reagent and antibodies

Antibody recognizing precursor sterol regulatory element binding protein-1 (SREBP-1) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-fatty acid synthase (FAS) and anti-nuclear form of SREBP-1 antibodies were supplied by BD Biosciences (Franklin Lakes, NJ, USA). Stearoyl-CoA desaturase-1 (SCD1), Phospho-AMP-activated protein kinase (p-AMPK), and acetyl-CoA carboxylase (ACC) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and T0901317 (T090) were supplied by Calbiochem (San Diego, CA, USA). Anti-β actin antibody, insulin, and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Docosahexaenoic acids (DHA) and Compound A (CpdA) were supplied by Cayman Chemical (Ann Arbor, MI, USA). GW1100 and AMG-1638 were kindly donated from LG Chemistry Institute (Seoul, South Korea).

Cell culture

Human hepatoma cell line HepG2 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 cells were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 50 U/ml penicillin and 50 μg/ml streptomycin. All cell lines were maintained at 37℃ in a humidified incubator with 5% CO_2 .

Animals

GPR120 knock out (KO) C57BL/6 mice were kindly donated from LG Chemistry Institute. The mice were housed in a pathogen-free animal facility under a standard 12 h light/dark cycle. Animal experiments were conducted under the guidelines of the Institutional Animal Use and Care Committee at Seoul National University.

Isolation of primary hepatocytes

After anesthetizing 8-week-old C57BL/6 mice (DBL, Eumseong, Korea) or GPR120 KO mice with zoletil and rompun, 24G catheter was cannulated to liver portal vein. After perfusion with Hanks' Balanced Salt solution medium (Life Technologies, Grand Island, NY) with 0.5 mM ethylene glycol tetraacetic acid and 25 mM

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), liver tissue was digested with low glucose DMEM with 1% penicillin/streptomycin, 15 mM HEPES and 1 mg/ml collagenase from *clostridium histolyticum* (Sigma-Aldrich, St.Louis, MO, USA). After tearing digested liver tissue, the isolated cells were washed three times with high glucose DMEM with 10% FBS, 1% penicillin/streptomycin, 15 mM HEPES, 10 nM dexamethasone. Other cells except primary hepatocytes were all removed by centrifugation.

mRNA isolation and real-time quantitative polymerase chain reaction (qPCR)

After washing with sterile PBS, total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA). mRNA was reverse transcripted to cDNA using Maxime RT Premix (iNtRON, Seongnam, South Korea). Amplified cDNA was analyzed by real-time PCR program (Bio-Rad, Hercules, CA, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad) and SYBR Select Master Mix (Life Technologies). Primer sequences used in experiments were:

5' -CTGTGCAGGAATGAGTGGAAG-3' (GPR120-forward)

5' -CTGATGGAGGGTACTGGAAATG-3' (GPR120-reverse)

5’ -TGGCGCGCCAGCCTGG-3’ (GPR120 KO-forward)

5’ -CCATATGAAAGCCAGCAGTGCC-3’ (GPR120 KO-reverse)

5’ -CGCCGTCGGCGCCGTG-3’ (GPR120 WT-forward)

5’ -CCATATGAAAGCCAGCAGTGCC-3’ (GPR120 WT-reverse)

5’ -TTTCATAAACCCGGACCTAGGA-3’ (GPR40-forward)

5’ -CCAGTGACCAGTGGGTTGAGT-3’ (GPR40-reverse)

5’ -TGGGCATCAACATACCCGTG-3’ (GPR40-forward)

5’ -GCCTCCTAGGTCCGGGTTTA-3’ (GPR40-reverse)

5’ -GTAACCCGTTGAACCCCATT-3' (18s rRNA-forward)

5’ -CCATCCAATCGGTAGTAGCG-3’ (18s rRNA-reverse)

Immunoblot analysis

After washing the cultured cells with PBS, cells were lysed in lysis buffer containing 20 mM TrisHCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2mM EDTA, 1mM sodium orthovanadate, 25 mM β-glycerophosphate, 2mM sodium pyrophosphate, 1 mM phenyl methyl sulfonyl fluoride, and 1 μg/ml leupeptin. Cells were then incubated in ice for 1 hr. The cell lysates were centrifuged at 10000g for 20 min to remove debris, and the protein samples were loaded on 8-15% SDS-PAGE gel and transferred to nitrocellulose membrane (GE healthcare Life Sciences, Chalfont, Buckinghamshire, UK). Membranes were

incubated for 1 hour with 5% skim milk (BD Bioscience, San Jose, CA) and reacted with primary antibodies overnight at 4℃. The membranes were washed and incubated with a second antibody for 1 hour at room temperature. Protein expression was visualized with LAS3000-mini (Fujifilm, Tokyo, Japan) using enhanced chemiluminescence (ECL) system reagent (EMD Millipore, Billerica, MA, USA).

Hematoxylin and eosin staining

The left lateral lobe of the liver was sliced, and tissue slices were fixed in 10% buffered-neutral formalin. The liver slices were stained with hematoxylin and eosin (H&E).

Detection of lipid droplets (LDs) by confocal fluorescence scanning microscopy

For Bodipy 493/503 staining (Thermo Fisher Scientific, Waltham, MA, USA), the 488 nm laser was used and signals were collected with a long pass filter 505 nm. Quantification of LD number and size was performed with MetaMorph, version 7.5(Molecular Devices, San Jose, CA, USA).

Statistical analysis

Densitometric analysis was performed by using Multiguage software (Fujifilm, Tokyo, Japan) and Image J 1.46r. Statistical analysis was performed by using student's t-test and Benjamini. A p value <0.05 was considered statistically significant.

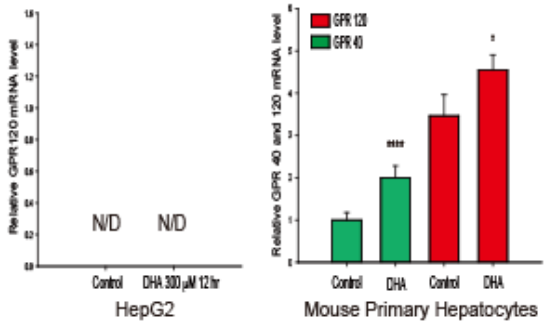
Results

1. Detection of GPR40 and GPR120 expression in mouse primary hepatocytes and public data analysis of hepatic lipogenesis genes after fish oil treatment

To investigate whether GPR40 and GPR120 are present in hepatocytes, qPCR analyses were performed in both HepG2 human hepatoma cell line and mouse primary hepatocytes. We failed to detect GPR120 mRNA in HepG2 cells (data not shown). However, mRNA levels of both GPR40 and GPR120 were detectable in mouse primary hepatocytes and greater mRNA levels of GPR120 were quantified compared to those of GPR40 in primary hepatocytes (Fig. 1A). Moreover, exposure of mouse primary hepatocytes to DHA for 12 hr enhanced the mRNA levels of both GPR40 and GPR120 (Fig. 1A). The protein expression of GPR40 and GPR120 was also confirmed by immunocytochemistry (Fig. 1B). To assess whether omega-3 fatty acids have functional benefits on the liver, microarray data comparing control mice with mice receiving fish oil for 2 weeks were analyzed. Gene ontology analyses revealed that the genes including lipid metabolic processes and cholesterol biosynthetic processes are mainly altered for similar functional

annotations in the fish oil-fed mouse (Fig. 1C). In addition, expression of genes involved in both lipid metabolism and fatty acid metabolic process were repressed in the liver from the fish oil-fed mouse (Fig. 1C). Moreover, a fish oil diet led to a notable reduction in the expression of many genes controlling triglyceride synthesis, including srebf1, acacb, fasn, and scd1 in the liver (Fig. 1D). Collectively, these data suggest that GPR40 and GPR120 sensing long chain fatty acids, are upregulated after DHA treatment in hepatocytes. Additionally, gene expression orchestrating lipid metabolism and fatty acid synthesis in the liver are attenuated following a fish oil diet.

A



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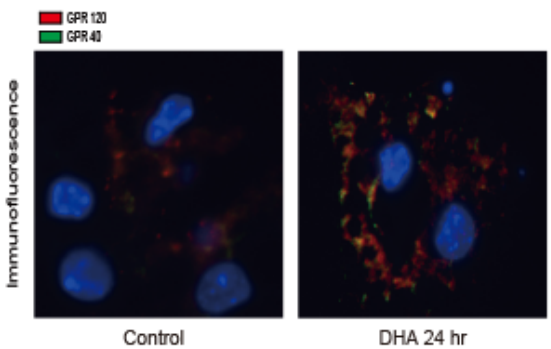


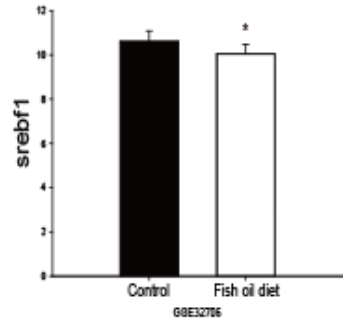
C

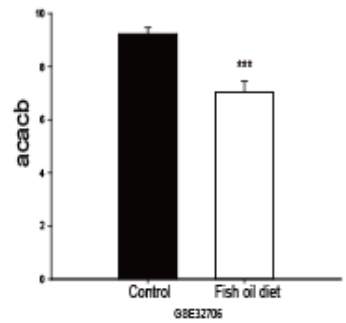
GO term	Benjamini
Lipid metabolic process	5.5E-42
Cholesterol biosynthetic process	6.5E-21
Oxidation-reduction process	7.6E-21
Sterol biosynthetic process	1.6E-20
Fatty acid metabolic process	3.3E-20
Steroid metabolic process	1.1E-18
Steroid biosynthetic process	5.4E-18
Fatty acid biosynthetic process	3.8E-15
Cholesterol metabolic process	5.9E-14
Metabolic process	6.4E-11

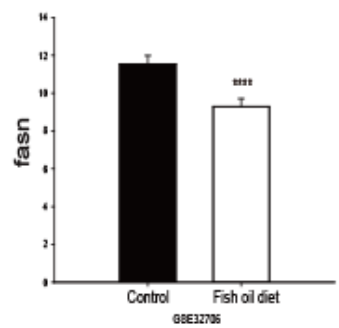
Omega-3 fatty acid repressed genes

GO term	Benjamini
Lipid metabolic process	5.2E-14
Fatty acid metabolic process	6.5E-14
Fatty acid metabolic process	2.7E-09
Fatty acid biosynthetic process	2.6E-07
Acetyl-CoA metabolic process	4.3E-06

D







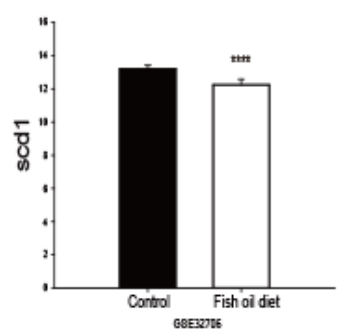


Fig. 1. Changes in GPR40 and GPR120 expression and lipogenesis proteins in mouse primary hepatocytes after DHA treatment.

(A) Both of the relative mRNA expressions of GPR40 and GPR120 levels were determined by real-time qPCR analyses in HepG2 cells and mouse primary hepatocytes. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01, ***P < 0.001.

(B) Immunocytochemistry of GPR40 and GPR120 in primary hepatocytes was performed. Immunofluorescent images of GPR40 and GPR120 at their basal levels and after DHA treatment are depicted.

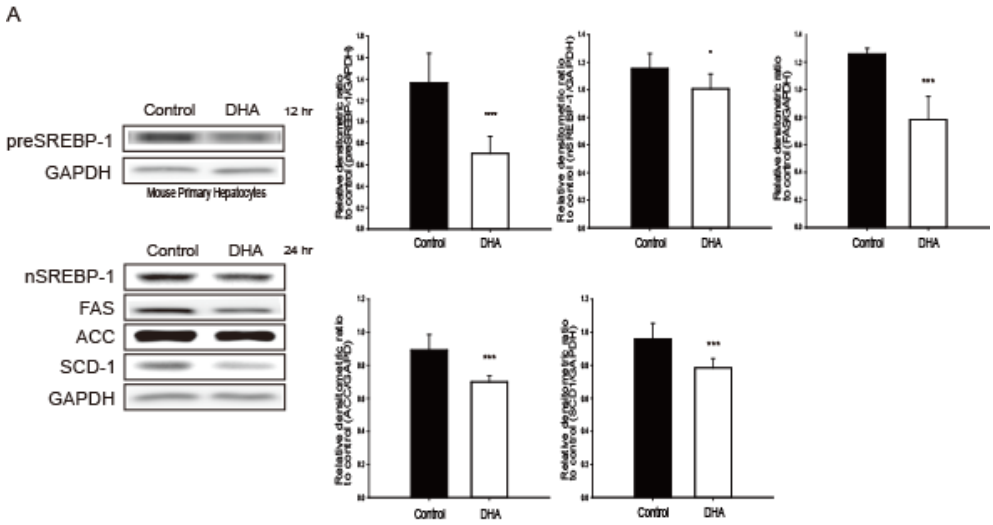
(C) Functional annotations associated with genes exhibiting lipid metabolism and cholesterol metabolism were altered in the fish oil-fed mouse. Also, functional annotations associated with genes exhibiting lipid and fatty acid metabolism were repressed by the fish oil diet in GSE32706.

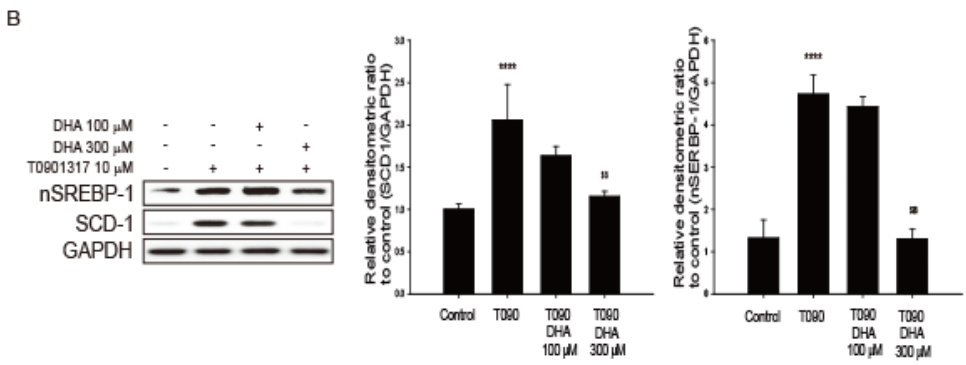
(D) Genes controlling synthesis of triglyceride including srebf1, acacb, fasn, and scd1 were reduced in the liver from the fish oil-fed mouse in GSE32706. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

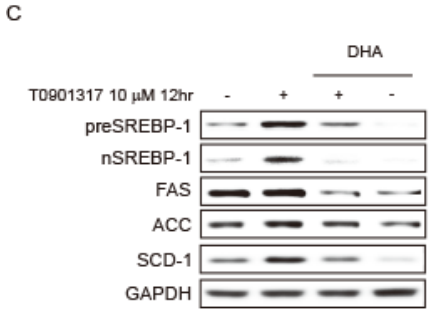
2. Downregulation of lipogenic proteins by DHA treatment in hepatocytes

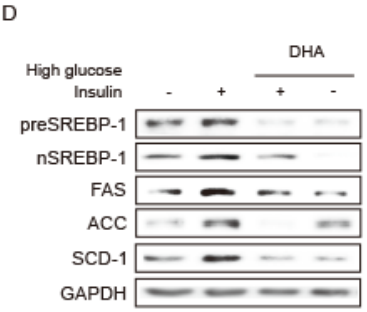
SREBP1 transcription factor is a critical regulator of fatty acid homeostasis. Activation of SREBP1 can control the expression of a range of lipogenic enzymes such as FAS, ACC, and SCD1[8]. Much of the existing evidence relating the role of omega-3 fatty acids in the regulation of lipogenic enzyme expression is somewhat limited in the hepatocytes. Therefore, we analyzed the effects of DHA on the expression of SREBP1 and its downstream lipogenesis enzymes. The protein level of a precursor form of SREBP-1 (PreSREBP-1) was reduced in primary hepatocytes incubated with DHA for 12 hours, and the levels of a nuclear form of SREBP-1 (nSREBP-1), FAS, ACC, and SCD1 were also diminished in hepatocytes treated with DHA for 24 hours (Fig. 2A). To gain further insight into DHA's anti-lipogenic properties, primary hepatocytes were treated with T0901317 (T090), an LXR agonist, to stimulate LXR-dependent SREBP-1 activation. Remarkably, increased protein levels of nSREBP-1 and SCD1 by the T090 compound were decreased by pretreatment with 300 μM DHA for 12 hours (Fig. 2B). Moreover, other lipogenic proteins such as preSREBP-1, FAS, and ACC were elevated by T090 treatment , and all the protein expression was also reduced by DHA (Fig. 2C).

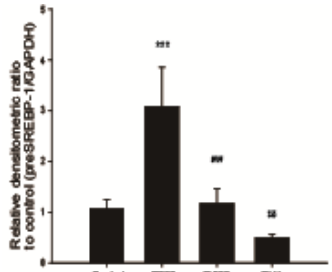
To confirm DHA's anti-lipogenic properties in metabolic dysfunction, the primary hepatocytes were exposed to high glucose and high insulin condition. As expected, the amounts of lipogenic proteins involved in TG synthesis such as preSREBP-1, nSREBP-1, FAS, ACC, and SCD1 were increased by the high glucose with insulin condition and the enhanced levels were recovered by DHA treatment (Fig. 2D). The data support the notion that long-term treatment with DHA amends the lipogenesis caused by either an LXR agonist or a high glucose with insulin.

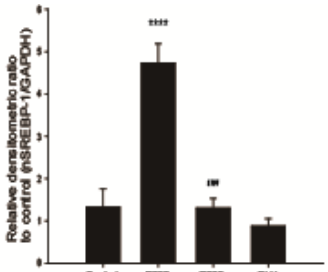

A
Control
DHA
12 hr
preSREBP-1
GAPDH
Mouse Primary Hepatocytes
Control
DHA
24 hr
nSREBP-1
FAS
ACC
SCD-1
GAPDH
Control
DHA

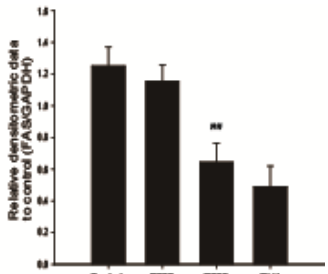

B
DHA 100 μM
DHA 300 μM
T0901317 10 μM
nSREBP-1
SCD-1
GAPDH
Relative densitometric ratio to control (SCD1/GAPDH)
Relative densitometric ratio to control (nSREBP-1/GAPDH)
Control
T090
T090 DHA 100 μM
T090 DHA 300 μM

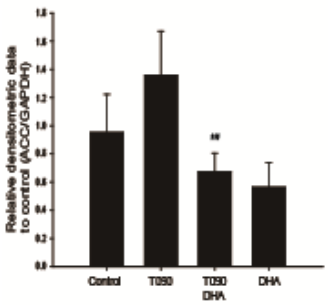

C
DHA
T0901317 10 μM 12hr
preSREBP-1
nSREBP-1
FAS
ACC
SCD-1
GAPDH

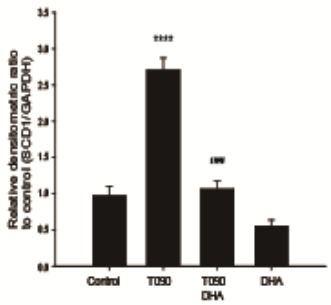

D
DHA
High glucose
Insulin
preSREBP-1
nSREBP-1
FAS
ACC
SCD-1
GAPDH

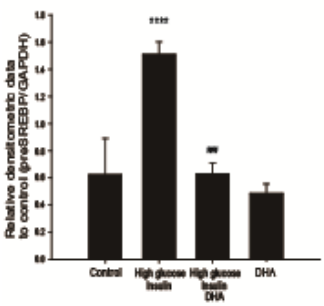

Relative densitometric ratio to control (preSREBP-1/GAPDH)
Control
T050
T050 DHA
DHA

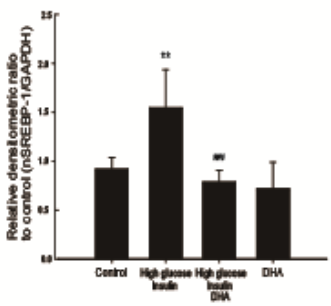

Relative densitometric ratio to control (nSREBP-1/GAPDH)
Control
T050
T050 DHA
DHA

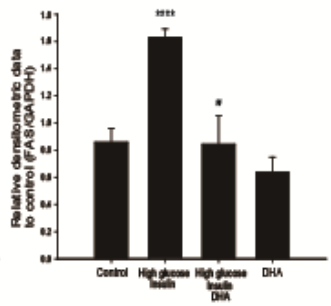

Relative densitometric data to control (FAS/GAPDH)
Control
T050
T050 DHA
DHA

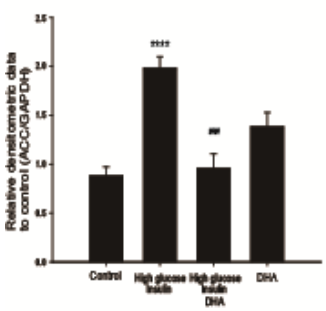

Relative densitometric data to control (ACC/GAPDH)
Control
T050
T050 DHA
DHA


Relative densitometric ratio to control (SCD1/GAPDH)
Control
T050
T050 DHA
DHA


Relative densitometric data to control (preSREBP/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA


Relative densitometric ratio to control (nSREBP-1/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA


Relative densitometric data to control (FAS/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA


Relative densitometric data to control (ACC/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA

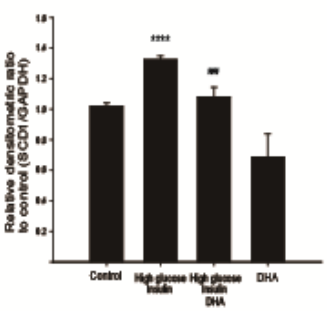

Relative densitometric ratio to control (SCD1/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA

Fig. 2. Decrease in lipogenesis enzymes after DHA treatment.

(A) To explore anti-lipogenic effects of DHA, primary hepatocytes were treated with DHA 300 μM for 12 and 24 hr, and protein levels of preSREBP-1, nSREBP-1 SCD1, FAS, and ACC were determined by immunoblottings. Data represent means ± SD (n=5), *P < 0.05, **P < 0.01, ***P < 0.001.

(B) In order to screen an optimal DHA concentration that exerts potent anti-lipogenic effects, primary hepatocytes were treated with DHA 100 μM and 300 μM for 12 hr followed by T0901317, LXR agonist for additional 12 hr. nSREBP-1 and SCD1 were detected by western blot Data represent means ± SD (n=5), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

(C) Primary hepatocytes were treated with DHA 300 μM for 12 hr followed by T0901317, LXR agonist for additional 12 hr to see anti-lipogenic effects of DHA on other lipogenic proteins. Data represent means ± SD (n=5), *P < 0.05, **P < 0.01, ***P < 0.001.

(D) Primary hepatocytes were treated with DHA 300 μM for 12 hr followed by 30 mM high glucose medium for 30 min and further incubation with 200 nM insulin for 24 hr. Data represent means ± SD (n=5), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3. Reduction of total area of lipid droplets in hepatocytes by DHA

Formation of intracellular lipid droplets (LDs) are known to actively interact with cytosolic proteins and other organelles to balance lipid homeostasis[9]. Abnormal accumulations of LDs in hepatocytes can lead to steatosis[10]. We explored how LDs behave in the hepatocytes with DHA treatment by using super resolution confocal fluorescence images of LDs. In hepatocytes treated with T090 as well as those incubated with high glucose/high insulin condition, a greater increase in LDs was observed (Fig. 3A). Contrastingly, there was no increase in LDs in DHA treated hepatocytes (Fig. 3A, B). Interestingly, there was no difference in the number of LDs per cell between hepatocytes treated with high glucose/high insulin and those co-treated with DHA (Fig. 3C). Nonetheless, number of LD size greater than 10 μm^2 representing pathologic lipid accumulation as well as total area of LD per cell decreased by DHA treatment (Fig. 3D & 3E). A recent study has reported that the decrease in size of LDs is connected with lower activity of SCD1[11]. With the western blot results showing a dramatic decrease in SCD1 with DHA treatment (Fig. 2A), these data demonstrated that DHA could not reduce total number of LDs per

cell but lower total area of LDs. Moreover, we can speculate that this decrease in LD size is due to diminished activity of the lipogenic protein, SCD1, but further research is needed.

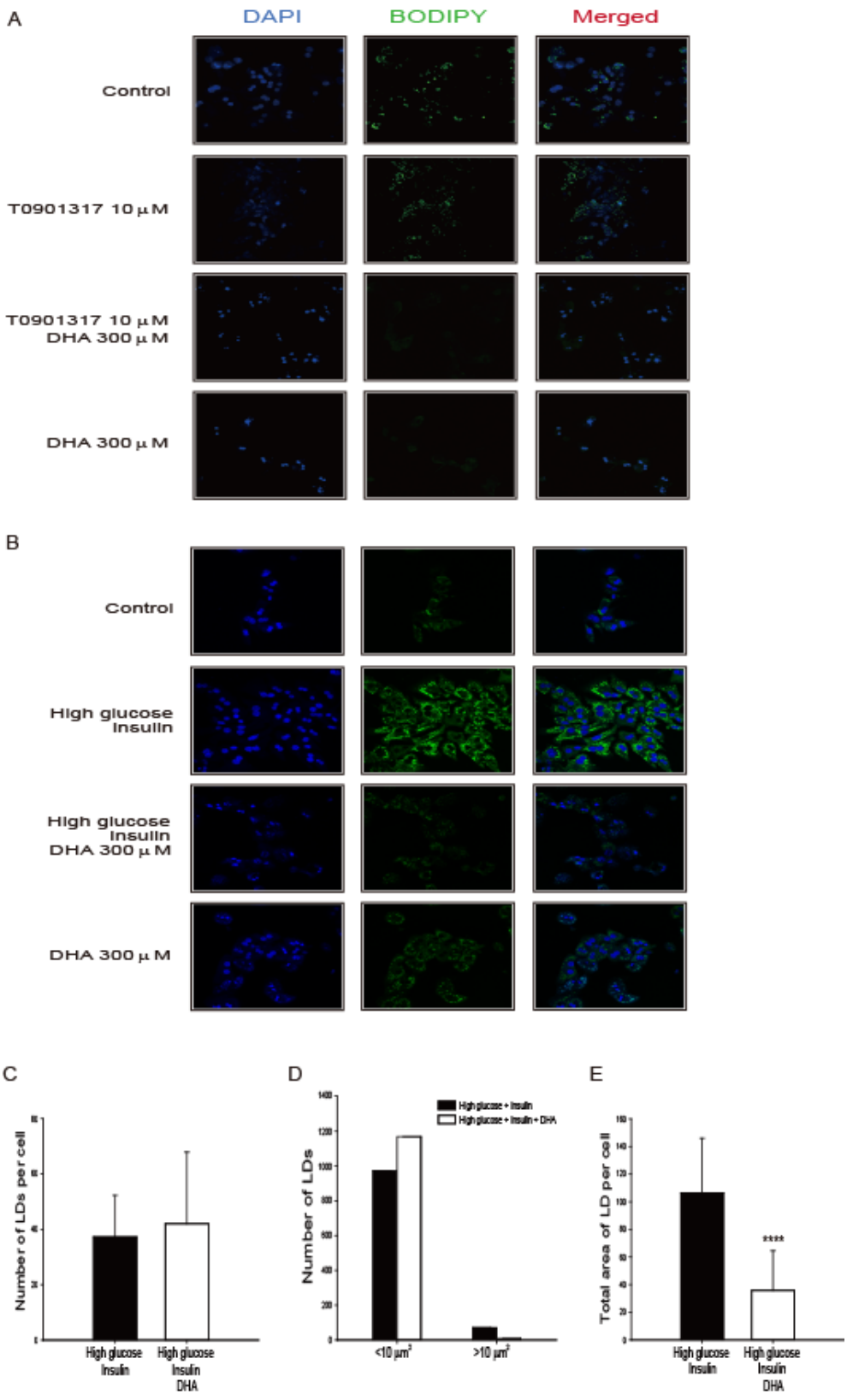

A
DAPI
BODIPY
Merged
Control
T0901317 10 μM
T0901317 10 μM
DHA 300 μM
DHA 300 μM
B
Control
High glucose
Insulin
High glucose
Insulin
DHA 300 μM
DHA 300 μM
C
Number of LDs per cell
High glucose
Insulin
High glucose
Insulin
DHA
D
High glucose + Insulin
High glucose + Insulin + DHA
Number of LDs
<10 μm²
>10 μm²
E
Total area of LD per cell
****
High glucose
Insulin
High glucose
Insulin
DHA

Fig. 3. Morphological changes in LD after DHA treatment

(A, B) LD formation in hepatocytes by T090 and high glucose with insulin condition. Mouse primary hepatocytes were treated with DHA for 12 hr followed by treatment of T090 for 12 hr or incubation with 30 mM high glucose for 30 min and further incubation with 200 nM insulin for 24 hr. LDs were stained with Bodipy 493/503 and visualized by confocal microscopy.

(C) Images of 16 random cells from each slides were captured and analyzed by MetaMorph to quantify number of LDs per cell.

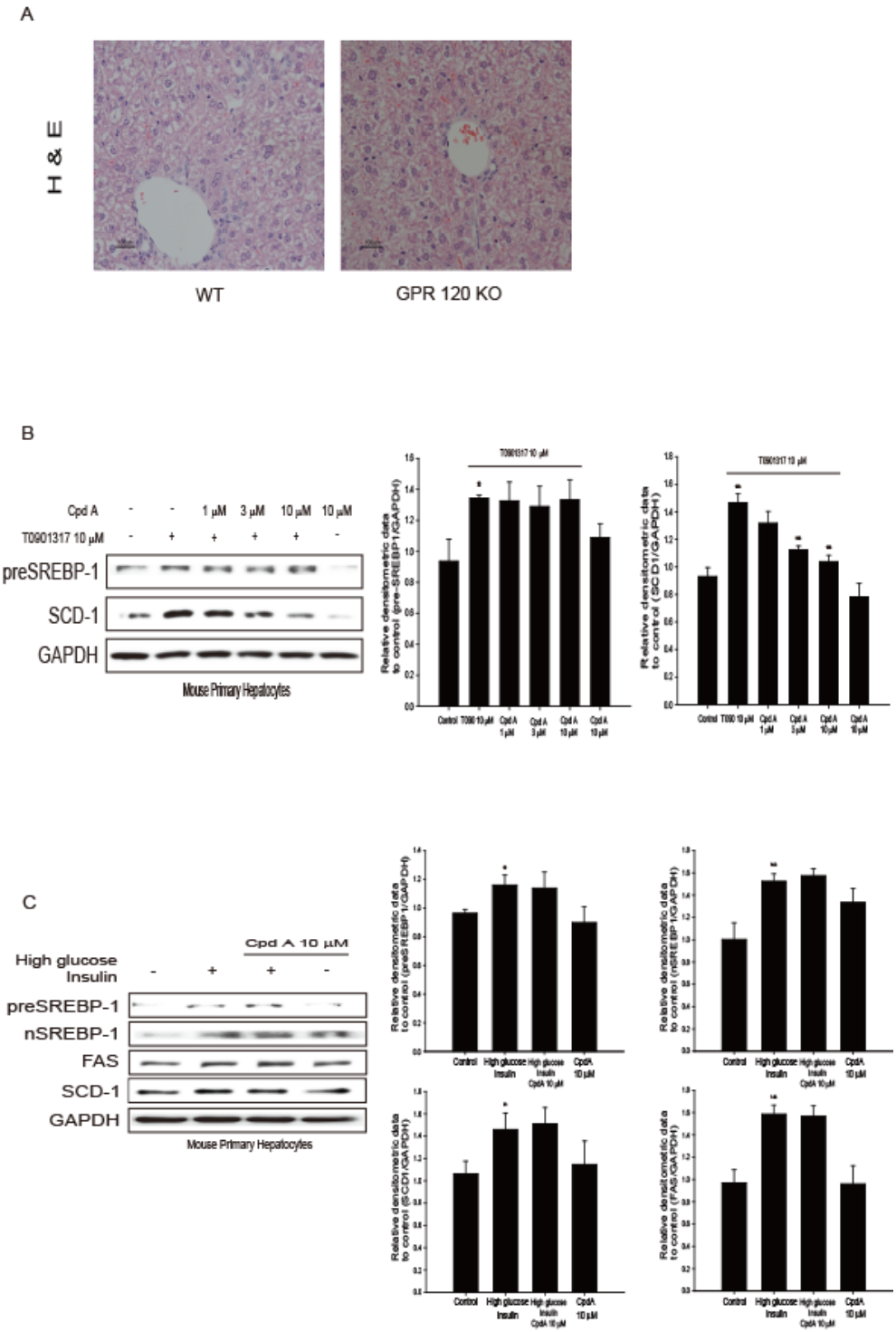
(D) Numbers of LDs with areas $\leq 10\ \mu m^2$ or $>10\ \mu m^2$ were counted and presented in bar graph form.

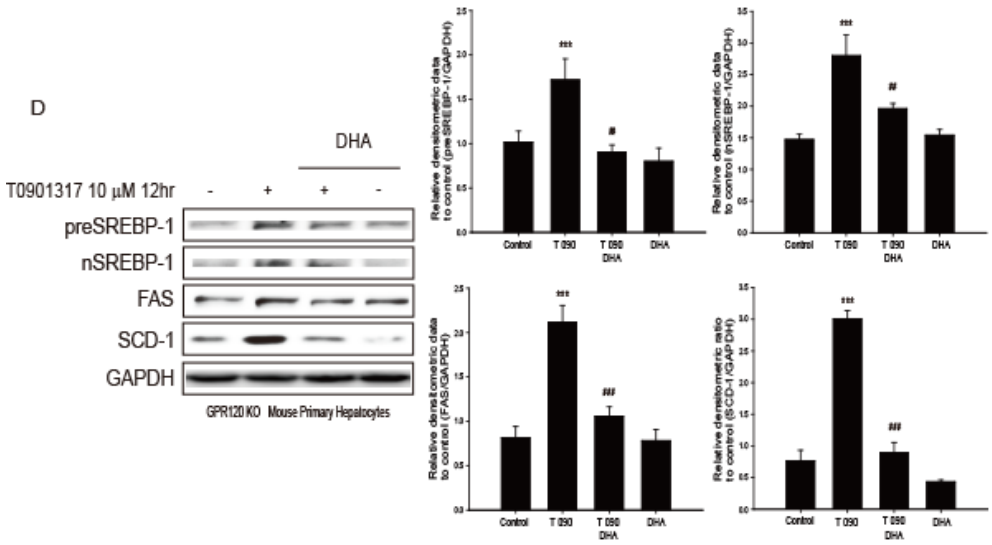
(E) Total area of LDs per cell were measured and presented as means ± SEM, ****$P < 0.0001$.

4. Limited role of GPR 120 in anti-lipogenesis effect of DHA

Because long chain fatty acids such as DHA act as an endogenous ligand on GPR120 and exerts metabolic and inflammation homeostasis in various cells[12], we sought to investigate the ramifications of GPR120 on hepatocytes. A specific GPR120 agonist, Compound A (CpdA), was used to clarify the function of GPR120 on anti-lipogenesis effects of DHA in T090-treated primary hepatocytes. SCD1, induced by the LXR agonist, was downregulated by CpdA in a concentration-dependent manner (Fig. 4A). Captivatingly, unlike DHA, CpdA could not lower the expression of preSREBP1 (Fig. 4A). In high glucose with high insulin condition, CpdA did not reduce the protein levels of preSREBP-1, nSREBP-1, FAS, and SCD1 in hepatocytes as well (Fig. 4B). Studies regarding LXR signaling propose direct interaction between LXR and SCD1[13,14]. Therefore, we presumed that downregulation of SCD1 by CpdA is through an unconventional manner rather than the SREBP-1 pathway. To comprehend the repercussions of CpdA, we further used GPR120 KO mice. GPR120 KO liver microarray data showed that lipogenic genes were elevated compared to WT liver (Fig. S1A). These elevations could represent the lipid buildup at baseline. Moreover, the pre-existence

of lipid accumulation in the liver could alter cellular inflammation and lipid metabolism[15,16]. Therefore, in order to rule out pre-existing histological complications between WT and GPR120 KO mouse, we performed pathological examination of 8 week-old WT and GPR120 KO liver tissues. Shown in Fig. 4C, there were no pathological differences between them. Astonishingly, decreasing effects of DHA on lipogenesis enzyme expression were not abolished in GPR120 KO hepatocytes and even tantamount to those shown in WT (Fig. 4D, E). The data suggest that the specific GPR120 agonist does not influence SREBP-1 expression and other lipogenic enzymes controlled by the SREBP-1 pathway. Moreover, anti-lipogenesis effects of DHA in hepatocytes persisted in the absence of GPR120.


A
H & E
WT
GPR 120 KO
B
Cpd A
T0901317 10 μM
preSREBP-1
SCD-1
GAPDH
Mouse Primary Hepatocytes
Relative densitometric data to control (pre-SREBP1/GAPDH)
Relative densitometric data to control (SCD1/GAPDH)
Control
T090 10 μM
Cpd A 1 μM
Cpd A 3 μM
Cpd A 10 μM
C
High glucose
Insulin
Cpd A 10 μM
preSREBP-1
nSREBP-1
FAS
SCD-1
GAPDH
Mouse Primary Hepatocytes
Relative densitometric data to control (nSREBP1/GAPDH)
Relative densitometric data to control (FAS/GAPDH)
Control
High glucose
High glucose Insulin
High glucose Insulin Cpd A 10 μM
Cpd A 10 μM


D
DHA
T0901317 10 μM 12hr - + + -
preSREBP-1
nSREBP-1
FAS
SCD-1
GAPDH
GPR120 KO Mouse Primary Hepatocytes
Relative densitometric data to control (preSREBP-1/GAPDH)
Relative densitometric data to control (nSREBP-1/GAPDH)
Relative densitometric data to control (FAS/GAPDH)
Relative densitometric ratio to control (SCD-1/GAPDH)
Control
T 090
T 090 DHA
DHA

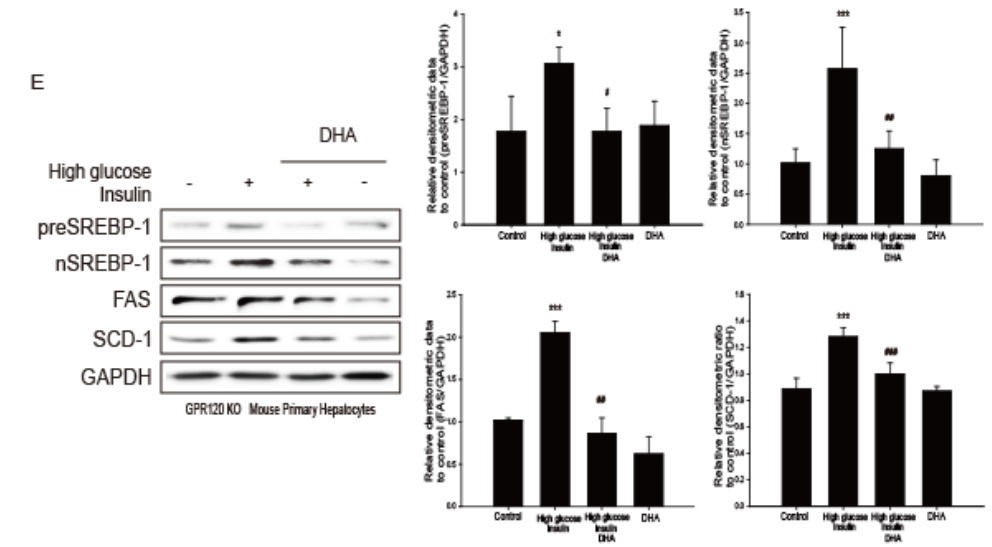

E
DHA
High glucose Insulin - + + -
preSREBP-1
nSREBP-1
FAS
SCD-1
GAPDH
GPR120 KO Mouse Primary Hepatocytes
Relative densitometric data to control (preSREBP-1/GAPDH)
Relative densitometric data to control (nSREBP-1/GAPDH)
Relative densitometric data to control (FAS/GAPDH)
Relative densitometric ratio to control (SCD-1/GAPDH)
Control
High glucose Insulin
High glucose Insulin DHA
DHA

Fig. 4. Role of GPR120 in expression of lipogenesis enzymes in hepatocytes.

(A) Liver sections of 8 week-old WT and GPR120 KO were stained with H&E staining. Original magnification setting of X200 for H&E was used..

(B) Primary hepatocytes were treated with CpdA 1 μM, 3 μM and 10 μM for 12 hr followed by T0901317, LXR agonist for additional 12 hr. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01.

(C) Primary hepatocytes were treated with CpdA 10 μM for 12 hr followed by 30 mM high glucose medium for 30 min and further incubation with 200 nM insulin for 24 hr. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01.

(D) GPR120 KO hepatocytes were treated with DHA for 12 hr followed by T0901317, LXR agonist for additional 12 hr. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01, ***P < 0.001.

(E) Primary hepatocytes were treated with DHA 300 μM for 12 hr followed by 30 mM high glucose medium for 30 min and further incubation with 200 nM insulin for 24 hr. Data represent means ± SD (n=3), *P < 0.05, **P < 0.01, ***P < 0.001

5. Involvement of GPR40 in anti-lipogenesis effect of DHA

The aforementioned findings led us to look at other G-protein coupled receptors sensing omega-3 fatty acids. The long chain fatty acid sensing receptor, GPR40, is mainly expressed in pancreatic β-cells and its agonism in hepatocytes are yet to be elucidated. One of the critical recent findings demonstrated that activation of GPR40 stimulated insulin secretion only in the presence of elevated glucose levels[17-19]. Thus, GPR40 has been an attractive potential therapeutic target for glucose homeostasis with little to no hypoglycemic risk. Moreover, activation of GPR40 is known to cause an influx of calcium into cells which lead to phosphorylation of AMPK, a potent regulator of fatty acid metabolism[20,21]. The GPR40 agonist, AMG-1638, is a well-studied compound in murine species and has shown specific full agonistic activities compared to several other candidates[22]. Therefore, in order to assess the role of GPR40 in hepatocytes, we used AMG-1638. Upregulated SREBP-1 and SCD1 by T090 were decreased by 3 μM AMG-1638 (Fig. 5A). Even in high glucose with insulin condition, AMG-1638 lowered the protein expression of lipogenic enzymes such as preSREBP-1, nSREBP-1, FAS, and SCD1 (Fig. 5B). To confirm if anti-lipogenic properties were mainly through

GPR40 activation, we next tested the effect of GPR40 antagonist, GW1100. When hepatocytes were co-incubated with both GW1100 and DHA, anti-lipogenic properties of DHA were abolished. The enhanced preSREBP-1 and SCD1 by T090 remained elevated in the presence of GW1100 (Fig. 4C). Moreover, these results were observed in GPR120 KO hepatocytes (Fig. 5D). Hence, we can speculate that GPR40 is essential for DHA's anti-lipogenic effects.

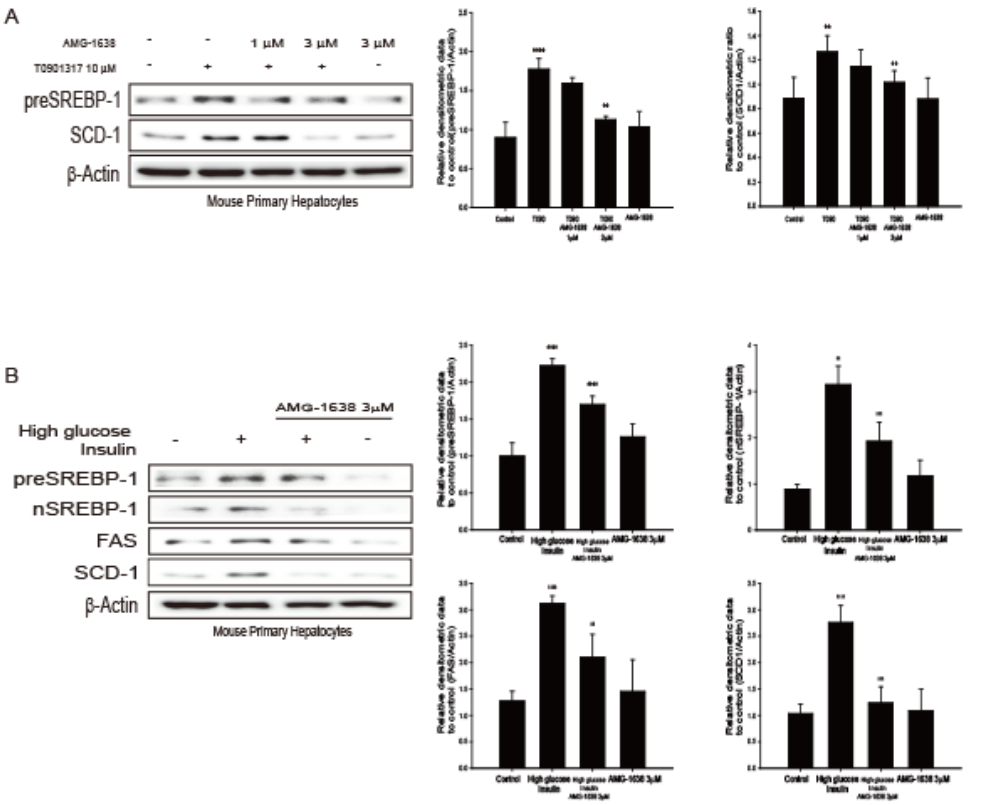

A
AMG-1638
T0901317 10 μM
1 μM
3 μM
3 μM
preSREBP-1
SCD-1
β-Actin
Mouse Primary Hepatocytes
B
AMG-1638 3μM
High glucose
Insulin
preSREBP-1
nSREBP-1
FAS
SCD-1
β-Actin
Mouse Primary Hepatocytes
C
DHA
GW1100 10 μM
T0901317 10 μM
preSREBP-1
SCD-1
GAPDH
Mouse Primary Hepatocytes
D
DHA
GW1100 10 μM
T0901317 10 μM
preSREBP-1
SCD-1
GAPDH
GPR 120 KO Mouse Primary Hepatocytes

Fig. 5. Effects of GPR40 agonist, AMG-1638 and GPR40 antagonist, GW1100 on the expression of lipogenesis enzymes in primary hepatocytes.

(A) Primary hepatocytes were treated with AMG-1638 1 μM and 3 μM for 12 hr followed by T0901317, LXR agonist for additional 12 hr. Concentration-dependently, lipogenic enzymes were lowered by AMG-1638. Data represent means ± SD (n=4), *P < 0.05, **P < 0.01, ***P < 0.001.

(B) Anti-lipogenic effects of AMG-1638 were also observed in high glucose and high insulin condition. Primary hepatocytes were treated with AMG-1638 3 μM for 12 hr followed by 30 mM high glucose medium for 30 min and further incubation with 200 nM insulin for 24 hr. Lipogenic enzymes were significantly lowered by AMG-1638. Data represent means ± SD (n=4), *P < 0.05, **P < 0.01, ***P < 0.001.

(C) Primary hepatocytes were treated with DHA and GW1100, GPR40 antagonist, 10 μM for 12 hr followed by 30 mM high glucose medium for 30 min and further incubation with 200 nM insulin for 24 hr. Data represent means ± SD (n=4), *P < 0.05, **P < 0.01, ***P < 0.001.

(D) In GPR120 KO hepatocytes, 10 μM GW1100 abrogated the anti-lipogenic effects of DHA. Data represent means ± SD (n=4), *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

Emerging evidence suggests that GPR40 and GPR120 are the omega-3 fatty acids' molecular targets. Since omega-3 fatty acids were elucidated as ligands for those receptors, many studies have been performed to explore both the receptors' potential effects in various cells and organs. However, in hepatocytes, possibly due to limited expression levels of GPR40 and GPR120 compared to other cells[23,24], there are limited studies providing a clear conclusion of which receptor is involved in the anti-lipogenic effects of omega-3 fatty acids. In this study, we investigated the effects of DHA on lipogenic enzyme expression in primary hepatocytes and sought to clarify the related lipid-sensing GPCR exerting its anti-lipogenic properties.

Both DHA and eicosapentaenoic acid (EPA) are under the umbrella of omega-3 fatty acids and act as ligands for GPR40 and GPR120. Even though they both have been shown to have anti-lipogenic properties, DHA is associated with a greater reduction in serum TG levels compared to EPA. In addition, DHA has a lipid control benefit over EPA by increasing high-density lipoprotein (HDL)[25]. Therefore, in this study, our aim was to focus on DHA and its anti-lipogenic properties.

Conventionally, when G-protein coupled receptors are persistently stimulated, cell receptors are downregulated to eschew repercussion effects caused by over-activation. Fascinatingly, Valerio Nobili et. al. reported that patients who were administered DHA developed increased levels of GPR120 in hepatocytes[26]. This result correlates with our findings that mRNA levels of both GPR120 and GPR40 are increased in mouse primary hepatocytes treated with DHA. There is an uncertainty in our data of whether the increased mRNA levels of GPR40 and GPR120 are due to direct stimulation of those receptors versus DHA's possible unknown targets. Regardless, in our data, GPR120 mRNA expression levels were higher than those of GPR40 at base line. However, with DHA treatment, even though the expression of both receptors increased, GPR40 mRNA levels were more significantly increased than those of GPR120.

Even though both GPR40 and GPR120 have different molecular structures, they share long chain fatty acids as their endogenous ligands and it is difficult to find highly specific ligands to discriminate both the receptors[27]. Before CpdA was designed by Merk, the TUG-891 was known to be the most selective compound for GPR120. Unfortunately, the TUG-891 compound loses its selectivity towards GPR120 in murine species[28]. Thus, in this study,

CpdA was used to target GPR120 in mouse primary hepatocytes. We found that CpdA could not lower the protein expression of lipogenic enzymes stimulated by high glucose with insulin milieu. Moreover, even in the GPR120 KO mouse, DHA's anti-lipogenic effects were sustained. From this finding, we can assert that, in hepatocytes, GPR120 is unnecessary or at least has a limited function in anti-lipogenic effects caused by DHA.

Because GPR40, a $G\alpha q_{/11}$-coupled receptor, is mainly expressed in pancreatic β-cells, previous research focused on its potential ability to stimulate insulin secretion. Moreover, GPR40 is negligibly expressed in the liver[29]. Thus, functional roles of GPR40 in hepatocytes have received less attention. In this study, to inspect if GPR40 plays a functional role in hepatocytes, we used a specific GPR40 full agonist, AMG-1638. The enhanced lipogenic protein levels by LXR agonist or high glucose with insulin milieu were diminished by AMG-1638 in primary hepatocytes. Furthermore, GPR40 antagonist, GW1100, abrogated the anti-lipogenic effects of DHA. These cell-based analyses using specific ligands suggest that the receptor may play an anti-lipogenic role in hepatocytes.

It has been shown that stimulation of GPR40 and GPR120 with long chain fatty acids cause a concentration-dependent increase in intracellular calcium in various cell types[30]. Hence, we

raised further questions of whether DHA's anti-lipogenic properties are mediated through activation of AMP-activated protein kinase (AMPK). As we suspected, AMPK was phosphorylated by DHA treatment in primary hepatocytes (Supplementary Fig. A).

From our discovery, even though GPR40 appears to be an omega-3 fatty acid's promising potential target, irreconcilable propositions contrary to our findings were issued recently. S Kang et. al. proclaimed DHA inhibits LXR-induced lipogenesis through GPR120 rather than GPR40[31]. These data somewhat correlates with our data using the CpdA as a specific ligands for GPR120 (Fig. 4A). As we mentioned earlier, direct linkage between LXR and SCD1 may play a role with GPR120. Therefore, more research is warranted to explore the relationship between GPR120 and LXR. In addition, we found set of publicly available liver microarray data regarding WT and GPR120 KO mice fed with either normal diet (ND) or high fat diet (HFD). Analysis of this microarray data revealed GPR120 KO mice fed with HFD have higher expression of lipogenic genes such as SREBF1, SCD1, ACACB, and FASN than that of WT (Supplementary Fig. B). However, since GPR40 and GPR120 are ubiquitously presented throughout the whole body and metabolic harmonies can only achieved by multiple orchestrating

organs, these increase in lipogenic genes in GPR120 KO mice fed with HFD could possibly interpret as knocking out GPR120 in the whole body, hindering lipogenic homeostasis.

Nevertheless, the findings made in this study present a plausible mechanism to by which omega-3 fatty acids can ameliorate lipid droplet accumulation in the hepatocytes. They also demonstrate upregulation of GPR40 and GPR120 following DHA treatment to the hepatocytes. Further investigation shows that DHA lowers lipogenic proteins in both the WT and GPR120 KO mouse. However, with the GPR40 antagonist, DHA's anti-lipogenic properties are abolished. Collectively, these findings may help to unravel how DHA can alleviate the burden caused by fatty acid accumulation in hepatocytes. Furthermore, these findings support the notion of using specific GPR agonists as an add-on therapy to manage metabolic syndrome and suggest that GPR40 merits further investigation as an adjuvant therapy for NAFLD.

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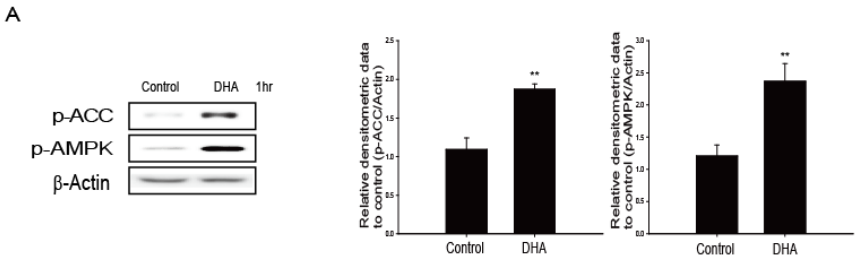
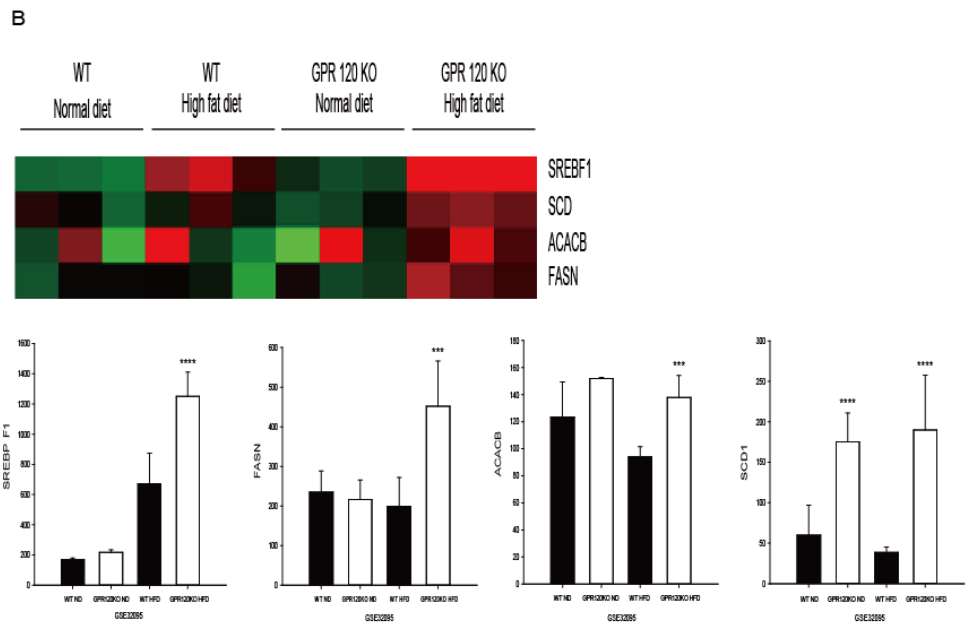
Supplemental Information





초록

Effects of Docosahexaenoic Acid on SREBP1-Mediated Lipogenic Enzyme Expression in Hepatocytes;
Role of Long Chain Fatty Acid Sensing GPCRs

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지방간(hepatic steatosis)은 간 질환 중 가장 빈번한 질환 중 하나이다. 미국 일차 병원의 초진 환자 중 대부분에서 간 손상 지표인 alanine aminotransferase (ALT), aspartate amino-transferase(AST)가 증가 되어있고 그 중 33%가 비알콜성 지방간질환(nonalcoholic fatty liver disease, NAFLD)으로 진단 받았다. 아시아인 같은 경우 5 년 동안 18세 이상의 집단을 추적 관찰 해 본 결과, 12%가 비알콜성 지방간질환으로 진단받았다. 이러한 NAFLD의 경우 뚜렷한 증상은 없지만 지방간(steatosis)을 시작으로 지방간염(non-alcoholic steatohepatitis, NASH), 섬유화증(fibrosis) 나아가 간 경변(cirrhosis)까지 진행될 수 있는 질환이다. 하지만 지방간에 대한 치료는 식이조절과 thiazolidinedione 계열 약물들 그리고 고농도 비타민 E

로 제한되어 있고 식이 조절을 제외한 약물 치료는 효과가 미미한 실정이다. omega-3 fatty acid는 최근 임상연구에서 관상동맥 질환, 뇌졸중 그리고 당뇨병의 예방과 치료에 효과가 없는 것으로 밝혀졌지만 임상적으로 중성지방을 낮춘다는 사실은 잘 알려져 있다. 그러나 실제 omega-3 fatty acid의 간 세포내 중성지방 합성과정에 영향을 미치는 표적 수용체 규명에 관한 연구는 제한적으로 수행되었으며, 아직도 논란이 되고 있다. 본 연구에서는 omega-3 fatty acid의 간세포내 지질 조절 기전을 1차 배양 간세포에서 연구하고자 하였다. Medium-long chain fatty acid 수용체인 GPR40과 GPR120은 omega-3 fatty acid 중 하나인 docosahexaenoic acid(DHA)의 수용체로 알려져 있다. 기존 연구결과에서 GPR40의 경우 베타 세포나 장내 분비 세포에 그리고 GPR120의 경우 대식 세포와 장에 많이 발현되어 있는 것으로 알려져 있으며, 이 두 수용체의 간 조직 내 발현은 거의 없는 것으로 알려져 있다. 그러므로 대부분의 GPR40과 GPR120의 연구가 간세포에서는 부족한 현실이다. 따라서 본 연구에서는 먼저 마우스의 1차 배양 간세포에서 GPR40와 GPR120의 기저 발현 양을 측정하였다. 평가 결과, GPR40 및 GPR120 두 수용체가 간 세포에 상시 발현되어 있을 뿐만 아니라 DHA에 의하여 그 발현이 증가됨을 PCR및 면역조직화학분석을 통하여 확인하였다. 다음으로 간 세포에서 omega-3 fatty acid의 효과를 알아보기위해 GEO 분석을 시행한 결과 2주 동안 생선 기름을 투여한 쥐 간의 지질 합성 효소 유전자 중 sterol regulatory element-binding transcription factor 1(srebf1), acetyl-CoA carboxylase beta(acacb),

fatty acid synthase(fasn) 그리고 stearoyl-CoA Desaturase(scd1)의 발현이 감소 된 것을 확인 하였다. 또한 마우스의 1차 배양 간세포에서 liver X receptor(LXR) 리간드 나 고당/고인슐린 노출에 의한 간세포의 sterol regulatory element binding protein-1(SREBP-1) 및 지질 합성 효소들의 발현의 증가가 DHA 처리에 의하여 억제되는 것을 확인 하였다. 이러한 DHA의 SREBP-1및 지질 합성 효소들의 억제 효과가 간에서 생성되는 지질 표현형에도 같은 효과가 있는 지 확인하기 위하여 본 연구에서는 Metamorph 프로그램을 사용 하여 지질의 개수와 면적을 확인 하였다. 고당/고인슐린에 노출된 마우스의 1차 배양 간세포에 DHA를 처리 하였을 경우 지질의 개수는 유의성 있게 변화되지 않았지만 병적 상태를 나타내는 면적 10 μm^2 이상의 지질이 DHA 처리 후 감소되었다. 그뿐만 아니라 각각의 간세포에서 지질의 총 면적 또한 감소되었다. 이것으로 보았을 때 DHA가 간 세포 내에 지질 방울의 숫자는 감소시키지 않지만 SREBP-1에 관여하여 지질의 면적을 줄이면서 비정상적인 간 내 지질 방울의 축적을 감소시킨다고 보여진다. DHA의 지질 합성 효소 억제 기전이 GPR40과 GPR120 수용체 중 어떤 수용체에 리간드로 작용하는지 알아보기 위해 GPR120 결손형(knock out, KO) 마우스의 1차 배양 간세포에서 LXR 리간드나 고당/고인슐린 처리에 의해 증가되는 SREBP-1과 지질 효소들을 확인하였다. GPR120 KO에서도 GPR120 야생형(wild type, WT)과 같이 DHA에 의한 SREBP-1 및 지질 효소의 억제 효과가 보여졌다. 흥미롭게도 WT 마우스의 1차 배양 간세포에서 GPR40 길항제인 GW1100을 DHA와 같이

처리 하였을 경우 DHA의 SREBP-1 억제 효과가 없어지는 것이 관찰되었다. GPR120 선택적 리간드인 Compound A를 농도 별로 처리해본 결과 LXR 리간드에 의해 증가된 SCD1만 Compound A에 의하여 농도 의존적으로 감소되는 것이 확인되었으며, SREBP-1 및 다른 지질 합성 단백질은 Compound A에 의해 감소되지 않는 것이 관찰되었다. 한편, GPR40 리간드인 AMG-1638을 처리하였을 때에는 LXR 리간드나 고당/고인슐린에 의해 증가된 SREBP-1 및 다른 지질 합성 단백질들이 감소되는 것을 확인하였다. 종합하면, 본 연구에서는 간세포에 GPR40과 GPR120이 발현되어 있을 뿐만 아니라, DHA에 의해 두 수용체가 유도됨을 밝혔다. 또한 DHA가 증가된 GPR40 수용체에 리간드로 작용하여 SREBP-1 및 다른 지질 합성 단백질 발현 억제를 일으켰으며, 지질 방울 면적의 감소 또한 일으켰다. 이는 DHA에 의한 간 세포내 지방축적 억제효과가 주로 GPR40 수용체의 작용으로 이루어진다는 사실을 시사한다.

주 요 어: GPR40, GPR120, DHA, Omega-3 fatty acid, SREBP-1, Hepatocytes

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Seungtae On