Master's Thesis of Medicine

Role of SENP2 on Fatty Acid Metabolism by Leptin in White Adipocyte and Cardiomyotube

렙틴에 의한 백색 지방 세포 및 심장 근육 세포에서의 지방산 대사에 대한 SENP2 의 역할

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Abstract

Role of SENP2 on Fatty Acid Metabolism by Leptin in White Adipocyte and Cardiomyotube

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In chronic metabolic diseases such as obesity and type 2 diabetes mellitus, increase of fatty acids in the blood and accumulation of fat in non-adipose tissues may worsen insulin resistance in peripheral tissues and lead to dysfunction of pancreatic β-cells. Leptin is an adipokine secreted from adipose tissue, which suppresses appetite from hypothalamus and increases fatty acid oxidation (FAO) in peripheral tissues. Although leptin was known to increase FAO through AMPK activation in skeletal muscle, it was recently found to increase FAO through the SUMO-specific protease 2 (SENP2)-PPAR pathway. However, it is not known whether SENP2 is involved in the effects of leptin in other tissues, such as adipose tissue and cardiac muscle. In this study, I investigated the role of SENP2 on fatty acid metabolism by

leptin in white adipocyte 3T3-L1 and cardiomyotube H9C2, as well as in adipocyte-specific SENP2 knock-out (SENP2 aKO) mice model.

When adipocytes and cardiomyotubes were treated with leptin, phospho-STAT3 and phospho-AMPK increased, and SENP2 expression also increased. In addition, expressions of FAO-associated genes, such as carnitine palmitoyl transferase 1b (CPT1b) and long-chain acyl-coenzyme A synthetase 1 (ACSL1) increased. AMPK and SENP2 knock-down experiments using small interfering ribonucleic acid showed that increase of FAO by leptin in adipocytes and cardiomyotubes was regulated by the AMPK pathway during the initial several hours, whereas the increase at 24 h was mainly dependent on the SENP2 pathway. Moreover, following the intraperitoneal injection of leptin into SENP2 aKO mice, FAO of visceral adipose tissue and subcutaneous adipose tissue and their levels of CPT1b and ACSL1 were not increased at 24 h after leptin injection, whereas soleus muscle and heart were not affected by knock-out and showed increase in FAO and FAO-related genes by leptin. Furthermore, SENP2 increased binding of PPARs on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment, which mediated prolonged increase of FAO.

Overall, the results of this study suggest that SENP2 plays an important role in leptin-induced fatty acid metabolism in white adipose tissue and cardiac muscle.

Keywords : SENP2, type 2 diabetes mellitus, fatty acid metabolism, insulin resistance, obesity, leptin

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List of Abbreviations

ACC: acetyl-coenzyme A carboxylase, acetyl-CoA carboxylase

ACO: acyl-CoA oxidase, ACOX

ACSL1: long-chain acyl-CoA synthetase 1

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, AMPK activator

AMP: 5' adenosine monophosphate-activated protein

AMPK: AMP-activated protein kinase

CPT1b: carnitine palmitoyl transferase 1b

FAO: fatty acid oxidation

FAS: fatty acid synthase

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

LepR: leptin receptor

OBRb: long form of LepR

PPAR: peroxisome proliferator-activated receptor

PPRE: peroxisome proliferator response element

SENP2: SUMO/sentrin-specific protease 2

STAT3: signal transducer and activator of transcription 3

SUMO: small ubiquitin-like modifier

JAK: Janus kinase

UCP2: mitochondrial uncoupling protein 2

Introduction

Type 2 diabetes mellitus (T2DM) is a worldwide epidemic. According to International Diabetes Federation, 463 million of age 20-79 years-old were living with diabetes in 2019, and the prevalence of diabetes globally is estimated to rise to 700 million by 2045 [1]. The prevalence of T2DM in the Republic of Korea was 14.4 % of Korean population in 2019, where 1 in 7 Koreans of age 30 years-old or older was diabetic or prediabetic [2,3]. Among patients with diabetes, obesity is another issue to solve, as half of them are obese with body mass index over 25 kg/m^2 [2]. Obesity increases insulin resistance, where the body does not fully respond to insulin, and with blood glucose levels keep rising, releasing more insulin [4]. Insulin resistance eventually exhausts the pancreatic β-cells, resulting in decline in insulin secretion, causing hyperglycemia and T2DM [4].

In the case of obesity, adipose tissue causes insulin resistance by releasing excess fatty acids that produce intracellular accumulation of lipid metabolites, leading to lipotoxicity, chronic low-grade inflammation, endoplasmic reticulum stress, failure of lipolysis suppression, and mitochondrial dysfunction [16]. Obesity is also characterized by a significant increase in adiposity from hypertrophy of pre-existing adipocytes or from hyperplasia by adipogenesis [51]. The excessive levels of glucose and free fatty acids are also known to induce tissue-specific cellular damage via increasing reactive oxygen species [26]. Insulin resistance in the adipose tissue is accompanied by reduced adiponectin level and increased resistin level, which are hormones that regulate 5' adenosine monophosphate-activated protein kinase (AMPK), insulin signaling, and glucose metabolism [16]. In addition,

inflammatory cytokines, such as interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 are known to increase in adipocytes from obese subjects [16]. Obesity also induces local inflammation and interleukin-6 in the heart, that inhibit AMPK and cause insulin resistance, leading to disturbed fatty acid oxidation (FAO), oxidative stress, and cell death [16,29]. Insulin resistance in heart eventually leads to diabetic cardiomyopathy, hypertension, and chronic heart failure [29].

Cellular resistance to insulin develops in multiple organs, such as skeletal muscle, liver, and adipose tissue as an important pathophysiologic abnormality of T2DM [6]. The skeletal muscle functions to uptake 70-90 % of glucose from exercise for blood glucose homeostasis, and insulin resistance in skeletal muscle is the primary defect in T2DM [5,7]. Previously, with skeletal muscle and C2C12 myotubes, the studies observed if reducing fat storage, or increasing FAO works to improve insulin resistance [6]. Treatment of C2C12 myotubes with palmitate has shown to increase FAO and FAO-related genes, including carnitine palmitoyl transferase 1b (CPT1b) and long-chain acyl-coenzyme A synthetase 1 (ACSL1) [6]. SUMOylation (small ubiquitin-like modifier modification) is responsible for various cellular processes, including signal transduction and gene expression, and it works by binding SUMO1-3 to lysine residues of proteins [31]. In humans, 6 isoforms of SUMO-, or sentrin-specific protease (SENP) were classified in 3 groups [18]. First group comprises SENP1-2, localized at the nuclear periphery, second group contains SENP3 and SENP5 in the nucleolus, and the third group consists of SENP6-7 in the nucleoplasm [18]. SENPs function to deconjugate SUMOylated proteins or process SUMO precursor activity [56]. Specifically, SENP2-mediated

deSUMOylation in skeletal muscle has been reported to increase peroxisome proliferator-activated receptor (PPAR) binding on CPT1b and ACSL1 promoters by deSUMOylating PPARδ/γ, which ultimately increases FAO [8].

Ever since the discovery of leptin in 1994, many research works have focused on leptin for its mechanism for obesity and leptin resistance [41]. Leptin is a 16-kDa fat-derived cytokine mainly produced from the adipose tissue and has a primary role in regulating food intake and controlling the body weight [20]. Leptin functions as a negative feedback signal for regulating energy balance; however, it also functions to regulate fatty acid and glucose homeostasis, reproduction, inflammation, tissue remodeling, bone physiology, and immunity [9,34]. The metabolic decision by leptin to either perform lipolysis or store triacylglycerol as fat occurs via leptin receptor (LepR) [38]. Lacking leptin can lead to development of various disorders in liporegulation, including lipoapoptosis, steatosis, lipotoxicity, and diet-induced obesity in non-adipose tissues that are not well-adapted for triacylglycerol storage [10].

Leptin functions by interacting with its LepR that belongs to cytokine class 1 receptor family on the plasma membrane [20]. There are multiple isoforms of LepR (OBRa-OBRe), where the long isoform OBRb is dominant over short isoforms OBRa, OBRc, and OBRd in mice, and over OBRa and OBRc in humans [12]. This OBRb is in fact, the only receptor that has a cytoplasmic portion that associates with Janus kinase 2 pathway, which in turn phosphorylates specific tyrosine residues on its receptor, recruiting other mediators such as signal transducer and activator of transcription (STAT) 1, 3, 5 and 6 to get activated and phosphorylated [9,12,19]. As STAT3 becomes phosphorylated by binding to OBRb, it dimerizes

and translocates to the nucleus to allow for transcription of leptin target genes [12]. OBRb is also expressed in peripheral tissues, where it is directly activated without passing through the central nervous system [12,39]. Leptin-stimulated FAO and fatty acid metabolism work in an acute manner via the AMPK pathway as rapid as 15 min after leptin treatment in skeletal muscle (Figure 1) [8,13]. The previous study in our laboratory showed that leptin increased AMPK activity, providing acute increase of FAO in C2C12 myotubes [8]. It also showed that leptin binding to OBRb stimulated for STAT3 phosphorylation and led to increase in SENP2 and FAO levels at prolonged state (24-48 h) in C2C12 myotubes, while knock-down of SENP2 explained reduction in leptin's chronic effect on FAO [8]. The role of SENP2 and leptin effects on FAO in skeletal muscle mentioned in previous experimental outcomes essentially function as a starting point for this related study (Figure 1) [8].

OBRb is also present in adipose tissue and cardiac muscle. Both OBRa and OBRb gene expressions increase during adipogenesis, and leptin has been reported to directly inhibit insulin-stimulated increase in fatty acid uptake in differentiated 3T3-L1 adipocytes [42,49]. In terms of leptin effects on fatty acid metabolism, leptin was found to decrease lipogenesis, increase triglyceride hydrolysis, and increase FAO in rat adipocytes [40]. Leptin also has shown to increase enzymes involved in fatty acid metabolism, including CPT1, PPARα, acyl coenzyme A oxidase and mitochondrial uncoupling protein 2 in isolated rat and mice adipocytes [25,45]. The previous research with intraperitoneal administration of leptin in wild type animals has shown to suppress expressions of lipogenic enzymes, including adenosine triphosphate-citrate lyase and fatty acid synthase in adipose tissues [55].

Moreover, the OBRb knock-out mice are known to be lighter in weight and have a blunted plasma insulin profile, while mice with OBRb are hyperleptinemic and hyperinsulinemic with a mild glucose intolerance, representing that leptin signaling is vital for body weight and glucose metabolism [24].

It is also reported that FAO enhancement in wild type skeletal muscle and cardiomyocytes occurs via the AMPK activation and phosphorylation that stimulate fatty acid translocase/cluster differentiating 36-mediated fatty acids and PPARα uptake [35]. Leptin is also recognized as a central regulator that selectively upregulates PPARβ/δ to suppress myocardial triacylglycerol accumulation, and hyperleptinemia is reported to act to protect the heart from lipotoxicity [57]. Likewise, although maintaining cardiac glucose metabolism is important for normal cardiac function, the mitochondrial FAO with branched chain amino acids is also crucial as the principal energy source for the normal heart [14]. In contrast, hypertrophic hearts or in the state of heart failure, a metabolic switch occurs in tricarboxylic acid (TCA) cycle, entering anabolic metabolism for heart to mainly use glucose instead of long-chain fatty acids for adenosine triphosphate [14,31]. Preservation of FAO, in turn, supports catabolic metabolism of TCA cycle and reduces anabolic substrate availability to prevent cardiomyocyte hypertrophy [58]. Leptin has antihypertrophic effects in addition to increased production of fibronectin, and for T2DM and diabetic heart failure with interstitial fibrosis, boosting FAO and normalizing mitophagy may be beneficial for mitochondrial homeostasis [37,47,52]. Leptin is also known to attenuate ischemia-reperfusion injury, or hypoxia-reoxygenation-induced or H_2O_2-induced apoptosis, suppress cardiac contractility, and to increase hyperplasia in the heart [15,28,53]. Leptin

levels are identified as elevated in the state of hypertension and obesity, and diabetic cardiomyopathy is in fact responsible for mortality and morbidity in T2DM patients [36].

In this study, the role of SENP2 on fatty acid metabolism by leptin in white adipocyte 3T3-L1 and cardiomyotube H9C2 was the focus of investigation. First, leptin was hypothesized to increase FAO and expressions of genes related to fatty acid metabolism in 3T3-L1 adipocytes and H9C2 cardiomyotubes. Second, as in skeletal muscle, the leptin-induced FAO was hypothesized to work via the AMPK pathway acutely or via the SENP2 pathway chronically in 3T3-L1 adipocytes and H9C2 cardiomyotubes. Lastly, in vivo study with adipocyte-specific SENP2 knock-out mice was explored to accompany in vitro experiments to confirm leptin's effect on FAO via SENP2.

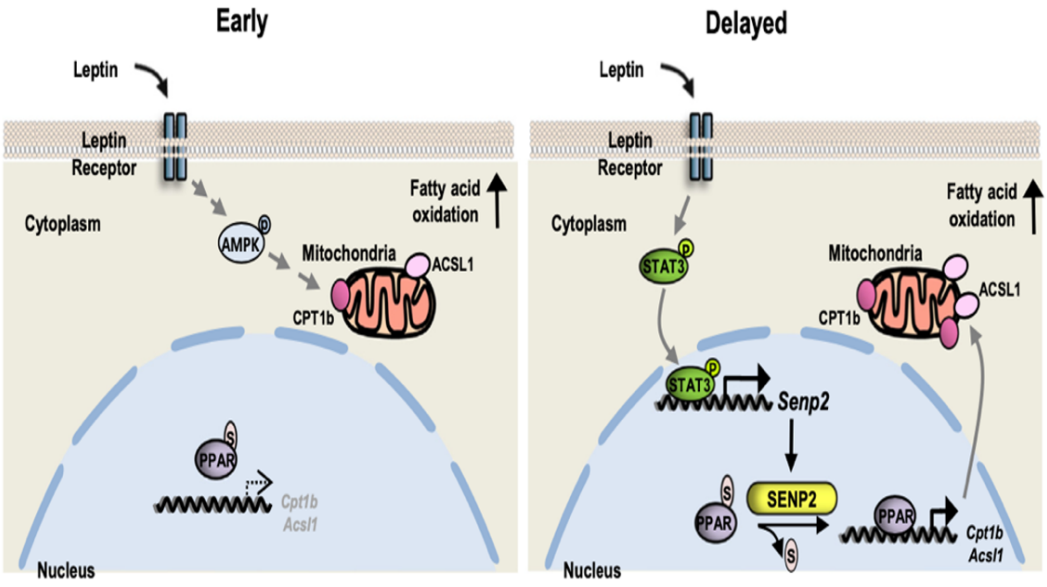


Figure 1. Key idea of the relationship between AMPK and SENP2 pathways and fatty acid oxidation from skeletal muscle study [8].

Materials and Methods

Cell culture and subculture

3T3-L1 mouse white adipose cell was maintained and seeded in high glucose Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % calf serum (Thermo Fisher Scientific, MA, USA) for cell to reach 100 % confluency. Using 12-well culture plate, 2 days after 100 % confluency, cell differentiation was initiated as Day 0. Cell was washed once with PBS and media was replaced with dexamethasone, 3-isobutyl-1-methylxanthine, and insulin in DMEM supplemented with 10 % fetal bovine serum (FBS). Cell was incubated in 37 °C for 2 days, and cell media was replaced with insulin in DMEM supplemented with 10 % FBS. Cell was incubated in 37 °C for 2 days, and media was replaced with 10 % FBS DMEM. Cell was maintained and seeded with 10 % FBS DMEM for 3 more days. On Day 7, cell was considered as fully differentiated and ready as adipocyte for experiment. Cell was serum starved for 24 h and treated with 50 ng/ml of leptin (Merck, Darmstadt, Germany).

H9C2 rat heart muscle cell (America Tissue Type Collection, VA, USA) plated on 6-well plate, was cultured in 10 % FBS DMEM supplemented with 1 % penicillin/streptomycin or 1 % antibiotic-antimycotic at 37 ^{o}C. Culture medium was changed every 2 days and cell was subcultured or initiated with differentiation upon reaching 80 % confluency to avoid loss of differentiation potential. Cell was washed with PBS once and cultured for 5 days in DMEM supplemented with 1 % FBS and 1 μM of *trans*-retinoic acid (RA) (Merck, Darmstadt, Germany) in dark. RA was added daily, and the cell medium was changed every other day. On the 6th day of differentiation, H9C2 cell was considered as differentiated as cardiomyotube for experiment [32]. On Day 7, cell was treated with 50 ng/ml of leptin.

For both 3T3-L1 and H9C2 cells, 100 mm dish was used for cell maintenance, and cells were washed with PBS and detached by using TrypLE^{TM} express 1X dissociation reagent (Thermo Fisher Scientific, MA, USA), and cells were counted by using haemacytometer.

siRNA treatment

Small interfering RNAs (siRNAs) of STAT3 and SENP2 were purchased from Dharmacon (Chicago, IL, USA), and nonspecific siRNA (NS, negative control) was purchased from BIONEER (Daejeon, Korea). For siRNA transfection in 3T3-L1, cell plated on 12-well plate was grown to fully differentiated adipocyte. siRNA stock solution was diluted with autoclaved water to concentration of 20 μM for use. With Lipofectamine RNAiMax-transfection reagent (Thermo Fisher Scientific, MA, USA), 2.5 μl for 50 nM, 5 μl for 100 nM, and 10 μl for 200 nM concentration were pipetted into Eppendorf (EP)-tube no.1 containing serum-free DMEM up to 50 μl, and complex was mixed by pipetting. From 20 μM siNS or siRNA, same step applied as above into EP-tube no.2 but without pipetting. Complex from EP-tube no.2 was mixed into no.1 by pipetting for volume of 100 μl and was incubated for 20 min at room temperature.

On Day 7 of differentiation, complex was treated to cell with 900 μl of serum-free DMEM while plate was tilted, and media was mixed by gently shaking and incubated for 24 h at 37 ºC. Cell medium was changed with serum-free DMEM the next day and was incubated for another 24 h at 37 ºC for total 48 h incubation for 3T3-L1 [30]. For siRNA transfection in H9C2, cell was plated on 6-well plate and grown to be fully differentiated cardiomyotube. Same procedure followed as above-mentioned but total volume of 200 μl complex was treated to cell plate with 800 μl of serum-free DMEM. After incubation at 37 ºC for 4 h, 1 ml of DMEM supplemented with 10 % FBS was added.

For AMPK inhibition in 3T3-L1 and H9C2, compound C (Tokyo Chemical Industry, Tokyo, Japan) of 10 μM was pre-treated for 1 h, followed by leptin treatment.

RNA preparation and Real-time quantitative polymerase chain reaction (PCR)

Total RNAs of differentiated 3T3-L1 and H9C2 cells were isolated using TRIzol (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instruction. To prepare cDNA for PCR, 5 μl of 5X FS buffer (Thermo Fisher Scientific, MA, USA), 2.5 μl of 0.1 M DTT (Thermo Fisher Scientific, MA, USA), 1.25 μl of 10 mM dNTP (Thermo Fisher

Scientific, MA, USA), 0.5 μl of Oilgo dT (Thermo Fisher Scientific, MA, USA), 0.25 μl of RNase inhibitor (Thermo Fisher Scientific, MA, USA), 1 μl of RTase/M-MLV RT buffer (Thermo Fisher Scientific, MA, USA), and 1 μg of 100 ng/μl RNA were mixed, and RNase free water was added up to 25 μl. The mixture was incubated at 25 °C for 10 min, 37 °C for 2 h, and at 85 °C for 5 min using PCR system. Expression levels of genes were determined by using SYBR-TB green premix ex taq (Takara, Otsu, Shiga, Japan) and QuantStudio™3 real-time PCR system (Applied Biosystem, CA, USA). The primer sequences for PCR of the genes were as indicated in Table 1.

Protein preparation, SDS-PAGE, and Western blotting

After cold PBS wash 3 times, cells were lysed with a lysis buffer containing radioimmunoprecipitation assay/NP-40 buffer, Halt™ protease-phosphatase inhibitor cocktail, ethylenediaminetetraacetic acid, and phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, MA, USA). The whole-cell lysates were vortexed 10 sec and incubated on ice for 5 min (repeated 6 times), and cell debris was removed by centrifugation (13,000 rpm) for 5 min at 4 °C. 30 μg of proteins were separated on the sodium dodecyl sulphate–polyacrylamide gel electrophoresis supplemented with 10 % gel. Separated proteins were transferred onto nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked with 5 % skim milk in Tween20-tris-buffered saline for at least 30 min at room temperature, and it was incubated with the specific primary antibody overnight at 4 °C. Next day, the membrane was probed with specific secondary antibody for 1 h at room temperature, and bands were visualized by enhanced x-ray film in dark. If needed, antibodies were stripped with Restore™ western blot stripping buffer (Thermo Fisher Scientific, MA, USA), followed by blocking and antibody attachment. The specific antibodies used were phospho-AMPKα (Cell Signaling Technology, MA, USA), AMPKα (Cell Signaling Technology, MA, USA), phospho-STAT3 (Cell Signaling Technology, MA, USA), STAT3 (Cell Signaling Technology, MA, USA), calsequestrin (Abcam, Cambridge, UK), and GAPDH (Merck, Darmstadt, Germany).

Measurement of fatty acid oxidation (FAO)

For measurement of FAO, 3T3-L1 cells, H9C2 cells or mouse tissues were homogenized in an ice-cold mitochondria isolation buffer (250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA). The lysates were incubated for 2 h with 0.2 mM [1-^{14}C] palmitate. $^{14}CO_2$ and ^{14}C-labeled acid-soluble metabolites were quantified using a liquid scintillation counter. Each radioactivity was normalized by protein amount of each lysate.

Plasmids, DNA transfection, and Luciferase/β-galactosidase assay

For DNA transfection, CPT1b and ACSL1 promotors were prepared [8]. The mouse Cpt1b promoter region from -1556 bp to 39 bp in the direction from the start codon was ligated to the pGL2-basic to generate mCpt1b(-1.5 Kbp)-Luc. The mouse Cpt1b promoter region from -342 bp to 39 bp in the direction from the start codon was ligated to the pGL2-basic to generate mCpt1b(-400 bp)-Luc. A PPAR response element between -263 bp and -250 bp in the mCpt1b promoter (5′-TGACCTTTTCCCT-3′) was mutated to 5′-TCTGCTTTTCCCT-3′ in the mCpt1b(-400 bp)mt-Luc. Similarly, the mouse Acsl1 promoter region from -1051 bp to -45 bp in the direction from the start codon was ligated to the pGL2-basic to generate mAcsl1(-1 Kbp)-Luc. A PPAR response element between -217 bp and -205 bp in the mAcsl1 promoter (5′-TTACCTTTAGCCC-3′) was mutated to 5′-TTACCTTTAGGGC-3′ in the mAcsl1(-1 Kbp)mt-Luc.

For luciferase reporter assays, 3T3-L1 pre-adipocytes plated on 12-well culture plate with 90 % confluency were transfected with each construct of CPT1b and ACSL1 promoters (300 ng/well), and RSV-β-gal (100 ng/well) for normalization in serum free DMEM using lipofectamine® with plus reagent (Thermo Fisher Scientific, MA, USA). After incubation at 37 °C for 30 min, DMEM media supplemented with 10 % fetal bovine serum (Thermo Fisher Scientific, MA, USA) was added. Following 2-3 h incubation at 37 °C, the media was changed with DMEM containing 10 % calf serum (Thermo Fisher Scientific, MA, USA), and cells were treated with 50 ng/ml of leptin for 24 h incubation at 37 °C. Reporter lysis buffer (Promega Corporation, WI, USA) was used for cell lysis, and the cell extract

was mixed with 20 μg of luciferase assay substrate in duplicate. Luciferase activity was measured by using Lumat LB 9507 machine (Berthold Technologies, Bad Wildbad, Germany), and its relative light unit was normalized by β–galactosidase activity.

Chromatin Immunoprecipitation (ChIP) with quantitative PCR

After 24 h serum starvation, 3T3-L1 adipocytes were treated with 50 ng/ml of leptin (Merck, Darmstadt, Germany) for 24 h. After crosslinking and DNA fragmentation, immunoprecipitation was performed with antibodies against PPARα, PPARδ, PPARγ (Santa Cruz Biotechnology, TX, USA), and STAT3 (Cell Signaling Technology, MA, USA). PCR was performed with primers flanking the PPRE (around −140 bp) in the Acsl1 promoter or the PPRE (around −409 bp) in Cpt1b promoter and with primers specific to STAT3 region in Cpt1b promoter. Primer sequences for PCR were as indicated in Table 1.

In vivo study with adipocyte-specific SENP2 knock-out mice

Animals were handled in compliance with the Guide for Experimental Animal Research from the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Bundang Hospital, Republic of Korea (Permit Number: BA-1805-248-044). Adipocyte-specific SENP2 knock-out mice (SENP2 aKO) were generated by crossing with adiponectin-Cre transgenic mice (Jackson lab, USA) previously in our laboratory [8]. After 4 h morning fast, body weight and blood glucose level were measured, and then recombinant murine leptin (3 mg/kg body weight) (Thermo Fisher Scientific, MA, USA) dissolved in saline was injected intraperitoneally into 10-weeks-old mice. For age- and weight-matched control group, sterile saline was injected. 24 h after, adipose tissues, soleus muscle, and heart were rapidly isolated, and FAO or mRNA levels were measured.

Statistical analysis

Statistical analysis was performed in GraphPad Prism version 8 and SPSS Statistics version 26 (SPSS Inc.). Statistical significance was tested using t-test and Mann-Whitney U test. A P-value below 0.05 was considered as statistically significant.

Table 1. Primer sequences for RT-qPCR

Gene	Forward primer sequence	Reverse primer sequence
(m)SENP2 exon3	5'-CAG TCT CTA CAA TGC TGC CA-3'	5'-CAG AAG GGG CCA CAT TC-3'
(m)CPT1b	5'-AAG TGT AGG ACC AGC CCC GA-3'	5'-TGC GGA CTC GTT GGT ACA GG-3'
(m)ACSL1	5'-CTG GTT GCT GCC TGA GCT TG-3'	5'-TTG CCC CTT TCA CAC ACA CC-3'
(m)STAT3	5'-GCT TCA GGG AGA GCA GCA AA-3'	5'-CTC CTC CTT GGG AAT GTC GG-3'
(m)PPARa	5'-TGT CGA ATA TGT GGG GAC AA-3'	5'-AAT CTT GCA GCT CCG ATC AC-3'
(m)PPARd	5'-GGA CCA GAA CAC ACG CTT CCT T-3'	5'-CCG ACA TTC CAT GTT GAG GCT G-3'
(m)PPARg	5'-GGA AGA CCA CTC GCA TTC CTT-3'	5'-GTA ATC AGC AAC CAT TGG GTC A-3'
(m)2nd OB Rb LepR	5'-GCA TGC AGA ATC AGT GAT ATT TGG-3'	5'-CAA GCT GTA TCG ACA CTG ATT TCT TC-3'
(m)GAPDH	5'-AGG TCG GTG TGA ACG GAT TTG-3'	5'-TGT AGA CCA TGT AGT TGA GGT CA-3'
(r)SENP2	5'-CCA GAT GAG ATT TTG AGC AGC G-3'	5'-TGA AGT GCT GGG TAG CCT TG-3'
(r)CPT1b	5'-TCG CAG GCG AAA ACA CAA TG-3'	5'-AGT GCT TGA CGG ATG TGG TT-3'
(r)ACSL1	5'-GTA CGT GCG TAC CCT TCC AA-3'	5'-TGG ACA GAT CAC ATG GTG GC-3'
(r)LepR OB Rb	5'-GCA TGC AGA ATC AGT GAT ATT TGG-3'	5'-CCC CTT GTG GAA TCT GGA GT-3'
(r)cTnT	5'-ATC CAC AAC CTA GAG GCC GA-3'	5'-CTT TGG CCT TCC CAC GAG TT-3'
(r)GAPDH	5'-TGG TGG ACC TCA TGG CCT AC-3'	5'-CAG CAA CTG AGG GCC TCT CT-3'
(m)UCP2	5'-ATG GTT GGT TTC AAG GCC ACA-3'	5'-TTG GCG GTA TCC AGA GGG AA-3'
(m)ACO	5'-GCC ATT CGA TAC AGT GCT GTG AG-3'	5'-CCG AGA AAG TGG AAG GCA TAG G-3'
(m)FAS	5'-CAC AGT GCT CAA AGG ACA TGC C-3'	5'-CAC CAG GTG TAG TGC CTT CCT C-3'
(m)ChIP Cpt1b PPRE	5'-GAG CAG CAG TGG TCC CTG AG-3'	5'-TGC TGG AAG GTC TGG GAC TG-3'
(m)ChIP Cpt1b STAT3	5'-GGA CTG ATG CAA CCA TGC TC-3'	5'-AGA ACA CCC TCC TTT TGG GG-3'
(m)ChIP ACSL1 PPRE	5'-GGT GAC TCT ACT CTC AGC TGC-3'	5'-CTT ACC AGG CTG CCA AGG TCT-3'

Abbreviation: (m) = mouse, (r) = rat

Results

Leptin effects in Adipocytes:

Leptin receptor expression in various tissues and cells.

As leptin receptor expression has been investigated previously in different types of muscle tissue, comprising soleus muscle, gastrocnemius muscle, and quadriceps muscle along with hypothalamus and C2C12 myotube [8], I wondered how OBRb expression would look for different types of adipose tissue. The experiment to measure OBRb was conducted via RT-qPCR with specific primers for OBRb in mice. Among various tissue types from wild type C57BL/6J mice, the expression level of leptin receptor long form OBRb was greatest in the hypothalamus, followed by subcutaneous adipose tissue, soleus muscle, liver, and then visceral adipose tissue and brown adipose tissue (Figure 2A). In terms of OBRb expression in various cell types, C2C12 myotube had the most expression, and then, 3T3-L1 adipocyte and H9C2 cardiomyotube showed expression of OBRb (Figure 2B).

Leptin increases fatty acid oxidation (FAO) in 3T3-L1 adipocytes.

As OBRb was detected in 3T3-L1 adipocyte, whether fatty acid oxidation increases in response to leptin was explored. 3T3-L1 adipocyte was serum starved for 24 h, and upon leptin treatment for 6 h or 24 h along with positive controls, consisting of AICAR, WY14643, and rosiglitazone, the level of FAO increased. The expression of FAO after leptin treatment for 6 h was almost 2-fold greater than that of vehicle (Figure 3A), and for 24 h, FAO level was about 1.75-fold greater than that of vehicle (Figure 3B).

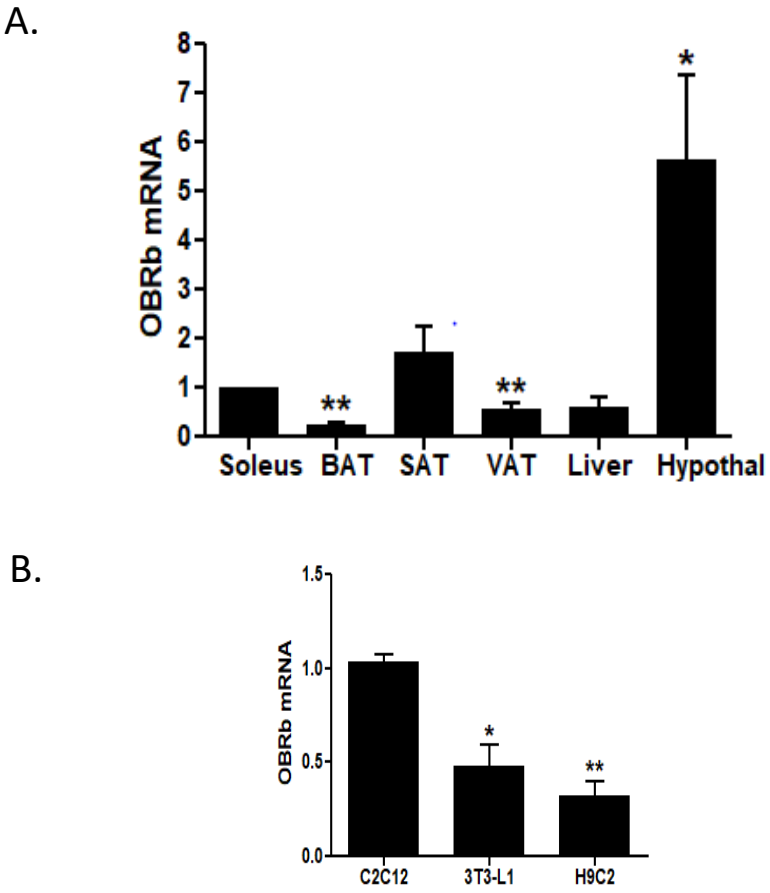


Figure 2. Leptin receptor expression in various tissues and cells

(A-B) The mRNAs of (A) several types of fat (BAT, SAT, VAT), hypothalamus, soleus muscle, and liver from wild type C57BL/6J mice, and (B) C2C12 myotubes, 3T3-L1 adipocytes, and H9C2 cardiomyotubes were subjected to RT-qPCR analysis using a leptin receptor (OBRb) primer. (A) The mRNA levels of soleus muscle and (B) C2C12 myotubes were expressed as 1, and the others were expressed as their relative values (n = 4-5). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. (A) soleus muscle or (B) C2C12 myotubes). BAT: brown adipose tissue, SAT: subcutaneous adipose tissue, VAT: visceral adipose tissue, Soleus: soleus muscle, Hypothal: hypothalamus.

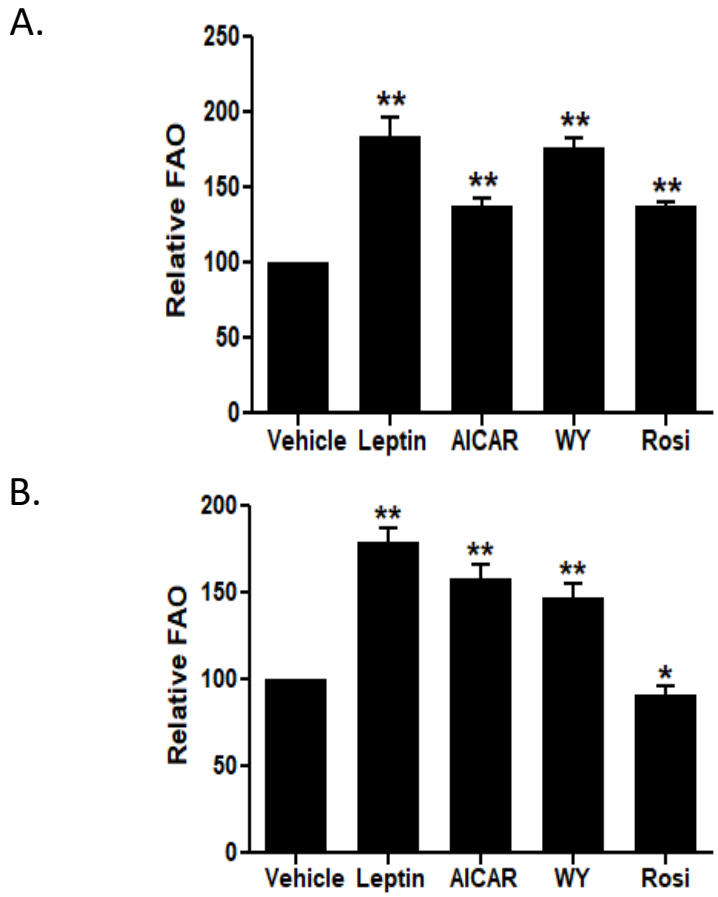


Figure 3. Fatty acid oxidation by leptin in 3T3-L1 cell

(A-B) After serum starvation for 24 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for (A) 6 h or (B) 24 h, and FAO was measured. AICAR (1 mM), WY14643 or rosiglitazone (10 μM) were used as positive controls. FAO level of vehicle without leptin treatment was expressed as 100, and the others were expressed as its relative values (n = 3-4). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. vehicle). WY: WY14643 (PPARα agonist), Rosi: rosiglitazone (PPARγ agonist).

AMPK pathway accounts for acute induction of FAO by leptin in 3T3-L1 adipocytes.

In the previous study, AMPK pathway was revealed as responsible for the acute induction of FAO by leptin in C2C12 myotubes [8]. Leptin treatment increased AMPK phosphorylated form (pAMPK), which is an active form, phosphorylating acetyl-coenzyme A carboxylase and reducing malonyl coenzyme A to acutely induce FAO in skeletal muscle [8,13]. I wondered if AMPK has a similar role in 3T3-L1 adipocytes, and thus, whether phosphorylation of AMPK increases upon leptin treatment was first observed. As fully differentiated 3T3-L1 adipocyte was serum starved for 24 h, and leptin treated by various concentrations (50, 100 or 200 ng/ml), phospho-AMPK increased (Figure 4A). As fully differentiated 3T3-L1 adipocyte was serum starved for 24 h, and leptin treated by various time intervals (3, 6, 10 or 24 h), phospho-AMPK increased (Figure 4B). For 3T3-L1 adipocytes, leptin's effect on increasing phosphorylation of AMPK was rapid and significant from treatment duration of 3 h (Figure 4B).

With the rise of phospho-AMPK by leptin, next, I went to investigate if AMPK pathway affects FAO level in short- or long-term leptin treatment in 3T3-L1 adipocytes. In order to do so, after pre-treatment with compound C for 1 h for AMPK inhibition, leptin was treated for 6 h or 24 h, and then FAO was measured in 3T3-L1 adipocytes (Figure 5). The results of FAO from use of compound C, the AMPK inhibitor, clearly indicate the reduced level of increase in FAO by leptin for 6 h (Figure 5A). In comparison, for 24 h, the effect of the AMPK inhibitor was lower (Figure 5B). Therefore, the AMPK pathway mainly accounts for acute induction of FAO by leptin in 3T3-L1 adipocytes.

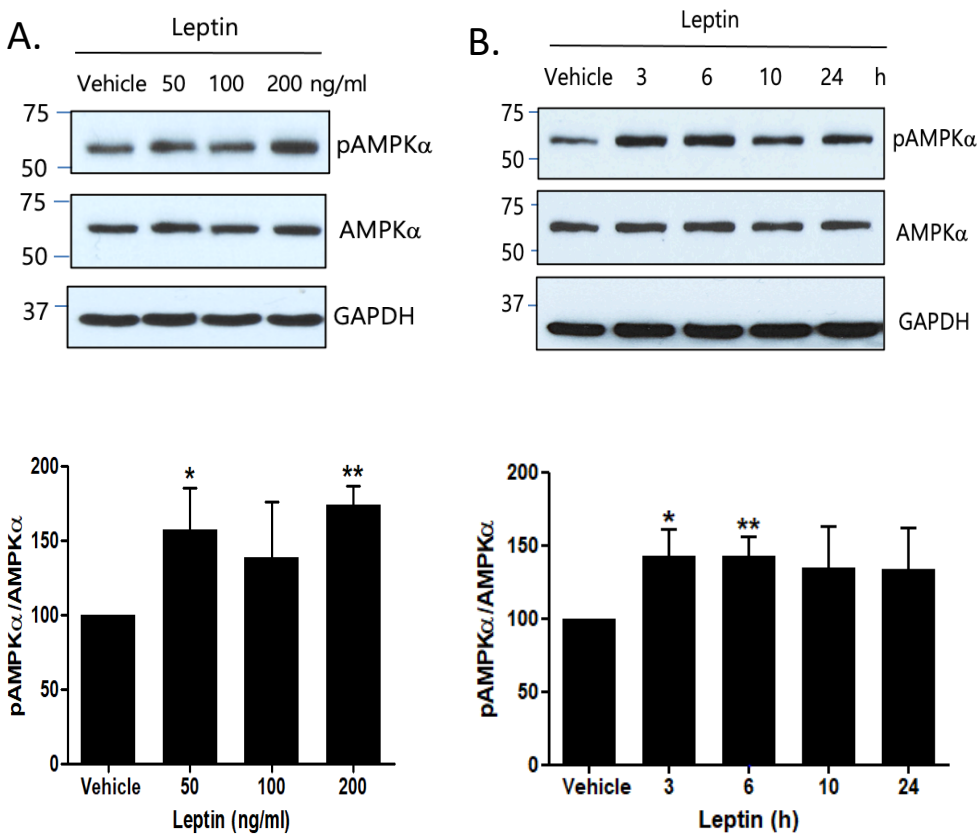


Figure 4. AMPK phosphorylation by leptin in 3T3-L1 cell

(A-B) After serum starvation for 24 h, 3T3-L1 adipocytes were treated (A) with leptin at different concentrations (50, 100 or 200 ng/ml) for 3 h or (B) with leptin (50 ng/ml) at different time points (3, 6, 10 or 24 h). Cell lysates were subjected to western blotting using antibodies against phospho-AMPK (pAMPK) and total AMPK (AMPK). The protein level of vehicle without leptin treatment was expressed as 100, and the others were expressed as its relative values (n = 3-5). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. vehicle).

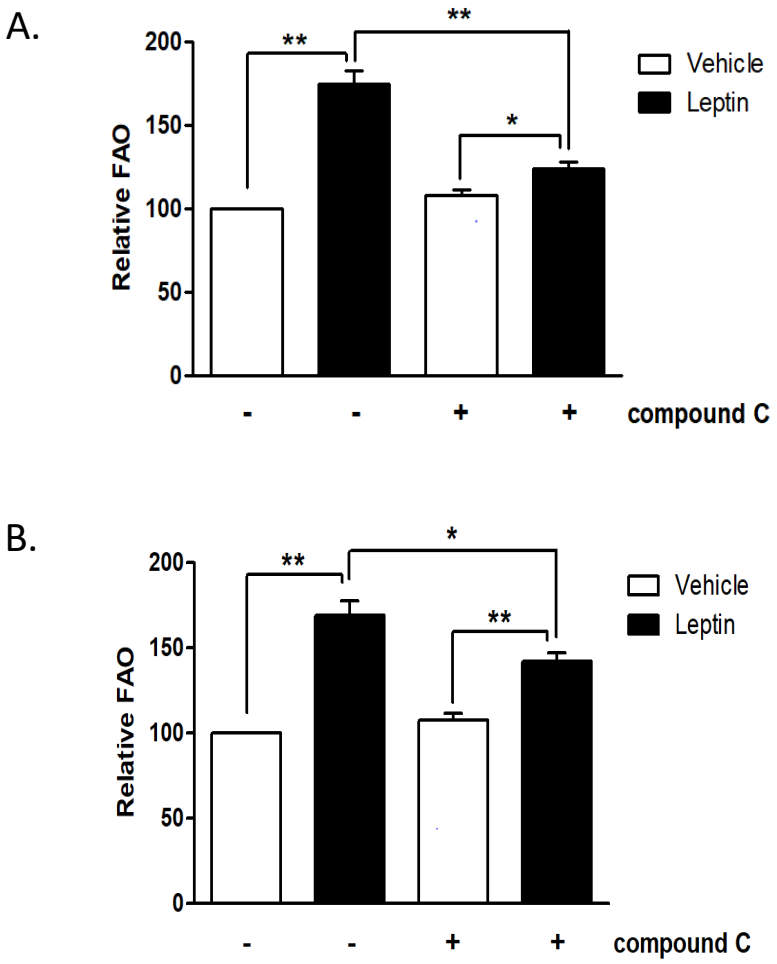


Figure 5. Fatty acid oxidation by leptin after AMPK inhibition in 3T3-L1 cell

(A-B) After pre-treatment with compound C (10 μM) for 1 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for (A) 6 h or (B) 24 h, and FAO was measured. (A-B) FAO levels of vehicle without compound C and without leptin treatment were expressed as 100, and the others were expressed as their relative values (n = 3). Data are presented as mean ± SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$)

Leptin increases SENP2 expression through STAT3 in 3T3-L1 adipocytes.

In the previous study, STAT3 was revealed as highly correlated with the role of SENP2 on chronic induction of FAO by leptin in C2C12 myotubes [8]. I wondered if STAT3 has a similar role in 3T3-L1 adipocytes, and thus, whether phosphorylation of STAT3 increases upon leptin treatment was observed. As fully differentiated 3T3-L1 adipocyte was serum starved for 24 h, and leptin treated by various concentrations (50, 100 or 200 ng/ml), the level of phospho-STAT3 increased at concentration of 50 ng/ml or higher (Figure 6A). As fully differentiated 3T3-L1 adipocyte was leptin treated by various time intervals (3, 6, 10 or 24 h), the level of phospho-STAT3 increased (Figure 6B). For 3T3-L1 adipocytes, as the duration of leptin treatment increased, phospho-STAT3 steadily increased. Leptin's effect on increasing phospho-STAT3 was significant from treatment duration of 3 h, and then, the level stayed at the increased level compared to total STAT3 in 3T3-L1 adipocytes (Figure 6B). Since SENP2 is known to interact with phospho-STAT3 to cause induction of FAO by leptin in C2C12 myotubes [8], I performed the RT-qPCR experiment with mouse SENP2-specific primers to observe if leptin increases SENP2 expression also in 3T3-L1 adipocytes. After serum starvation for 24 h, 3T3-L1 adipocyte was leptin (50 ng/ml) treated for different time intervals (3, 6, 10 or 24 h), and SENP2 expression increased steadily by time and reached its peak at 24 h (Figure 7).

To determine if SENP2 expression is increased in 3T3-L1 adipocytes via STAT3, STAT3 was knocked down using siRNA. Upon treatment of 3T3-L1 adipocytes with different concentrations (50, 100 or 200 nM) of siRNA specific to STAT3 (siSTAT3) for 48 h, the mRNA expression of STAT3 decreased as compared to the vehicle (Figure 8A). After STAT3 expression was knocked down with siSTAT3, leptin was treated for 24 h, and then mRNA expression of SENP2

was measured in 3T3-L1 adipocytes (Figure 8B). The knock-down of STAT3 caused approximately 90 % reduction in the rise of SENP2 by leptin (Figure 8B). These results suggest that leptin increases SENP2 expression via STAT3 in 3T3-L1 adipocytes.

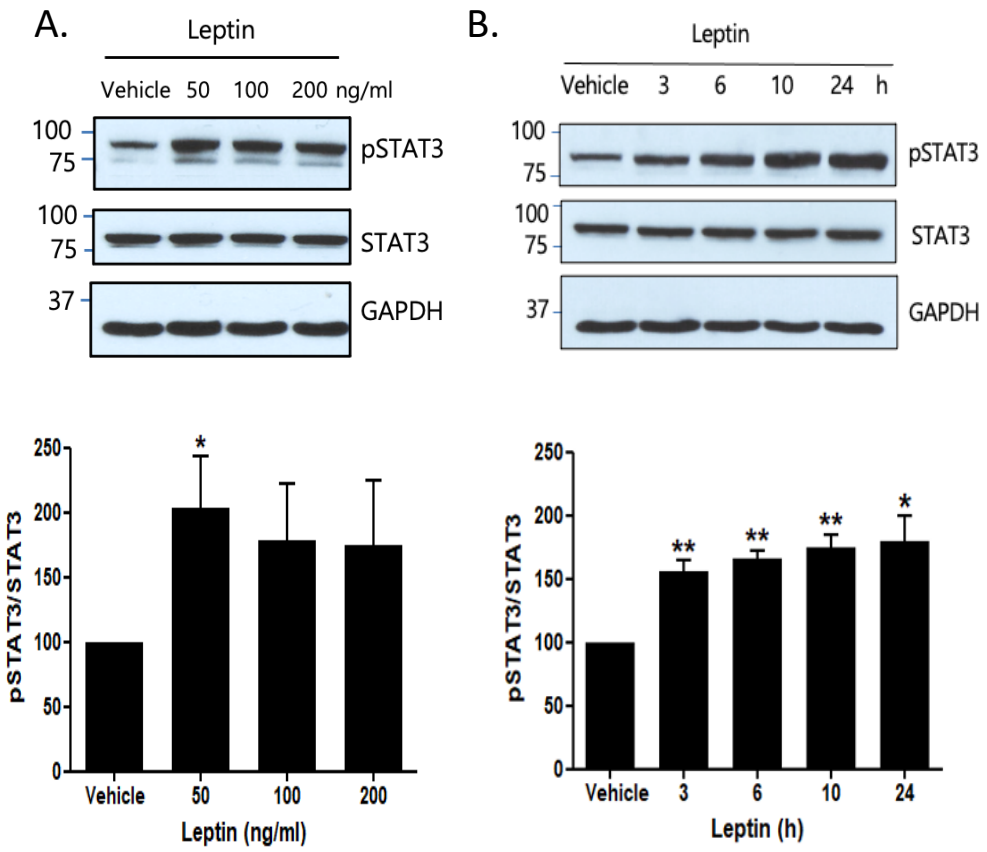


Figure 6. STAT3 phosphorylation by leptin in 3T3-L1 cell

(A-B) After serum starvation for 24 h, 3T3-L1 adipocytes were treated (A) with leptin at different concentrations (50, 100 or 200 ng/ml) for 3 h or (B) with leptin (50 ng/ml) at different time points (3, 6, 10 or 24 h). Cell lysates were subjected to western blotting using antibodies against phospho-STAT3 (pSTAT3) and total STAT3 (STAT3). The protein level of vehicle without leptin treatment was expressed as 100, and the others were expressed as its relative values (n = 3-5). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. vehicle).

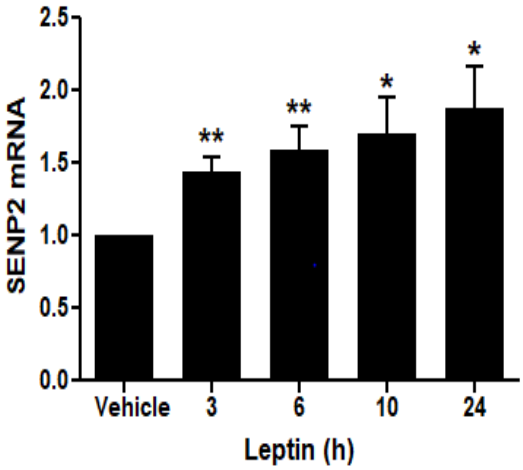


Figure 7. SENP2 expression by leptin in 3T3-L1 cell

After serum starvation for 24 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) at different time points (3, 6, 10 or 24 h). RT-qPCR analysis was run using a SENP2 primer. The mRNA level of vehicle without leptin treatment was expressed as 1, and the others were expressed as its relative values ($n = 5$). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. vehicle).

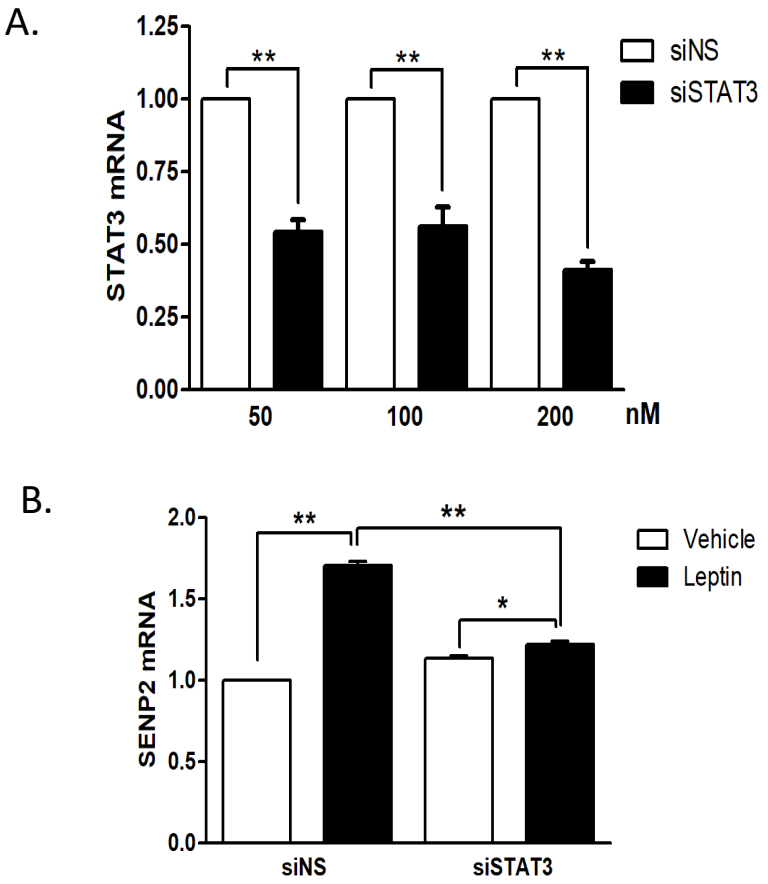


Figure 8. SENP2 expression by leptin after STAT3 knock-down in 3T3-L1 cell

(A) For STAT3 knock-down, 3T3-L1 adipocytes were treated with siNS or siSTAT3 (50, 100, 200 nM) for 48 h. (B) After siNS or siSTAT3 (100 nM) treatment for 48 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for 24 h. The mRNA expression levels for adipocytes treated (A) with siNS or (B) with siNS but without leptin treatment were expressed as 1, and the others were expressed as their relative values (n = 3-6). Data are presented as mean ± SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$)

Leptin increases expression of fatty acid oxidation-associated genes through SENP2 in 3T3-L1 adipocytes.

After observing the rise of SENP2 mRNA expression by leptin, I was curious if leptin also increases expression of genes related to FAO, carnitine palmitoyl transferase 1b (CPT1b) and long-chain acyl-coenzyme A synthetase 1 (ACSL1). As 3T3-L1 adipocyte was serum starved for 24 h and treated with leptin (50 ng/ml) for different duration of time (3, 6, 10 or 24 h), the gene expressions of CPT1b and ACSL1 increased and reached their peak at 24 h (Figure 9). The mRNA expression of CPT1b quickly rose from 3 h of leptin treatment, while mRNA expression of ACSL1 begun to rise from 10 h of leptin treatment (Figures 9A,9B).

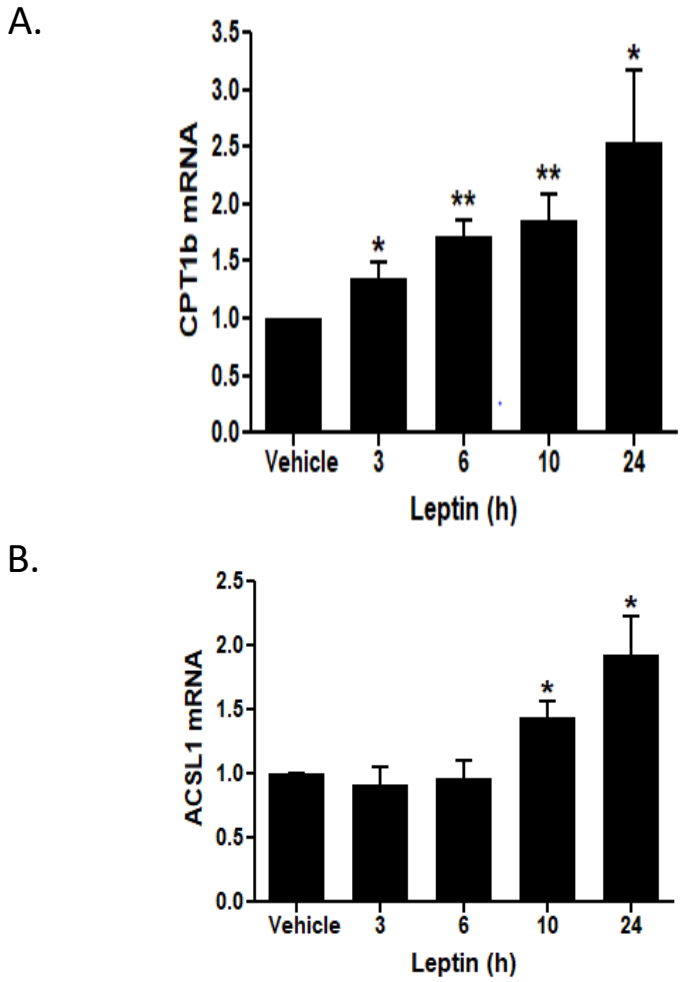


Figure 9. FAO-related genes' expressions by leptin in 3T3-L1 cell

(A-B) After serum starvation for 24 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) at different time points (3, 6, 10 or 24 h). RT-qPCR analysis was run using (A) CPT1b primer and (B) ACSL1 primer. The mRNA levels of vehicle without leptin treatment were expressed as 1, and the others were expressed as their relative values ($n = 5$). Data are presented as mean ± SEM. ($^{*}P < 0.05$ or $^{**}P < 0.01$ vs. vehicle).

Next, I tested if SENP2 mediates leptin-induced expression of genes related to FAO in 3T3-L1 adipocytes as with the previous study of C2C12 myotubes [8]. SENP2 was first knocked down and the efficiency of knock-down was confirmed. Upon treatment of 3T3-L1 adipocytes with siSENP2 with different concentrations (50, 100 or 200 nM) for 48 h, the mRNA expression of SENP2 decreased as compared to the vehicle (Figure 10). The gene expression of SENP2 was knocked down for approximately 60 % when treated with siSENP2 concentration of 200 nM (Figure 10). SENP2 knock-down effects on FAO-associated genes, such as CPT1b and ACSL1 were also studied. After SENP2 gene was knocked down with siSENP2, leptin was treated for 24 h, and then gene expressions of SENP2, CPT1b, and ACSL1 were measured in 3T3-L1 adipocyte by RT-qPCR (Figure 11). SENP2 gene expression was knocked down by more than 50 % compared to siNS vehicle even in the presence of leptin treatment (Figure 11A). For siSENP2-treated group, the increasing levels of CPT1b and ACSL1 by leptin were significantly reduced compared to leptin-treated siNS group in 3T3-L1 (Figures 11B,11C). These results suggest that leptin-induced FAO-related gene expression is mainly mediated by SENP2.

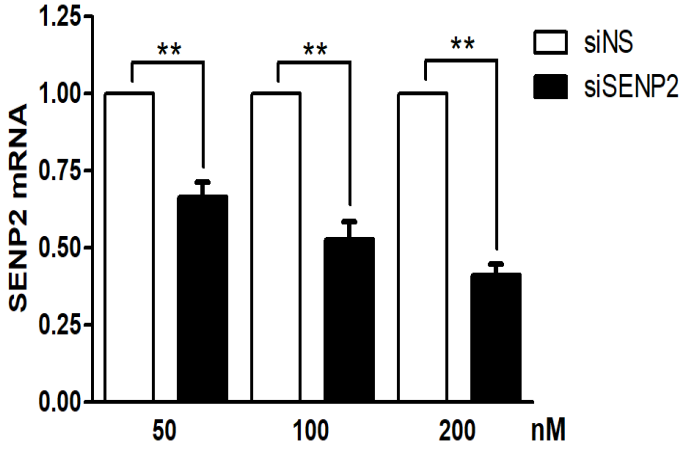


Figure 10. SENP2 level of knock-down in 3T3-L1 cell

3T3-L1 adipocytes were treated with siNS or siSENP2 (50, 100 or 200 nM) for 48 h, and then adipocytes were subjected to RT-qPCR analysis using SENP2 exon 3 primer. The mRNA expression levels of gene for adipocytes treated with siNS were expressed as 1, and the others were expressed as their relative values (n = 6). Data are presented as mean ± SEM. ($^{**}P < 0.01$)

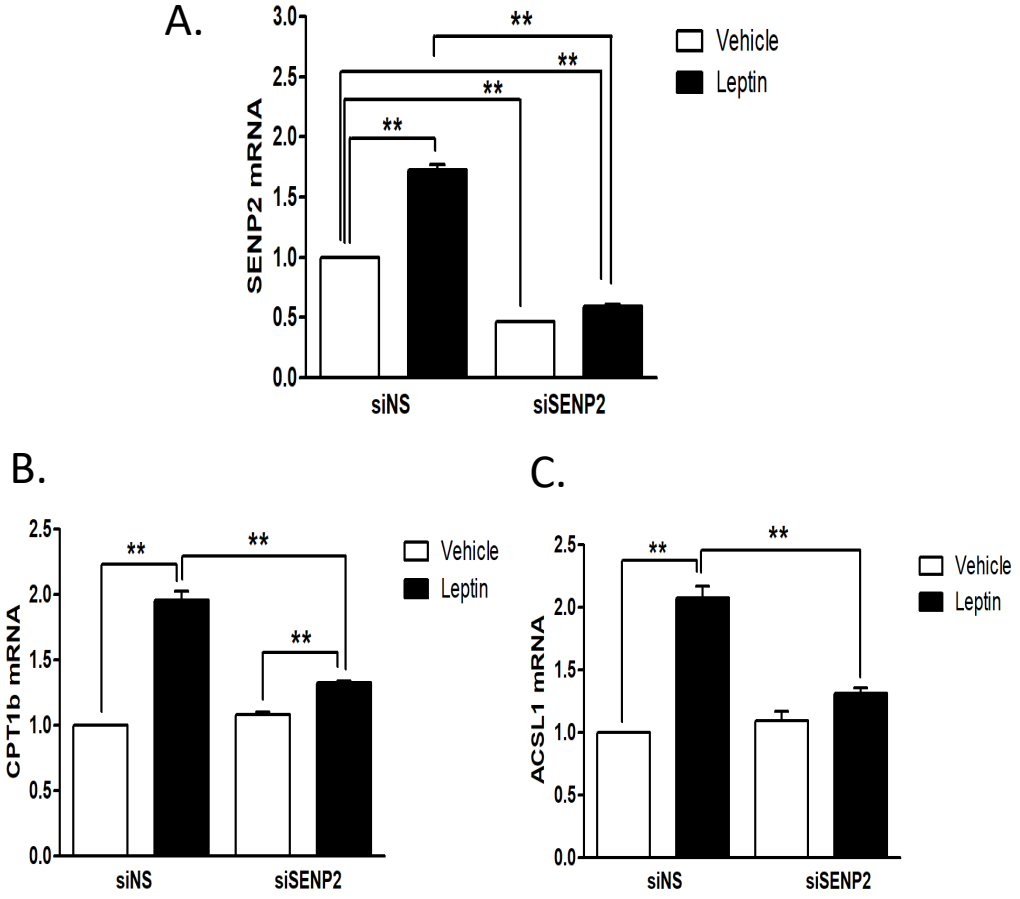


Figure 11. Expressions of SENP2 and genes related to fatty acid oxidation by leptin after SENP2 knock-down in 3T3-L1 cell

(A-C) After siNS or siSENP2 (200 nM) treatment for 48 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for 24 h and were subjected to RT-qPCR analysis using (A) SENP2 primer, (B) CPT1b primer or (C) ACSL1 primer. The mRNA expression levels for adipocytes treated with siNS but without leptin treatment were expressed as 1, and the others were expressed as their relative values (n = 3). Data are presented as mean ± SEM. ($^{**}P < 0.01$)

SENP2 pathway accounts for prolonged, delayed increase of FAO by leptin in 3T3-L1 adipocytes.

Then, I investigated whether SENP2 is responsible for FAO induction by leptin in 3T3-L1 adipocytes. After SENP2 was knocked down, leptin was treated for 6 h or 24 h, and then FAO was measured in 3T3-L1 adipocyte (Figure 12). The FAO experimental results showed that siSENP2 treatment completely removed the increase in FAO by leptin for 24 h leptin-treated group (Figure 12B). For 6 h leptin-treated group, the effect of SENP2 knock-down for lowering the rise of FAO by leptin was much lower (Figure 12A). Therefore, the experimental outcomes reveal that the chronic rise of FAO by leptin occurs mainly via SENP2-induced increase of FAO-related enzymes in 3T3-L1 adipocytes.

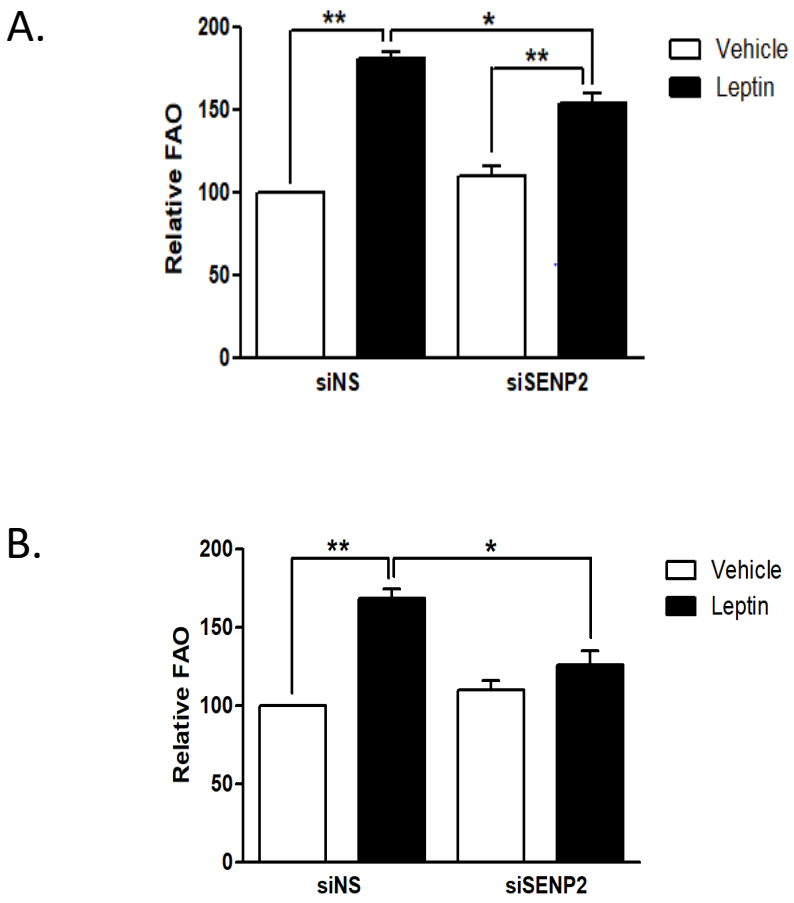


Figure 12. Fatty acid oxidation by leptin after SENP2 knock-down in 3T3-L1 cell

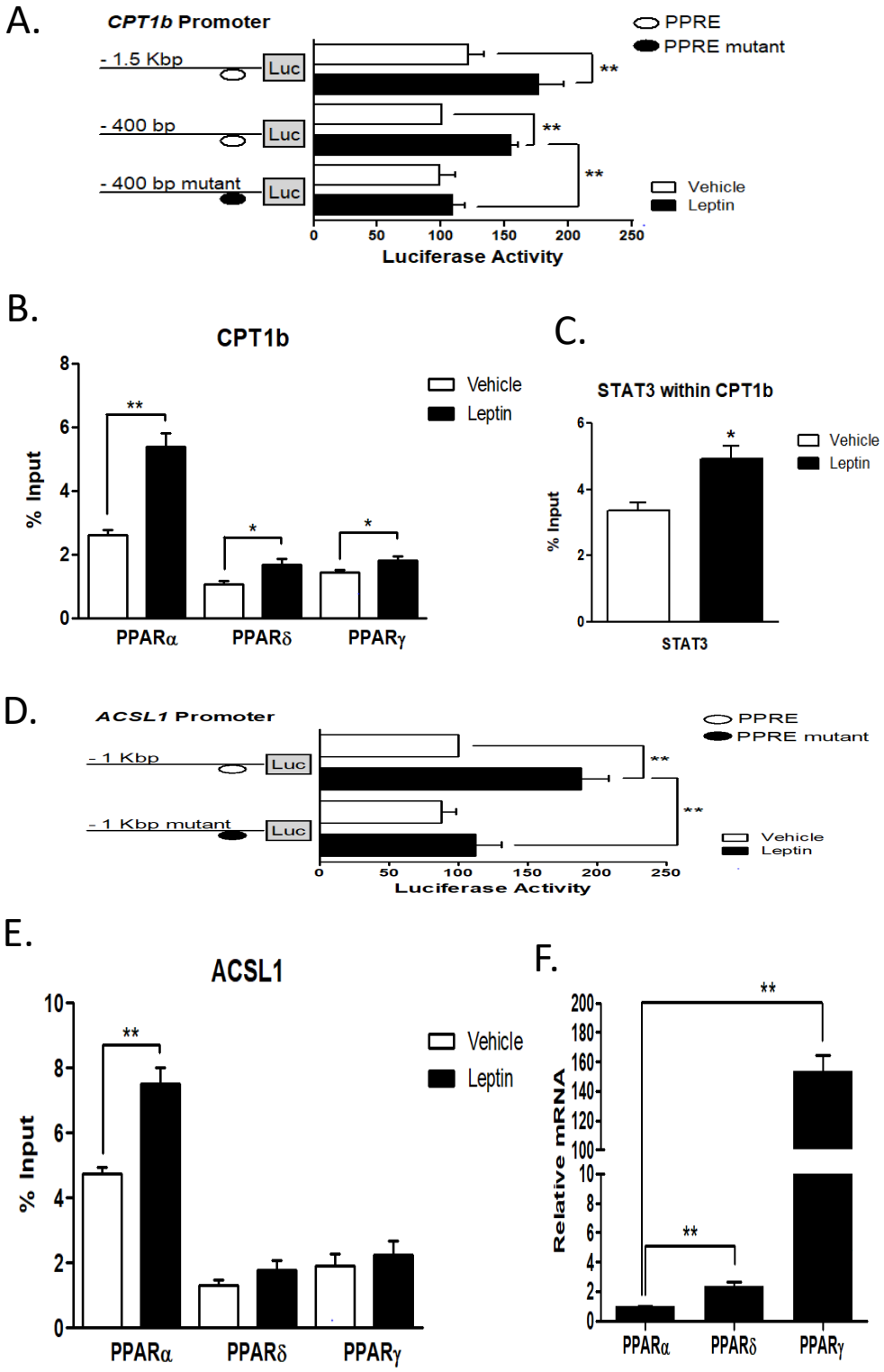
(A-B) After siNS or siSENP2 (200 nM) treatment for 48 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for (A) 6 h or (B) 24 h, and FAO was measured. FAO level of vehicle without leptin treatment in adipocytes treated with siNS was expressed as 100, and the others were expressed as its relative values (n = 3). Data are presented as mean ± SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$)

SENP2 increases binding of PPARs on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment in 3T3-L1 adipocytes.

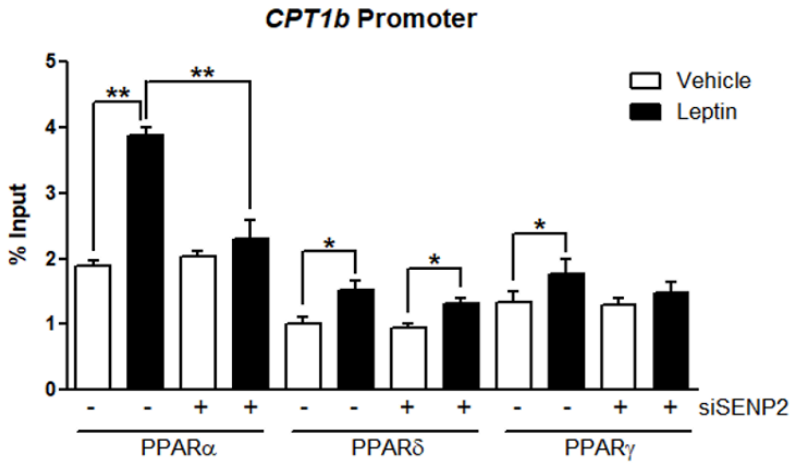
To determine whether peroxisome proliferator-activated receptors (PPARs) are responsible for SENP2's action on FAO likewise with the previous study of C2C12 [8], the luciferase activity on PPRE sites of CPT1b and ACSL1 promoters were measured. Leptin treatment led to 1.5-fold increase in the luciferase activity for 3T3-L1 pre-adipocytes transfected with mCpt1b(-1.5 Kbp)-luc or with mCpt1b(-400 bp)-luc. On the other hand, the luciferase activity upon leptin treatment was not as significant for 3T3-L1 pre-adipocytes transfected with mCpt1b(-400 bp)mt-luc containing mutations at a potential PPRE site at -260 bp (Figure 13A). Likewise, leptin treatment led to almost 2-fold increase in the luciferase activity for 3T3-L1 pre-adipocytes transfected with mAcsl1(−1 Kbp)-luc, while it was reduced to 1.3-fold increase for 3T3-L1 pre-adipocytes transfected with mAcsl1(−1 Kbp)mt-luc containing mutations at PPRE site at −210 bp (Figure 13D). The rise of luciferase activity revealed that PPARs binding on PPRE sites of CPT1b and ACSL1 promoters is necessary for the increase of CPT1b and ACSL1 expressions upon leptin treatment in 3T3-L1 adipocytes.

Moreover, leptin treatment led to over 2-fold increase in the ChIP activity for 3T3-L1 adipocytes treated with PPARα, over 1.6-fold increase with PPARδ, and about 1.3-fold increase with PPARγ antibodies on PPRE sites of CPT1b promoter (Figure 13B). ChIP activity for STAT3 region of CPT1b promoter also increased for about 1.5-fold by leptin, as a possibility for STAT3 directly binding to CPT1b promoter (Figure 13C). Similarly, ChIP activity increased by leptin for over 1.6-fold with PPARα, over 1.4-fold with PPARδ, and about 1.2-fold increase with PPARγ antibodies on PPRE sites of ACSL1 promoter (Figure 13E). Although 3T3-L1 adipocytes have PPARγ expression as major, followed by PPARδ and

PPARα (Figure 13F), the luciferase activity and ChIP experimental outcomes (Figures 13A-B, 13D-E, 13G-H) explain that SENP2's action on FAO may be due to deSUMOylation of PPARs, mainly PPARα, and then PPARδ/γ on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment in 3T3-L1 adipocytes.


A.
CPT1b Promoter
- 1.5 Kbp
- 400 bp
- 400 bp mutant
Luc
PPRE
PPRE mutant
Vehicle
Leptin
**
0
50
100
150
200
250
Luciferase Activity
B.
CPT1b
% Input
Vehicle
Leptin
PPARα
PPARδ
PPARγ
C.
STAT3 within CPT1b
STAT3
D.
ACSL1 Promoter
- 1 Kbp
- 1 Kbp mutant
Luciferase Activity
E.
ACSL1
F.
Relative mRNA

G.


CPT1b Promoter
% Input
Vehicle
Leptin
siSENP2
PPARα
PPARδ
PPARγ

H.

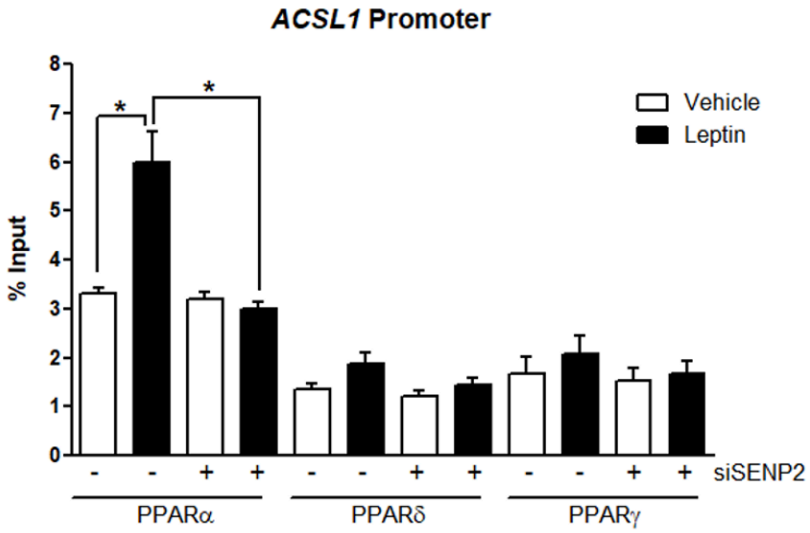

ACSL1 Promoter
% Input
Vehicle
Leptin
siSENP2
PPARα
PPARδ
PPARγ

Figure 13. SENP2 increases binding of PPARs on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment in 3T3-L1 adipocytes

(A) 3T3-L1 pre-adipocytes were transfected with mCpt1b(-1.5 Kbp)-luc, mCpt1b(-400 bp)-luc or mCpt1b(-400 bp)mt-luc containing mutations at a potential PPRE (-260 bp), and then treated with leptin (50 ng/ml) for 24 h for luciferase activity analysis. (B-C,E) After serum starvation for 24 h or (G-H) after siNS or siSENP2 (200 nM) treatment for 48 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for 24 h and were subjected to chromatin immunoprecipitation with quantitative PCR analysis using antibodies (B,E,G-H) against PPARα, PPARδ, and PPARγ and (B,G) CPT1b PPRE primer or (E,H) ACSL1 PPRE primer or (C) using antibodies against STAT3 and primer specific to STAT3 within CPT1b. (D) 3T3-L1 pre-adipocytes were transfected with mAcsl1(−1 Kbp)-luc or mAcsl1-(−1 Kbp)mt-luc containing mutations at a potential PPRE (−210 bp), and then treated with leptin (50 ng/ml) for 24 h. (F) 3T3-L1 adipocytes were subjected to RT-qPCR analysis using PPARα, PPARδ, and PPARγ primers. (A,D) Luciferase activity of cells transfected with mCpt1b(-400 bp)-luc or mAcsl1(−1 Kbp)-luc without leptin treatment was expressed as 100, and the others were expressed as its relative values (n = 5). (B-C,E,G-H) ChIP activity was expressed as its % input (n = 3-5). (F) PPARα expression of 3T3-L1 adipocytes was expressed as 1, and the others were expressed as its relative values (n = 3). Data are presented as mean ± SEM. ($^{**}P < 0.01$, $^{*}P < 0.05$) ⬭, a PPRE site; ⬬, a mutation at the PPRE site.

SENP2 is involved in the effect of leptin on expressions of genes related to fatty acid metabolism in 3T3-L1 adipocytes.

In the previous study, leptin was found to increase mitochondrial uncoupling protein 2 (UCP2), peroxisome proliferator-activated receptor alpha (PPARα), and acyl-coenzyme A oxidase (ACO), while decreasing fatty acid synthase (FAS) level in epididymal white adipose tissue [21,45]. I wanted to investigate leptin effects on these genes related to fatty acid metabolism in 3T3-L1 adipocytes, and to discover the role of SENP2 in these gene expressions by leptin. Thus, SENP2 gene was knocked down with siSENP2, leptin was treated for 24 h, and then gene expressions of UCP2, PPARα, ACO, and FAS were measured in 3T3-L1 adipocytes (Figure 14). In the siSENP2-treated group, there was no significant effect of leptin on genes related to fatty acid metabolism, while there was a significant 1.5-fold increase in the levels of UCP2, PPARα and ACO mRNA expression and decrease in FAS level in leptin-treated siNS group in 3T3-L1 (Figures 14A-D). Consequently, these results reveal that leptin also increases UCP2, PPARα, and ACO, while decreasing FAS level in 3T3-L1 adipocytes via SENP2.

There is no chronic increase of FAO by leptin in adipose tissues of adipocyte-specific SENP2 knock-out mice.

Next, to double-check the significance of in vitro experimental results, in vivo study was conducted. At 10-weeks old, the body weight- and age-matched adipocyte-specific SENP2 knock-out (SENP2 aKO) mice and the control group mice were each injected with leptin (3 mg/kg) or saline intraperitoneally (n = 3) after 4 h morning fast. 24 h later, mice were sacrificed, and their FAO and FAO-related gene expressions were measured (Figures 15,16).

The adipose tissues including visceral adipose tissue and subcutaneous adipose tissue, soleus muscle, and heart were obtained from each group of mice, and FAO experiment was conducted to observe whether SENP2 functions for the rise of FAO by leptin in vivo. The FAO experimental results show that adipose tissues of leptin-injected SENP2 aKO mice had no rise of FAO in contrast to 1.8-fold increase of FAO by leptin in adipose tissues of leptin-injected control mice group (Figure 15). On the other hand, soleus muscle and heart of SENP2 aKO mice group and control mice group both had 2-fold increase of FAO by leptin, indicating that SENP2 accounts for induction of FAO by leptin 24 h after its injection in adipose tissues (Figure 15).

In addition to check on FAO levels, I wanted to verify SENP2 knock-down effects on FAO-related genes CPT1b and ACSL1. SENP2 expression was increased by leptin more than 2-folds in adipose tissues, soleus muscle, and heart of leptin-injected control mice group (Figure 16A). Both leptin- and saline-injected groups of visceral adipose tissue and subcutaneous adipose tissue of SENP2 aKO mice showed reduction in the rise of SENP2 mRNA expression compared to adipose tissues of control mice (Figure 18A). The rise of CPT1b and ACSL1 expression by leptin was not detected in adipose tissues of SENP2 aKO mice, while CPT1b and ACSL1 expressions were increased by leptin in soleus muscle and heart for both SENP2 aKO mice group and control mice group (Figures 16B,16C). These results suggest that SENP2 accounts for increase of FAO-associated enzyme expressions by leptin also in mice. Accordingly, the in vivo study results reflect on in vitro study results, and SENP2 is responsible for the chronic rise of FAO by leptin in adipose tissues.

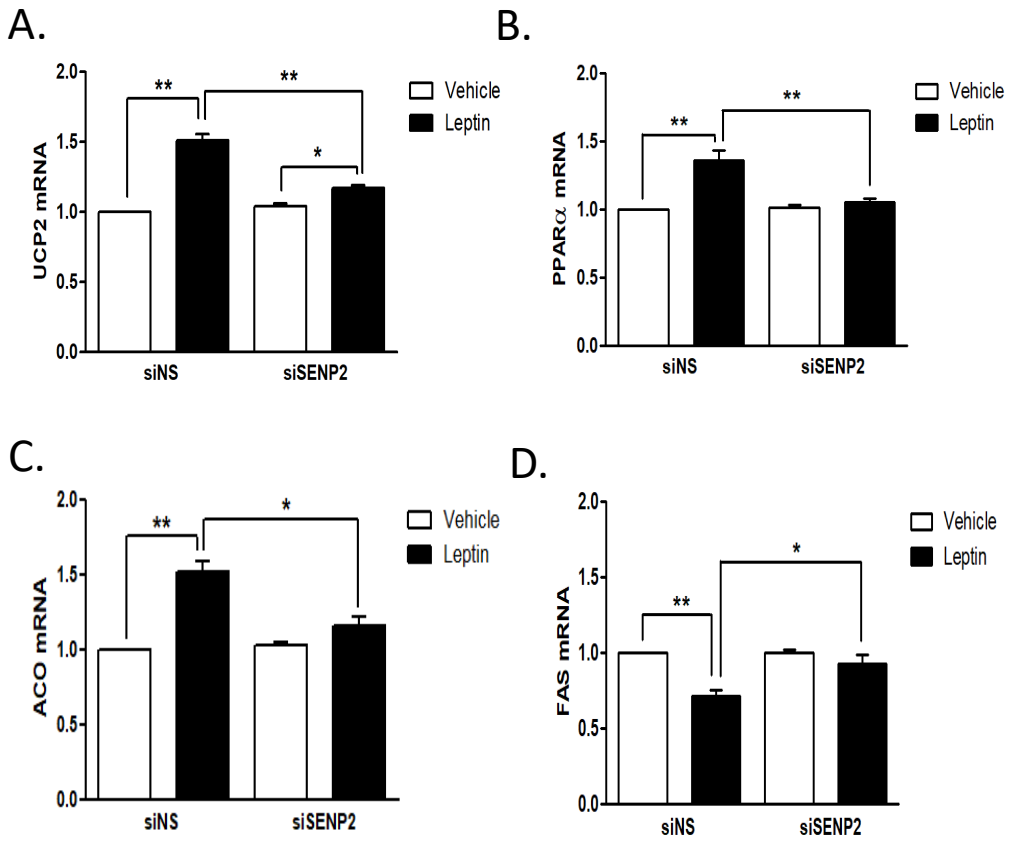


Figure 14. Expressions of genes related to fatty acid metabolism by leptin after SENP2 knock-down in 3T3-L1 cell

After siNS or siSENP2 (200 nM) treatment for 48 h, 3T3-L1 adipocytes were treated with leptin (50 ng/ml) for 24 h and were subjected to RT-qPCR analysis using (A) UCP2 primer, (B) PPARα primer, (C) ACO primer or (D) FAS primer. The mRNA expression levels for adipocytes treated with siNS but without leptin treatment were expressed as 1, and the others were expressed as their relative values (n = 3). Data are presented as mean ± SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$)

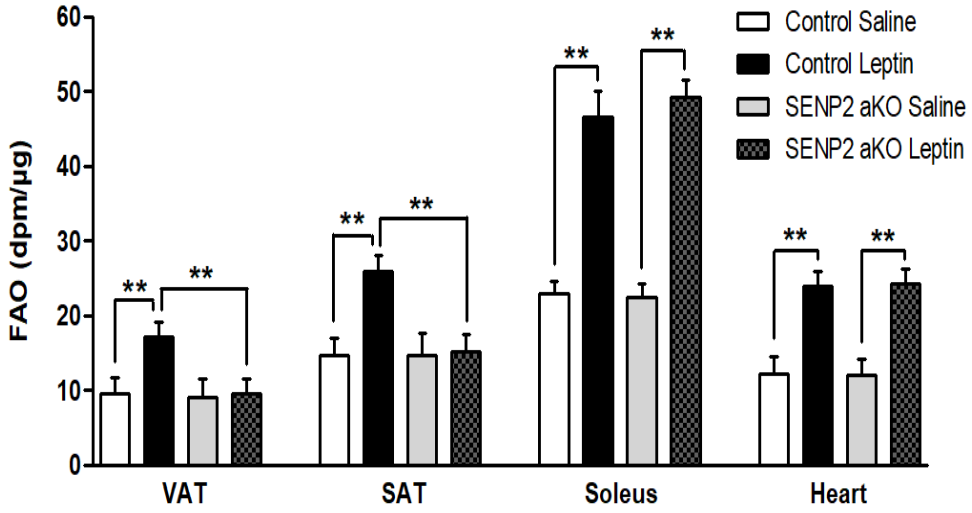


Figure 15. Fatty acid oxidation by leptin in adipocyte-specific SENP2 knock-out mice

At 10 weeks old, leptin (3 mg/kg) or saline was injected intraperitoneally. 24 h later, mice tissues were obtained, and FAO was measured. FAO level of each group was expressed as dpm/protein concentration (n = 3). Data are presented as mean ± SEM. VAT: visceral adipose tissue, SAT: subcutaneous adipose tissue, Soleus: soleus muscle. SENP2 aKO: adipocyte-specific SENP2 knock-out. ($^{**}P < 0.01$)

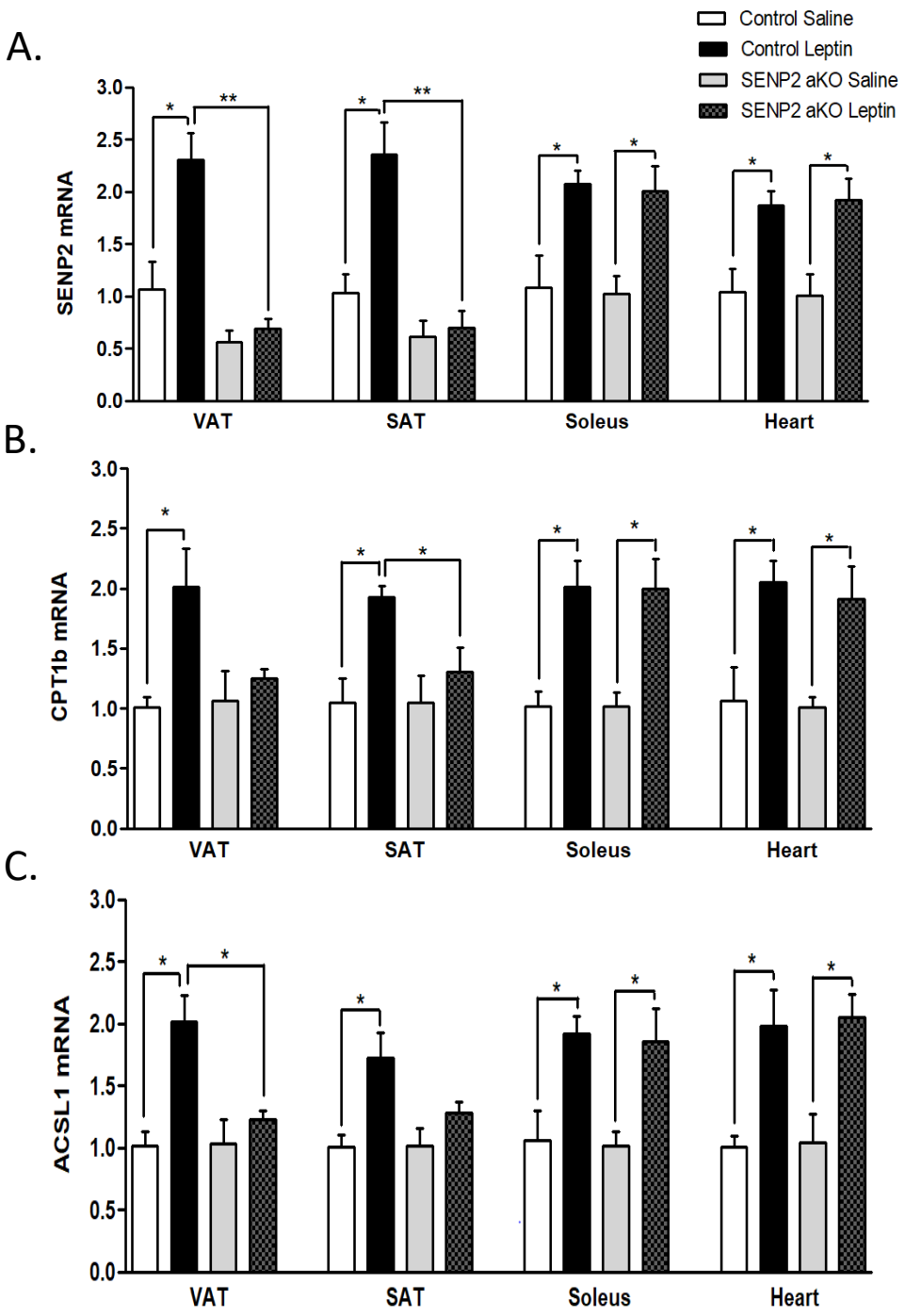

A.
Control Saline
Control Leptin
SENP2 aKO Saline
SENP2 aKO Leptin
SENP2 mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
VAT
SAT
Soleus
Heart
B.
CPT1b mRNA
C.
ACSL1 mRNA

Figure 16. SENP2 and FAO-related genes' expressions by leptin in adipocyte-specific SENP2 knock-out mice

(A-C) At 10 weeks old, leptin (3 mg/kg) or saline was injected intraperitoneally. 24 h later, mice tissues were obtained, and they were subjected to RT-qPCR analysis using (A) SENP2 primer, (B) CPT1b primer or (C) ACSL1 primer. The mRNA expression level for control injected with saline was expressed as 1, and the others were expressed as its relative values (n = 3). Data are presented as mean ± SEM. VAT: visceral adipose tissue, SAT: subcutaneous adipose tissue, Soleus: soleus muscle. SENP2 aKO: adipocyte-specific SENP2 knock-out. ($^{*}P < 0.05$, $^{**}P < 0.01$)

Leptin effects in Cardiomyotubes:

Leptin increases fatty acid oxidation in H9C2 cardiomyotubes.

As the study has confirmed SENP2's role on fatty acid metabolism by leptin in 3T3-L1 adipocytes, I also wanted to test the same hypothesis on H9C2 cardiac muscle cell line. Since H9C2 is a muscle cell line, I had an assumption that it would have similar results as with C2C12 from previously [8]. First, to determine whether differentiated H9C2 cardiomyotubes have a more cardiac-like phenotype than the cardiomyoblasts, the expressions of calsequestrin-cardiac and troponin T were measured. The differentiated H9C2 cardiomyotubes showed greater expressions of calsequestrin and troponin T than the cardiomyoblasts (Figures 17A,17B). Thus, H9C2 fully differentiated as cardiomyotubes were chosen for experiments. The first point of interest to check was whether leptin treatment increases FAO in H9C2, and indeed, leptin increased FAO level in H9C2 cardiomyotubes (Figure 18). Among the PPAR agonists that played as positive controls in the FAO experiment, the PPARβ/δ agonist (GW501516) was most active, next to PPARα agonist (WY14643) while the PPARγ agonist (rosiglitazone) was less significant in FAO and possibly also in cardiac fatty acid metabolism (Figure 18A). H9C2 cardiomyotubes was leptin treated by different concentrations (10, 25, 50, 100 or 200 ng/ml), and FAO increased by 1.5-fold upon leptin treatment from 50 ng/ml (Figure 18B). H9C2 cardiomyotubes was also leptin treated by different time points (6, 12, 24 or 48 h), and FAO increased steadily to reach its peak at 24 h and downregulated afterwards (Figure 18C).

Leptin increases SENP2 and FAO-related enzyme expressions in H9C2 cardiomyotubes.

To investigate whether leptin treatment increases SENP2 expression in H9C2 cardiomyotubes, leptin was treated by different concentrations (50, 100 or 200 ng/ml) and by different time points (6, 12, 24 or 48 h), and RT-qPCR was conducted using rat SENP2-specific primers. SENP2 mRNA expression increased by 1.5-fold upon leptin treatment from 50 ng/ml (Figure 19A). In terms of duration of leptin treatment, SENP2 expression increased steadily to reach its peak at 24 h (Figure 19B).

To also explore whether leptin treatment increases FAO-related CPT1b and ACSL1 expressions in H9C2 cardiomyotubes, leptin was treated by different concentrations (50, 100 or 200 ng/ml) and by different time points (6, 12, 24 or 48 h), and RT-qPCR was conducted using rat CPT1b- and ACSL1-specific primers. For both CPT1b and ACSL1, mRNA expression levels increased by over 1.5-fold dose- and time-dependently upon leptin treatment (Figures 19A,19B).

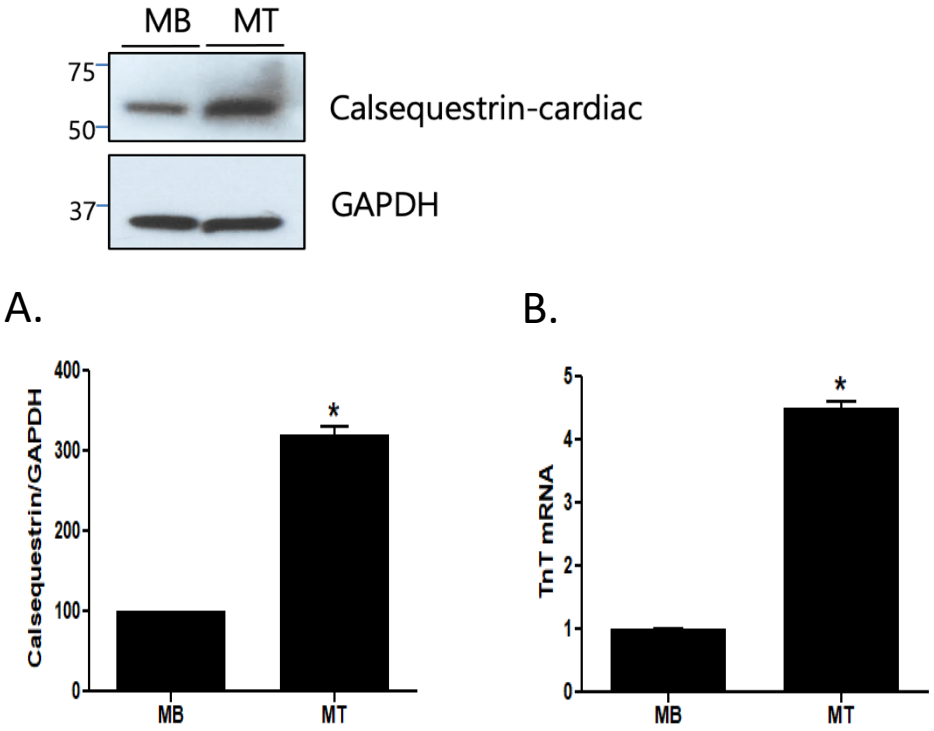


Figure 17. Cardiac-like phenotype in H9C2 cell

(A) Cell lysates of H9C2 cardiomyoblasts in DMEM supplemented with 10 % fetal bovine serum and H9C2 cardiomyotubes in DMEM supplemented with 1 % fetal bovine serum and *trans*-retinoic acid (1 μM) were subjected to western blotting using antibodies against calsequestrin. (B) H9C2 cardiomyoblasts and cardiomyotubes were subjected to RT-qPCR analysis using troponin T (TnT) primer. (A-B) The protein level of cardiomyoblasts was expressed as 100, the mRNA expression level of cardiomyoblasts was expressed as 1, and the others were expressed as their relative values ($n = 3$). Data are presented as mean ± SEM. MB: cardiomyoblast, MT: cardiomyotube. ($^{*}P < 0.05$)

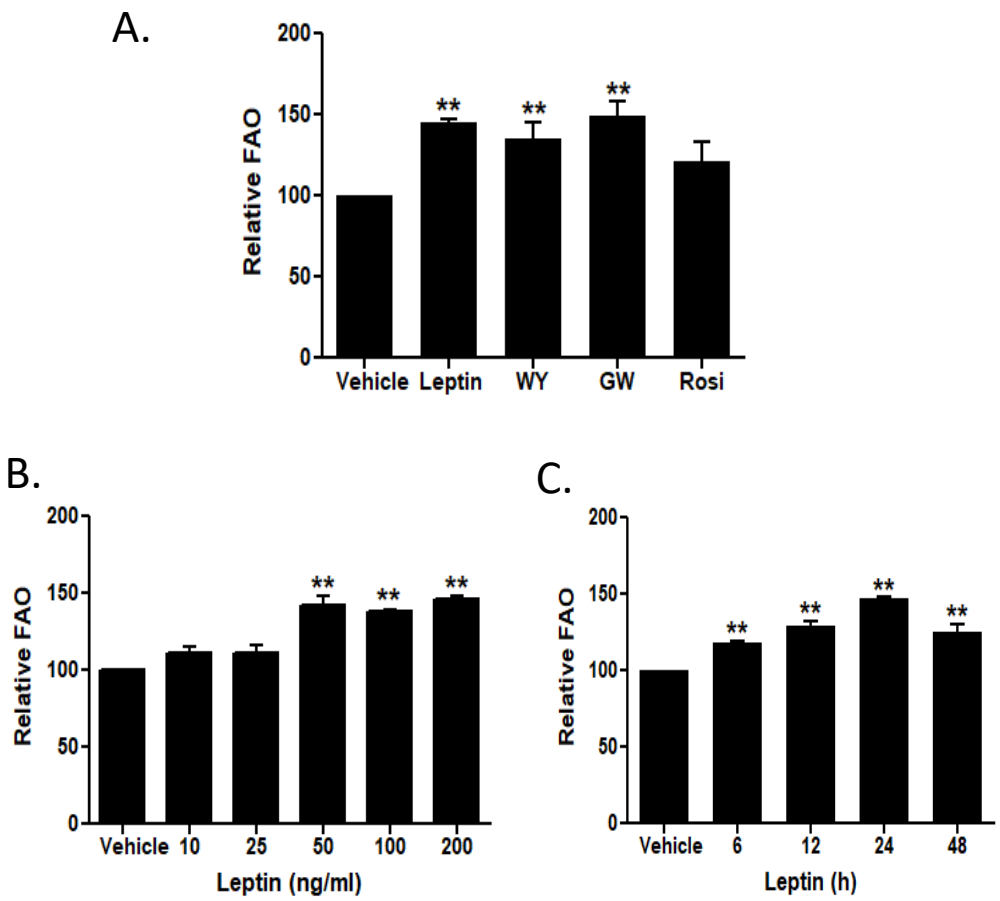


Figure 18. Fatty acid oxidation by leptin in H9C2 cell

(A-C) H9C2 cardiomyotubes were treated (A) with leptin (50 ng/ml), PPARα agonist WY14643 (10 μM), PPARβ/δ agonist GW501516 (10 μM) or with PPARγ agonist rosiglitazone (10 μM) for 24 h, (B) with different concentrations of leptin (10, 25, 50, 100 or 200 ng/ml) for 24 h or (C) with leptin (50 ng/ml) at different time points (6, 12, 24 or 48 h), and FAO was measured. FAO level of vehicle without leptin treatment was expressed as 100, and the others were expressed as its relative values (n = 3-7). Data are presented as mean ± SEM. ($^{**}P < 0.01$ vs. vehicle).

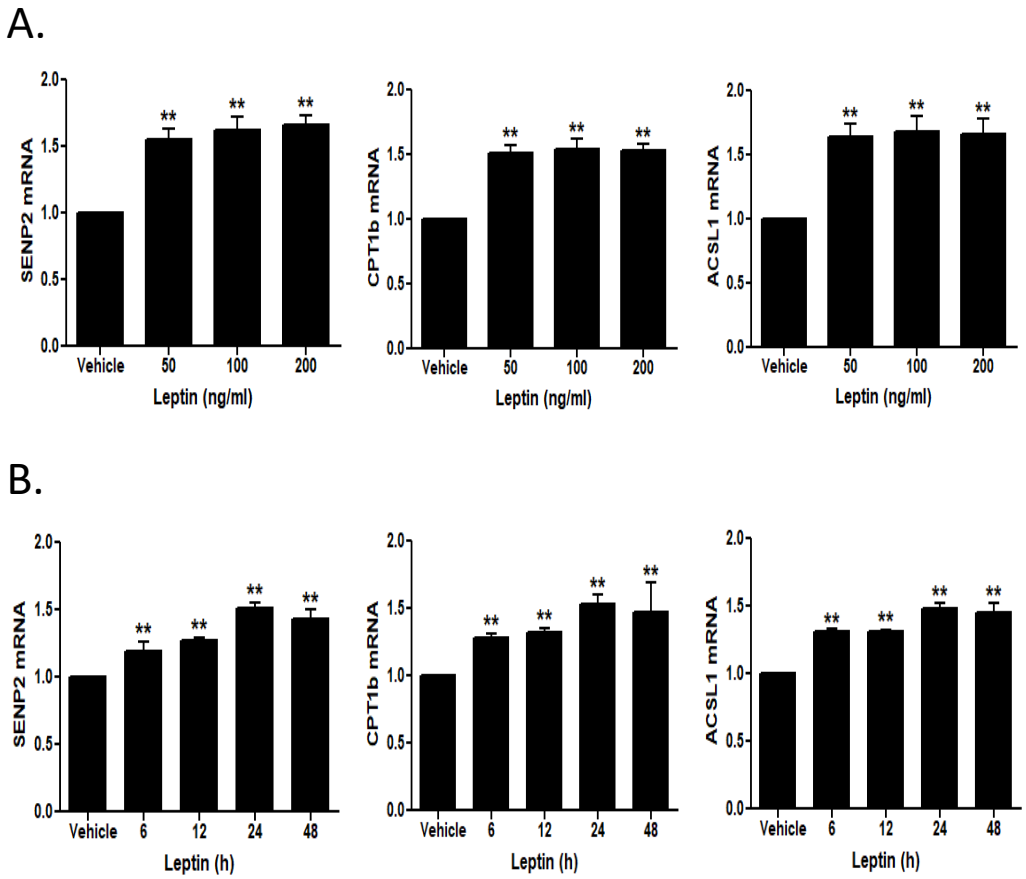


Figure 19. SENP2 and FAO-related genes' expressions by leptin in H9C2 cell

(A-B) H9C2 cardiomyotubes were treated with (A) different concentrations of leptin (50, 100 or 200 ng/ml) for 24 h or (B) with (50 ng/ml) leptin at different time points (6, 12, 24 or 48 h), and then RT-qPCR analysis was run using SENP2 primer, CPT1b primer or ACSL1 primer. The mRNA levels of vehicle without leptin treatment were expressed as 1, and the others were expressed as their relative values (n = 3-6). Data are presented as mean ± SEM. ($^{**}P < 0.01$ vs. vehicle).

AMPK pathway accounts for acute induction of FAO by leptin in H9C2 cardiomyotubes.

In order to test whether AMPK pathway is responsible for increase of FAO by leptin in H9C2 cardiomyotubes, AMPK was inhibited with compound C. After compound C treatment for 1 h, leptin was treated for 6 h or 24 h, and then FAO was measured in H9C2 cardiomyotubes (Figure 20). The results of FAO show that compound C, the AMPK inhibitor clearly removed the increase of FAO by leptin for 6 h leptin-treated group (Figure 20A). In contrast, for 24 h, the effect of AMPK inhibition was much lower (Figure 20B).

SENP2 pathway accounts for prolonged, delayed increase of FAO by leptin in H9C2 cardiomyotubes.

Likewise, to test whether SENP2 pathway is responsible for increase of FAO by leptin in H9C2 cardiomyotubes, SENP2 was knocked down using siRNA. Upon treatment of H9C2 cardiomyotubes with siNS or siSENP2 with different concentrations (50, 100 or 200 nM) for 24 h, expression of SENP2 decreased in siSENP2-treated group compared to siNS-treated group (Figure 21A). The gene expression of SENP2 was knocked down effectively for more than 75 % when treated with siSENP2 for all concentrations (50-200 nM) (Figure 21A).

For measurement of FAO, siSENP2 (50 nM) was treated for 24 h, leptin was treated for 6 h or 24 h, and then FAO was measured in H9C2 cardiomyotubes (Figures 21B,21C). The results of FAO show that siSENP2 treatment significantly lowered the rise of FAO by leptin for 24 h from 1.6-fold to 1.1-fold increase (Figure 21C). In contrast, for 6 h, there was almost no effect of SENP2 knock-down on the rise of FAO by leptin (Figure 21B). Accordingly, the rapid induction of FAO by

leptin is run by the AMPK pathway, while the delayed rise of FAO by leptin is regulated by the SENP2 pathway in H9C2 cardiomyotubes.

Leptin effects via AMPK-dependent pathway and the SENP-dependent pathway.

Taken altogether, leptin induces FAO in adipocytes and cardiomyotubes via two pathways, the AMPK pathway and the SENP2 pathway (Figure 22). The AMPK pathway works as the following: As leptin binds to OBRb, AMPK gets phosphorylated, and FAO increases in acute manner. On the other hand, the SENP2 pathway functions as the following: As leptin binds to OBRb, STAT3 gets phosphorylated, causing increase of SENP2. SENP2 deSUMOylates PPARs, (mainly PPARα, and then PPARδ/γ for adipocytes) on PPRE sites of CPT1b and ACSL1 promoters to increase expressions of CPT1b and ACSL1. In addition, genes related to fatty acid metabolism UCP2, ACO, and PPARα increased, and FAS decreased by leptin via SENP2. Then, increase of FAO-related enzymes causes increase of FAO in chronic manner. These two pathways, the AMPK pathway and the SENP2 pathway work in conjunction in both adipose tissue and cardiac muscle to increase FAO in response to leptin.

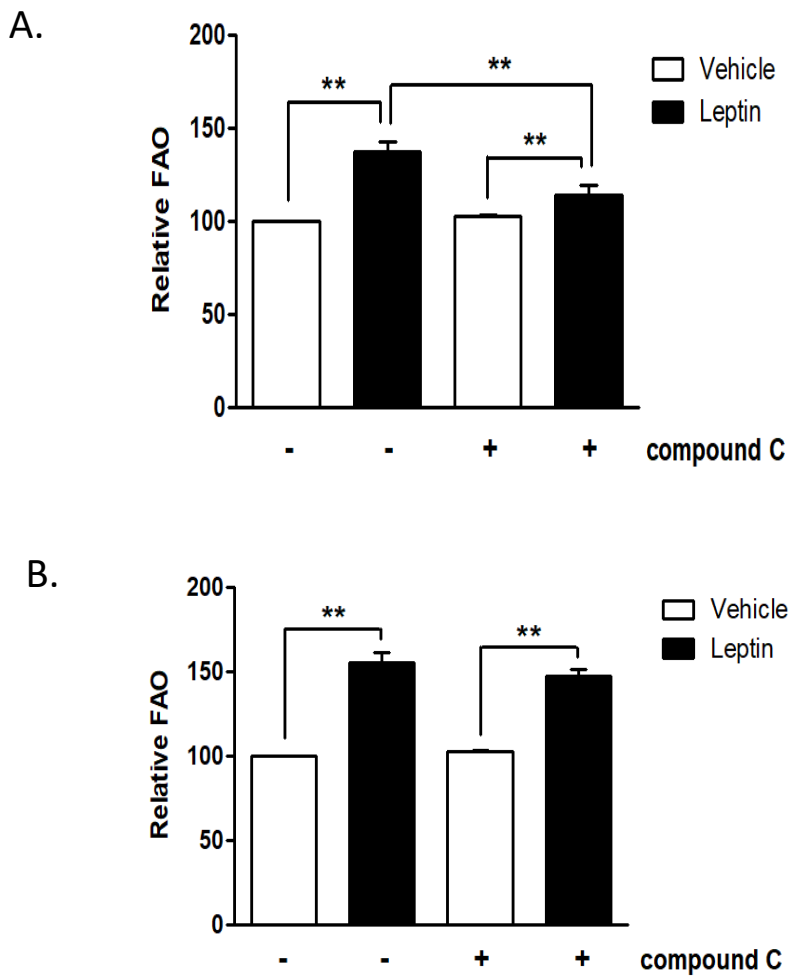


Figure 20. Fatty acid oxidation by leptin after AMPK inhibition in H9C2 cell

(A-B) After pre-treatment with compound C (10 μM) for 1 h, H9C2 cardiomyotubes were treated with leptin (50 ng/ml) for (A) 6 h or (B) 24 h, and FAO was measured. (A-B) FAO levels of vehicle without compound C and without leptin treatment were expressed as 100, and the others were expressed as their relative values (n = 3). Data are presented as mean ± SEM. ($^{**}P < 0.01$)

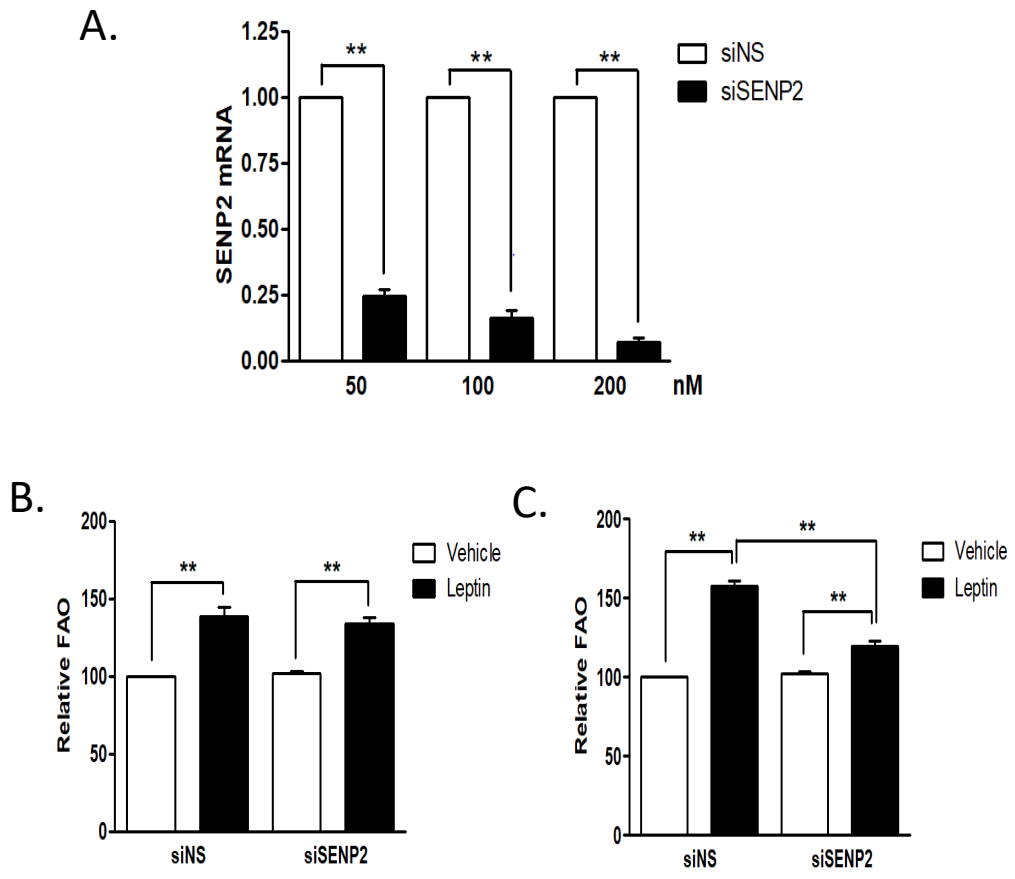


Figure 21. Fatty acid oxidation by leptin after SENP2 knock-down in H9C2 cell

(A) H9C2 cardiomyotubes were treated with siNS or siSENP2 (50, 100 or 200 nM) for 24 h. The mRNA expression levels of SENP2 gene for cardiomyotubes treated with siNS were expressed as 1, and the others were expressed as their relative values (n = 3). (B-C) After siNS or siSENP2 (50 nM) treatment for 24 h, H9C2 cardiomyotubes were leptin (50 ng/ml) treated for (B) 6 h or (C) 24 h, and FAO was measured. FAO level of vehicle without leptin treatment in siNS-treated cardiomyotubes was expressed as 100, and the others were expressed as its relative values (n = 4). Data are presented as mean ± SEM. ($^{**}P < 0.01$)

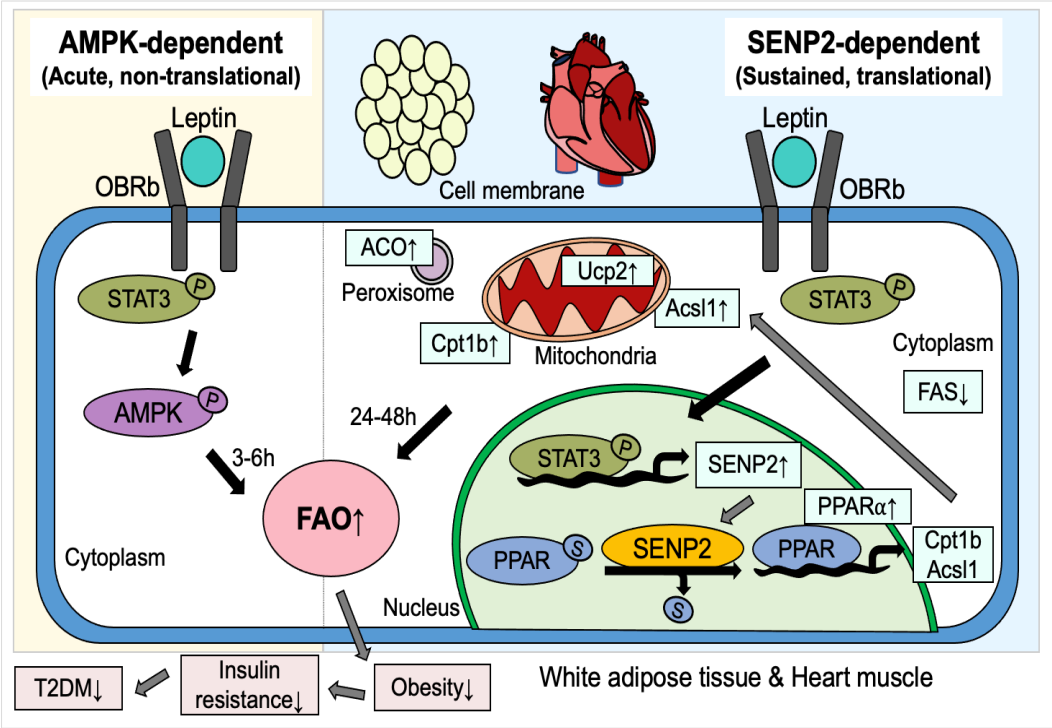


Figure 22. Schematic model showing the role of SENP2 on fatty acid metabolism by leptin in adipose tissue and cardiac muscle

Upon leptin binding to its leptin receptor (OBRb), AMPK and STAT3 get phosphorylated. Phosphorylation of STAT3 increases the cellular level of SENP2 for deSUMOylation of PPARs. These transcription factors are then recruited to the promoters of FAO-associated enzymes CPT1b and ACSL1, which leads to increase of FAO. Leptin treatment also increases expressions of ACO, UCP2, and PPARα, while decreasing expression of FAS via SENP2. Leptin effects on FAO-related genes work to regulate fatty acid metabolism by increasing FAO, to improve obesity, insulin resistance, and T2DM. In adipose tissue and cardiac muscle, rapid induction of FAO is mainly regulated by the AMPK pathway, and prolonged increase of FAO is mainly mediated by the SENP2 pathway. S, SUMO; P, Phosphorylation.

Discussion

From this study, leptin was revealed to increase fatty acid oxidation (FAO) in adipocytes and cardiomyotubes via SUMO-specific protease 2 (SENP2). While 5' adenosine monophosphate-activated protein kinase (AMPK) regulated the acute induction of FAO by leptin, SENP2 mainly mediated for the chronic increase of FAO by leptin. In vivo study with adipocyte-specific SENP2 knock-out (SENP2 aKO) mice also confirmed the role of SENP2 on sustained rise of FAO in response to leptin. When AMPK was inhibited by compound C, FAO level for 6 h leptin treatment showed a more significant reduction in the rise of FAO than 24 h leptin treatment in 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figures 5,20). Compound C was used as it was known as a competitive inhibitor that inhibited AICAR-induced AMPK activation by binding to same ligand site as AMP or AICAR [11]. The increased phospho-AMPK for the initial several hours by leptin (Figure 4B) and the rapidly increased carnitine palmitoyl transferase 1b (CPT1b) expression by leptin at 3-6 h (Figures 9A,19B) possibly from phosphorylation of acetyl-coenzyme A carboxylase (ACC) [8] also explained the AMPK-dependent mechanism for fast and acute rise of FAO by leptin in 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figure 22). In terms of SENP2, FAO results for siSENP2 group showed no increase of FAO for leptin treatment for 24 h, while there was a significant 2-fold increase of FAO in leptin-treated siNS group in both 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figures 12B,21C). The prolonged rise of FAO by leptin in 3T3-L1 adipocytes and H9C2 cardiomyotubes, where leptin would suppress lipogenesis, increase triglyceride hydrolysis, and cause fatty acid and glucose oxidation to alleviate mitochondrial dysfunction in long-term, was mainly mediated by SENP2 as with our previous skeletal muscle study [8].

As a way to supplement and verify the significance of the cell study, in vivo study with SENP2 aKO mice was conducted, and the animal study results further explain leptin's effect on FAO via SENP2 in adipose tissues. The mice were morning fasted for 4 h and had intraperitoneal injection of leptin. 24 h later, there was no increase of FAO by leptin in visceral adipose tissue and subcutaneous adipose tissue of SENP2 aKO mice, whereas FAO increased in response to leptin in tissues of control mice (Figure 15). In addition, expressions of FAO-associated enzymes CPT1b and long-chain acyl-coenzyme A synthetase 1 (ACSL1) were not increased in adipose tissues of the leptin-injected SENP2 aKO mice in contrast to the leptin-injected control mice (Figures 16B,16C). Nonetheless, FAO, SENP2, and FAO-related CPT1b and ACSL1 expressions in other tissues, such as soleus muscle and heart were increased by over 2-folds in response to leptin in both groups (Figures 15,16). Consequently, the in vivo study results supplementary to the in vitro experimental outcomes illustrated SENP2 as a responsible factor for the prolonged rise of FAO by leptin in adipose tissues.

This study also found that as leptin bound to leptin receptor long form (OBRb), it increased SENP2 expression through signal transducer and activator of transcription 3 (STAT3). The rise in phosphorylation of AMPK and phosphorylation of STAT3 by leptin in 3T3-L1 adipocytes (Figures 4,6) was confirmed. Then, the significant 2-fold increase of SENP2 expression by leptin was observed in 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figures 7,19). Followed by STAT3 knock-down, the rise of SENP2 gene expression in leptin-treated siSTAT3 group reduced by 90% in 3T3-L1 adipocytes (Figure 8). The increased phospho-STAT3 and slow but complete translation of SENP2 by leptin at 24 h (Figures 6,7,19) explained the SENP2-dependent mechanism for delayed

and chronic rise of FAO by leptin in 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figure 22).

Moreover, this study observed that leptin increased expressions of CPT1b and ACSL1 that have peroxisome proliferator response element (PPRE) sites, to increase FAO in 3T3-L1 adipocytes and H9C2 cardiomyotubes. CPT1b and ACSL1 increased by leptin in 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figures 9,19), in contrast to ACC that was previously found to decrease by leptin [54]. These FAO-associated genes were the rate-limiting enzymes that transported cytosolic long-chain acyl coenzyme A molecules into mitochondria for oxidation; and thus, increase of expressions of these genes also meant increase of FAO [43]. Recent study from our laboratory has shown that prolonged leptin-stimulated FAO in C2C12 myotubes occurred via deSUMOylation of PPARδ/γ by the SENP2 pathway, while rapid induction of FAO by leptin was due to the activation of CPT1b by the AMPK pathway [8]. This study revealed that leptin increased binding of PPARs to PPRE sites of the promoters of CPT1b and ACSL1 for deSUMOylation of PPARs by SENP2 [23] to ultimately lead to increase of FAO by leptin in 3T3-L1 adipocytes (Figures 13A,13D,22). Although PPARγ gene expression was most abundant among PPARs in adipocytes (Figure 13F) [33], the highest FAO level was found in PPARα agonist WY14643 (Figure 3), and PPARα binding to PPRE sites on CPT1b and ACSL1 promoters was major, followed by PPARδ/γ in response to leptin treatment (Figures 13B,13E). For H9C2 cardiomyotubes, PPARβ/δ agonist GW501516 had the highest FAO level, followed by PPARα agonist (Figure 18A).

Accordingly, SENP2 increases binding of PPARs on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment in adipocytes, and whether the long-

term leptin-induced FAO in adipocytes occurs from deSUMOylation of mainly PPARα via SENP2 remains to be discovered. Likewise, it remains unanswered whether deSUMOylation of mainly PPARβ/δ by SENP2 is responsible for the prolonged increase of FAO by leptin in cardiomyotubes. Or perhaps, there may be a direct relationship between STAT3 response element on CPT1b promoter and leptin, since there was a rapid increase of CPT1b gene expression from 3 h of leptin treatment (Figure 9A), as well as increase of STAT3 binding on STAT3 region on CPT1b promoter (Figure 13C). These study outcomes are relatable to the breast cancer stem cell research that reported that STAT3 could directly activate FAO by leptin through transcription of CPT1b [17], and also to the skeletal muscle studies that showed that ACC phosphorylation and CPT1b activation occur by leptin to increase FAO [8,13].

This study also showed that leptin functions in the heart in similar manner as in adipose tissue. H9C2 cardiomyotubes also had OBRb (Figure 2B), and since heart is another site of leptin production [27], the role of SENP2 on fatty acid metabolism by leptin in cardiomyotubes was also explored. H9C2 cardiac muscle cell line derived from BDIX rat cardiac tissue possessed the capability of differentiating like C2C12 [48], and since fully differentiated cardiomyotubes had more cardiomyocyte phenotypes with increased gene expressions of cardiac sarcomeric protein troponin T and calcium transporter calsequestrin than cardiomyoblasts, cardiomyotubes were used (Figure 17). Although leptin is known to increase cardiomyocyte hyperplasia by extracellular signal-regulated kinase-/phosphatidylinositol 3-kinase-dependent signaling pathways, the pathogenesis of diabetic heart failure in obese individuals remains unclear [28], and whether leptin increases FAO in H9C2 cardiomyotubes has not been reported until this study.

Upon leptin treatment, FAO level increased along with positive controls (AICAR or WY14643, GW501516, and rosiglitazone), which are AMPK activator or PPAR agonists [22] in both 3T3-L1 adipocytes and H9C2 cardiomyotubes (Figures 3,18A). Not only are PPARs important for adipose tissues, PPARs are critical for the regulation of cardiac energy homeostasis [31].

Furthermore, it was noticed that leptin also affects expressions of genes other than CPT1b and ACSL1 that are related to fatty acid metabolism via SENP2. In addition to CPT1b and ACSL1, leptin increased the expressions of mitochondrial uncoupling protein 2 (UCP2), PPARα, and acyl-coenzyme A oxidase (ACO), while it decreased expression of fatty acid synthase (FAS) (Figures 11,14,19), and SENP2 knock-down lowered leptin's effect on rise or fall of these gene expressions [21,45]. Nevertheless, further studies could aim to explain the mechanism behind the different expressions of CPT1b and ACSL1 by leptin, as well as the importance of UCP2, PPARα, ACO, and FAS in terms of FAO that this study found as dependent on SENP2.

Maintaining energy metabolism by leptin is important, and a crosstalk among adipose tissue, liver, and muscle is vital for homeostasis [50]. From this study, leptin effects on fatty acid metabolism in adipose tissue and heart muscle were revealed as significant. First, leptin increased FAO via SENP2 in chronic manner. For the initial several hours of leptin administration, the AMPK pathway played a major role and the SENP2 pathway was minor for rapid induction of FAO by leptin, whereas for 24 h of leptin, the SENP2 pathway was major and the AMPK pathway was minor for prolonged stimulation of FAO by leptin. Second, leptin increased SENP2 expression through STAT3. Third, leptin increased expressions of CPT1b and ACSL1 that have PPRE sites, to increase FAO. In muscle, it was

PPARδ/γ [8], while it was suggested as PPARα that SENP2 deSUMOylated for the chronic increase of FAO in adipose tissue. Fourth, leptin also functioned in the heart similarly as in adipose tissue to induce FAO. Fifth, leptin also worked to increase expressions of UCP2, PPARα, and ACO, while it decreased expression of FAS via SENP2.

Now, this study revealed that SENP2's action on FAO may be due to increase of PPAR binding, mainly PPARα, and then PPARδ/γ on PPRE sites of CPT1b and ACSL1 promoters upon leptin treatment in adipose tissue (Figures 13,14B). Accordingly, leptin was found to increase FAO gene expression through possibly PPARα deSUMOylation by SENP2 in adipose tissue (Figures 13G-H). Likewise, future studies could be planned to conduct experiments with chromatin immunoprecipitation and SUMO detection in PPAR to reveal specific types of PPAR that were deSUMOylated by SENP2 in other tissues for increase of FAO by leptin. Moreover, the role of SENP2 in relation to leptin-induced thermogenesis, where it was known to be acutely activated in response to increased mitochondrial uncoupling protein 1 gene expression by leptin in both brown adipose tissue and white adipose tissue [46] may be fascinating to study. Besides, leptin was also reported to upregulate adipose triglyceride lipase expression via Janus kinase/STAT and mitogen-activated protein kinase signaling pathways, along with PPARγ [44], and leptin effects on additional enzymes related to fatty acid metabolism via SENP2 could be investigated.

Therefore, this study signified that the role of SENP2 on fatty acid metabolism by leptin in adipose tissue and heart muscle is crucial for improvement of obesity and insulin resistant-T2DM (Figure 22).

Bibliography

[1] International Diabetes Federation. IDF diabetes atlas. 9th ed. Belgium: International Diabetes Federation; 2019.

[2] Korean Diabetes Association. Diabetes fact sheet in Korea 2020. Korea: Korean Diabetes Association; 2020.

[3] Kim MK, Ko S-H, Kim B-Y, Kang ES, Noh J, Kim S-K, et al. 2019 Clinical practice guidelines for type 2 diabetes mellitus in Korea. Diabetes Metab J. 2019;43:398-406.

[4] International Diabetes Federation. Type 2 diabetes. Belgium: International Diabetes Federation; 2020.

[5] DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care. 2009;32(suppl 2):157-9.

[6] Koo YD, Choi JW, Kim M, Chae S, Ahn BY, Oh BC, et al. SUMO-specific protease 2 (SENP2) is an important regulator of fatty acid metabolism in skeletal muscle. Diabetes. 2015;64:2420-31.

[7] Evans PL, McMillin SL, Weyrauch LA, Witczak CA. Regulation of skeletal muscle glucose transport and glucose metabolism by exercise training. Nutrients. 2019;11:2432.

[8] Koo YD, Lee JS, Lee S-A, Quaresma PGF, Bhat R, Haynes WG, et al. SUMO-specific protease 2 mediates leptin-induced fatty acid oxidation in skeletal muscle. Metabolism. 2019;95:27-35.

[9] Harris RBS. Direct and indirect effects of leptin on adipocyte metabolism. Biochimica et Biophysica Acta. 2014;1842:414-23.

[10] Unger RH. Lipotoxic Diseases. Annu Rev Med. 2002;53:319-20.

[11] Chen D, Wang Y, Wu K, Wang X. Dual effects of metformin on adipogenic differentiation of 3T3-L1 preadipocyte in AMPK-dependent and independent manners. Int J Mol Sci. 2018;19:1547.

[12] Pereira S, Cline DL, Glavas MM, Covey SD, Kieffer TJ. Tissue-specific effects of leptin on glucose and lipid metabolism. Endocr Rev. 2020;42:1-28.

[13] Minokoshi Y, Kim Y-B, Peroni OD, Fryer LG, Müller C, Carling D, et al. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature. 2002;415:339-43.

[14] Rech M, Luiken JJ, Glatz JF, van Bilsen M, Schroen B, Nabben M. Assessing fatty acid oxidation flux in rodent cardiomyocyte models. Sci Rep. 2018;8:1.

[15] Dong F, Zhang X, Ren J. Leptin regulates cardiomyocyte contractile function through endothelin-1 receptor–NADPH oxidase pathway. Hypertension. 2006;47:222-9.

[16] Gray S, Kim JK. New insights into insulin resistance in the diabetic heart. Trends Endocrinol Metab. 2011;22:394-403.

[17] Wang T, Fahrmann JF, Lee H, Li Y-J, Tripathi SC, Yue C, et al. JAK/STAT3-regulated fatty acid β-oxidation is critical for breast cancer stem cell self-renewal and chemoresistance. Cell Metab. 2018;27:136-50.

[18] Mendes AV, Grou CP, Azevedo JE, Pinto MP. Evaluation of the activity and substrate specificity of the human SENP family of SUMO proteases. Biochim Biophys Acta Mol Cell Res. 2016;1863:139.

[19] Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Pernin A, Chin WW, et al. Direct effects of leptin on brown and white adipose tissue. J Clin Invest. 1997;100:2858-64.

[20] Zhou J, Lei W, Shen L, Luo H-S, Shen Z-X. Primary study of leptin and human hepatocellular carcinoma in vitro. World J Gastroenterol. 2008;14:2900-4.

[21] Gan L, Liu Z, Cao W, Zhang Z, Sun C. FABP4 reversed the regulation of leptin on mitochondrial fatty acid oxidation in mice adipocytes. Sci Rep. 2015;5:1-12.

[22] Dolinar K, Jan V, Pavlin M, Chibalin AV, Pirkmajer S. Nucleosides block AICAR-stimulated activation of AMPK in skeletal muscle and cancer cells. Am J Physiol Cell Physiol. 2018;315:803-17.

[23] Chung SS, Ahn BY, Kim M, Kho JH, Jung HS, Park KS. SUMO modification selectively regulates transcriptional activity of peroxisome-proliferator-activated receptor γ in C2C12 myotubes. Biochem J. 2010;433:155-61.

[24] Pereira S, O'Dwyer SM, Webber TD, Baker RK, So V, Ellis CE, et al. Metabolic effects of leptin receptor knockdown or reconstitution in adipose tissues. Sci Rep. 2019;9:1-17.

[25] Ceddia RB, Curi R. Leptin controls the fate of fatty acids in isolated rat white adipocytes. J Endocrinol. 2002;175:735-6.

[26] Wei C-D, Li Y, Zheng H-Y, Tong Y-Q, Dai W. Palmitate induces H9c2 cell apoptosis by increasing reactive oxygen species generation and activation of the ERK1/2 signaling pathway. Mol Med Rep. 2013;7:855-61.

[27] Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ Res. 1991;69:1476-86.

[28] Tajmir P, Ceddia RB, Li R-K, Coe IR, Sweeney G. Leptin increases cardiomyocyte hyperplasia via extracellular signal-regulated kinase- and

phosphatidylinositol 3-kinase-dependent signaling pathways. Endocrinol. 2004;145:1550-5.

[29] Ljubkovic M, Gressette M, Bulat C, Cavar M, Bakovic D, Fabijanic D, et al. Disturbed fatty acid oxidation, endoplasmic reticulum stress, and apoptosis in left ventricle of patients with type 2 diabetes. Diabetes. 2019;68:1924-33.

[30] Lim A, Park S-H, Sohn J-W, Jeon J-H, Park J-H, Song D-K, et al. Glucose deprivation regulates K_{ATP}Channel trafficking via AMP-activated protein kinase in pancreatic β-cells. Diabetes. 2009;58:2813-9.

[31] Mendler L, Braun T, Müller S. The ubiquitin-like SUMO system and heart function. Circ Res. 2016;118:132-44.

[32] Patten V, Chabaesele I, Sishi B, Van Vuuren D. Cardiomyocyte differentiation: experience and observations from 2 laboratories. SA Heart. 2017;14:96-107.

[33] Tamori Y, Masugi J, Nishino N, Kasuga M. Role of peroxisome proliferator-activated receptor- in maintenance of the characteristics of mature 3T3-L1 adipocytes. Diabetes. 2002;51:2045-55.

[34] Paz-Filho G, Mastronardi CA, Licinio J. Leptin treatment: facts and expectations. Metabolism. 2015;64:146.

[35] Momken I, Chabowski A, Dirkx E, Nabben M, Jain SS, McFarlan JT, et al. A new leptin-mediated mechanism for stimulating fatty acid oxidation: a pivotal role for sarcolemmal FAT/CD36. Biochem J. 2016;474:149-62.

[36] Sun H-J, Xiong S-P, Wu Z-Y, Cao L, Zhu M-Y, Moore PK, et al. Induction of caveolin-3/eNOS complex by nitroxyl (HNO) ameliorates diabetic cardiomyopathy. Redox Biol. 2020;32:101493.

[37] Barouch LA, Berkowitz DE, Harrison RW, O'Donnell CP, Hare JM. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. Circ. 2003;108:754-9.

[38] Zhang Y, Chua S. Leptin function and regulation. Compre Physiol. 2017;8:351-69.

[39] D'souza AM, Neumann UH, Glavas MM, Kieffer TJ. The glucoregulatory actions of leptin. Mol Metab. 2017;6:1052-65.

[40] Stern JH, Rutkowski JM, Scherer PE. Adiponectin, leptin, and fatty acids in the maintenance of metabolic homeostasis through adipose tissue crosstalk. Cell Metab. 2016;23:770-4.

[41] Pan H, Guo J, Su Z. Advances in understanding the interrelations between leptin resistance and obesity. Physiol Behav. 2014;130:157-69.

[42] Machinal-Quélin F, Dieudonné MN, Leneveu MC, Pecquery R, Giudicelli Y. Proadipogenic effect of leptin on rat preadipocytes in vitro: activation of MAPK and STAT3 signaling pathways. Am J Physiol Cell Physiol. 2002;282:853-61.

[43] Muoio DM, Newgard CB. Fatty acid oxidation and insulin action: when less is more. Diabetes. 2008;57:1455-6.

[44] Li Y-cheng, Zheng X-li, Liu B-ting, Yang G-she. Regulation of ATGL expression mediated by leptin in vitro in porcine adipocyte lipolysis. Mol Cell Biochem. 2009;333:121-8.

[45] Wang M-Y, Lee Y, Unger RH. Novel form of lipolysis induced by leptin. J Biol Chem. 1999;274:17541-4.

[46] Commins SP, Watson PM, Frampton IC, Gettys TW. Leptin selectively reduces white adipose tissue in mice via a UCP1-dependent mechanism in brown adipose tissue. Am J Physiol Endocrinol Metab. 2001;280:372-7.

[47] Shao D, Kolwicz SC, Wang P, Roe ND, Villet O, Nishi K, et al. Increasing fatty acid oxidation prevents high-fat diet–induced cardiomyopathy through regulating Parkin-mediated mitophagy. Circ. 2020;142:983-4.

[48] Ricotti L, Polini A, Genchi GG, Ciofani G, Iandolo D, Vazão H, et al. Proliferation and skeletal myotube formation capability of C2C12 and H9c2 cells on isotropic and anisotropic electrospun nanofibrous PHB scaffolds. Biomed Mater. 2012;7:035010.

[49] Ho M, Foxall S, Higginbottom M, Donofrio DM, Liao J, Richardson PJ, et al. Leptin-mediated inhibition of the insulin-stimulated increase in fatty acid uptake in differentiated 3T3-L1 adipocytes. Metab. 2006;55:8-11.

[50] Kim S-H, Hong SH, Park Y-J, Sung J-H, Suh W, Lee KW, et al. MD001, a novel peroxisome proliferator-activated receptor α/γ agonist, improves glucose and lipid metabolism. Sci Rep. 2019;9:1-12.

[51] Chang E, Kim Y. Vitamin D decreases adipocyte lipid storage and increases NAD-SIRT1 pathway in 3T3-L1 adipocytes. Nutrition. 2016;32:702-8.

[52] Yang J, Savvatis K, Kang JS, Fan P, Zhong H, Schwartz K, et al. Targeting LOXL2 for cardiac interstitial fibrosis and heart failure treatment. Nat Commun. 2016;7:1-2.

[53] Shin E-J, Schram K, Zheng X-L, Sweeney G. Leptin attenuates hypoxia/reoxygenation-induced activation of the intrinsic pathway of apoptosis in rat H9c2 cells. J Cell Physiol. 2009;221:490-7.

[54] Yamagishi S-I, Edelstein D, Du X-L, Kaneda Y, Guzmán M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. J Biol Chem. 2001;276:25096-100.

[55] Jiang L, Wang Q, Yu Y, Zhao F, Huang P, Zeng R, et al. Leptin contributes to the adaptive responses of mice to high-fat diet intake through suppressing the lipogenic pathway. PLoS ONE. 2009;4:6884.

[56] Wilson NR, Hochstrasser M. Function and regulation of SUMO proteases. Nat Rev Mol Cell Biol. 2012;13:759.

[57] Mora C, Pintado C, Rubio B, Mazuecos L, López V, Fernández A, et al. Central leptin regulates heart lipid content by selectively increasing PPAR β/δ expression. J Endocrinol. 2018;236:43-56.

[58] Ritterhoff J, Young S, Villet O, Shao D, Neto FC, Bettcher LF, et al. Metabolic remodeling promotes cardiac hypertrophy by directing glucose to aspartate biosynthesis. Circ Res. 2020;126:182-3.

초 록

제 2 형 당뇨병 및 비만과 같은 만성 대사질환에서의 혈액 내 지방산의 증가와 비지방조직의 지방 축적은 말초 조직의 인슐린 저항성을 악화시키고, 췌장 베타세포의 기능 장애를 유발할 수 있다. 렙틴은 지방조직에서 분비되는 아디포카인으로 시상하부에서 식욕을 억제할 뿐 아니라 말초조직에서 지방산 산화를 증가시킨다. 렙틴은 골격근에서 AMPK 활성화를 통해 지방산 산화를 증가시킨다고 알려져 있었으나 최근 SUMO-specific protease 2 (SENP2)-PPAR 경로를 통해 지방산 산화를 증가시키는 것이 밝혀졌다. 그러나 지방조직과 심장근육 등 다른 조직에서 렙틴의 효과에 SENP2 가 관여하는지는 알려져 있지 않다. 저자는 본 연구에서 백색 지방 세포인 3T3-L1 과 심장 근육 세포인 H9C2 에서, 그리고 지방 세포 특이적 SENP2 유전자가 제거된 녹아웃 쥐 모델에서 렙틴에 의한 지방산 대사 효과 중 SENP2 의 역할을 연구하였다.

지방 세포와 심장 근육 세포를 렙틴으로 처리하면 STAT3 및 AMPK 의 인산화 발현이 증가하였으며 SENP2 의 발현도 증가하였다. 또한 CPT1b, ACSL1 과 같은 지방산 산화 관련 유전자들의 발현이 증가하였다. siRNA 를 이용한 AMPK 와 SENP2 녹다운 실험을 통해 지방 세포 및 심장 근육 세포에서의 렙틴에 의한 지방산 산화의 증가는 초기 수시간동안은 AMPK 경로에 의해 조절되는 반면, 24 시간째 증가는 주로 SENP2 경로 의존적임을 알 수 있었다. 더불어, 지방 세포 특이적 SENP2 녹아웃 쥐에 렙틴을 복강 내 주입한 결과, 내장 지방 조직 및 피하 지방 조직의 지방산 산화와 CPT1b 및 ACSL1 의 수준은 24 시간의 렙틴 처리에 의해 증가하지 않는 반면, 가자미 근과 심장은 녹아웃의 영향을 받지 않았으며 렙틴에 의한 지방산 산화 그리고 지방산 산화 관련 유전자들의 증가를 보였다. 더 나아가, SENP2 는 렙틴 처리 시 CPT1b 및 ACSL1

프로모터의 PPRE 부위에 대한 PPAR 의 결합을 증가시켜 FAO 의 장기간 증가를 중재하여 성립시켰다.

본 연구 결과를 통해 백색 지방 및 심장 근육에서 렙틴에 의해 유도된 지방산 대사에 있어서 SENP2 가 중요한 역할을 한다는 것을 알 수 있었다.

주요어 : SENP2, 제 2 형 당뇨병, 지방산 대사, 비만, 렙틴

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