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Role of SENP2 in leptin-induced fatty acid oxidation in skeletal muscle

Leptin에 의한 골격근 지방산산화에 대한 SENP2의 역할

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Abstract

Leptin is an adipokine produced predominantly in adipose tissue, and plays an important role in maintaining adipose mass by regulating both food intake and energy expenditure. Leptin binds to leptin receptor long form (LepRb) and enhances Janus kinase 2 (JAK2)/ signal transducer and activator of transcription 3 (STAT3) signaling. Leptin binds to LepR in hypothalamus and inhibits food intake by promotion of anorexigenic factors and inhibiting orexigenic factors. In arcuate region of hypothalamus, pro-opiomelanocortin (POMC) neurons activate peptide which is a hormone inhibiting food intake, and POMC/ cocaine and amphetamine-regulated transcript (CART) is known for decreasing food intake. Leptin increases energy expenditure in skeletal muscle by activation of AMP-activated protein kinase and resultant increase in fatty acid oxidation. Our group recently demonstrated that leptin increases SUMO specific protease 2 (SENP2) expression through leptin receptor/STAT3 signaling in C2C12 myotubes. SENP2 desumoylates peroxisome proliferator-activated receptors (PPAR) δ and PPARγ, which increases transcription of PPAR target genes to produce fatty acid oxidation(FAO)-associated enzymes such as acyl-CoA synthetase 1 (ACSL1) and carnitine palmitoyl transferase-1 (CPT1b). In this study, I aimed to verify the role of SENP2 on the effect of leptin in skeletal muscles *in vivo*. Intraperinoteal injection of leptin (3mg/kg, body weight) significantly increased mRNA levels of SENP2 in soleus muscle but not in gastrocnemius muscles (GM) of C57BL/6J mice. Leptin did not increase SENP2 expression in soleus muscle of db/db mice which have defective leptin long form receptors. Incubation of isolated soleus muscles of C57BL/6J mice with leptin (3mg/kg, body weight) increase phosphorylation of STAT3 from 30 minutes to 2 hours. These results suggest that leptin increases SENP2 expression through the leptin receptor/STAT3 signaling pathway in skeletal muscle. Leptin administration also increased the mRNA levels of ACSL1 and CPT1b and FAO in soleus muscles of C57BL/6J mice. However, leptin did not increase FAO-associated enzymes expression and FAO in GM muscle of C57BL/6J mice as well as soleus muscles of db/db mice. These data suggest that leptin increases FAO in skeletal

muscle and leptin induced SENP2 expression contribute to in vivo effect of leptin on FAO.

Keywords: SUMO-specific protease 2, Leptin, Leptin receptor, fatty acid oxidation, obesity

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List of Abbreviations

LepR or ObR : Leptin receptor

LepRb: leptin receptor long form

JAK2: Janus kinase 2

STAT3 :signal transducer and activator of transcription 3

SENP2: SUMO specific protease 2

PPAR : peroxisome proliferator-activated receptor

FAO : fatty acid oxidation

ACSL1 : acyl-CoA synthetase 1

CPT1b : carnitine palmitoyl transferase-1

GM : gastrocnemius muscles

AMPK: AMP-activated protein kinase

ACC :Acetyl-CoA carboxylase

SUMO : Small ubiquitin like modifier

CEBP : CCAAT-enhancer-binding protein

RT-PCR : real-time polymerase chain reaction

IP : intraperitoneal

GAPDH : glyceraldehyde 3-phosphate dehydrogenase

RPS2 : ribosomal protein S2

QM : quadriceps muscle

i.v. injection: intravenous injection

i.h.p. injection: intrahypothalamic injection

MCR: melanocortin receptor

VMH: ventromedial hypothalamus

ERK: extracellular signal-regulated kinase

IRS-2: insulin receptor substrate 2

PI3 kinase: phosphatidylinositol-3 kinase

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Introduction

Leptin

Leptin is a hormone released by adipocytes related to accumulation of triglycerides [1]. This hormone, 16kDa non-glycosylated protein in cytokine class 1 super family, is encoded by *ob* gene [2,3]. In brain, leptin manages energy balance by reducing food intake and expanding energy expenditure in promotion of anorexigenic factors and inhibiting orexigenic factors [2]. And leptin also influences many functions containing glucose metabolism, glucocorticoid synthesis, cytokine secretion, phagocytosis, CD4+ T-lymphocyte proliferation, reproduction, angiogenesis and hypothalamic pituitary-adrenal axis regulation. For biological activity, leptin binds to class 1 cytokine receptor encoded by LEPR in humans and Lepr in mice [3,4].

Leptin receptor

By positional cloning experiment, ob gene comprised of a 4500bp sequence was reported to encode leptin. And leptin or Ob protein described as an adipose tissue-derived signaling factor regulates body weight homeostasis. [5, 6, 8]. Leptin receptor (LepR or ObR), a single membrane-spanning receptor, has six isoforms. These LepR isoforms (LepRa-LepRf) are constructed by alternative splicing or ectodomain shedding, and having a general leptin binding domain with different intracellular domains. The six isoforms are categorized into long form (LepRRb), short form (LepRa, LepRc, LepRd and LepRf) and soluble form (LepRe) [9, 10].

db/db mutation

In db/db mice, 2bp after 106nt insertion is different from young control mice. And paralleled to consensus sequence, this G→T point mutation produces new splice donor site. In addition, the 106nt insertion with stop codon inhibits production of long intracellular domain. In an anticipation, the long intracellular domain form is important for beginning of intracellular

signal transduction, and obese phenotype of db/db mice is shown when this form is not able to be generated [6]. db/db mice have diabetic phenotypes such as obesity, high glucose levels in fasting and high insulin levels [7].

Leptin signaling pathway in central nervous system

In neuron, leptin binds to the long form of leptin receptor (LepRb) and intracellular JAK2 activity is occurred acutely. And this JAK2 activity results in increased tyrosine phosphorylation of LepRb. After binding of STAT3 proteins to Y1138 of leptin receptor, phosphorylation of the STAT3 proteins is processed by JAK2, and the STAT3 proteins configurate dimers after separation in cytoplasm. Furthermore, the STAT3 proteins are translocated to nucleus for controlling other gene transcription [11].

In brain, leptin regulates food intake and energy expenditure by arcuate nucleus neurons: pro-opiomelanocortin (POMC) and agouti-related protein (AGRP) neurons. POMC neurons which manufacture melanocortin receptors (MCRs) promote energy expenditure and decrease food intake, while AGRP with neuropeptide Y (NYP) and neurotransmitter GABA express orexigenic (increasing appetite) peptides. Therefore, leptin elevates POMC expression and inhibits AGRP activity in its neuronal signaling [1].

Leptin signaling pathway by AMPK phosphorylation in skeletal muscle

AMPK plays a role for leptin-induced fatty acid metabolism in muscle [13-19].

In skeletal muscle, leptin selectively increases phosphorylation of α2 catalytic subunit of AMPK and activated AMPK subsequently phosphorylate acetyl-CoA carboxylase (ACC). The phosphorylated ACC blocks ACC activity. And this prevention of ACC activity decreases malonyl CoA content but carnitine palmitoyltransferase I is elevated that fatty acid oxidation is promoted as a result [12]. By these mechanisms, leptin promotes fatty acid oxidation in muscle (Figure 1). Leptin activates AMPK directly in skeletal muscle at 15min of leptin administration

while leptin activate AMPK via hypothalamic-sympathetic nervous system axis in late stage [12].

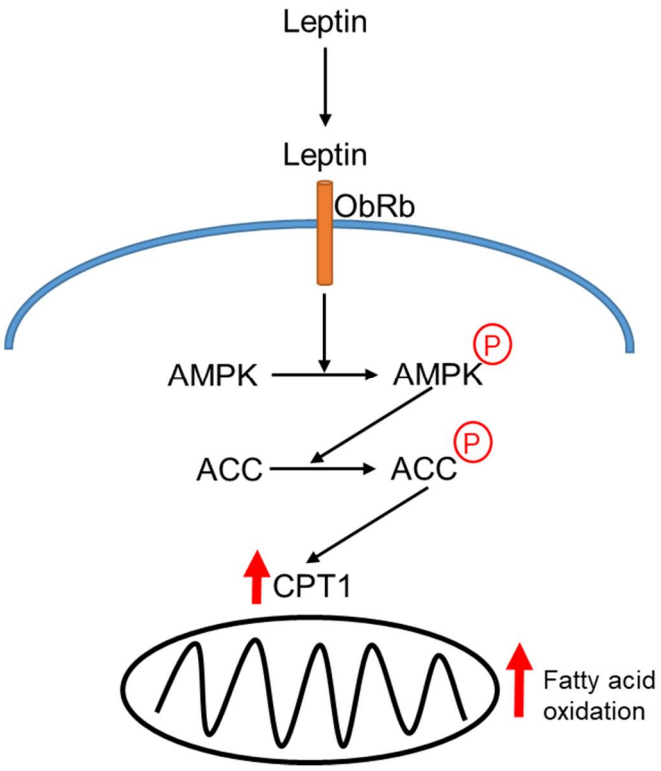

Figure 1. Leptin signaling by AMPK phosphorylation in skeletal muscles

Chronic leptin influence on lipid oxidation in C2C12 myotubes

Chronic leptin influence on lipid oxidation in C2C12 myotubes was reported. In differentiated C2C12 myotubes, leptin elevates lipid oxidation in concentration and time-dependent manner, and the lipid oxidation is definitely promoted after 6 hours of leptin treatment. Leptin increased JAK2 and STAT3 phosphorylation and leptin did not increase lipid oxidation after JAK2 inhibitor and STAT3 siRNA. Therefore, in skeletal muscles, JAK2 and STAT3 signaling pathway is important for chronic effect of leptin to induce lipid oxidation [20].

Role of SENP2 in fatty acid oxidation in C2C12 myotubes

Palmitate treatment increases SENP2 expression through TLR4-MyD88-nuclear factor-κB pathway in C2C12 myotubes. SENP2 desumoylates peroxisome proliferator-activated receptors (PPAR)δ and PPARγ and increased PPAR transcription complex bound to the promoters of fatty acid oxidation (FAO)-related genes like acyl-CoA synthetase 1 (ACSL1) and carnitine palmitoyl transferase-1 (CPT1b) and increases fatty acid oxidation in skeletal muscle [21].

Sumoylation

Small ubiquitin like modifier (SUMO) is a small protein and can be covalently conjugated to other proteins, which is called sumoylation. Sumoylation influences many cellular activities of the proteins [22]. Sumoylation pathway is similar with ubiquitination. In the first step, E1 class activating enzyme activates mature SUMO polypeptide depending on ATP hydrolysis. And then transfer of SUMO to E2 conjugating enzyme promotes target protein covalent SUMO modification. SUMO E3 ligases help SUMO binding to target protein specifically, and increase sumoylation efficiency [23]. SUMO-specific proteases, which is belonged to cysteine proteases family, accelerate desumoylation [24]. SENPs are identified as six (SENP1, 2, 3, 5, 6, and 7)

with distinct cellular localization and substrate specificities [25, 26].

Role of SENP2 in adipogenesis

In early stage of adipogenesis, SENP2 levels were increased. And this mechanism depends on PKA activation. When SENP2 knockdown is occurred in early stage of adipogenesis, adipogenesis is not processed because sumoylation of C/EBPβ is increased. And as a result, C/EBPβ is degraded. Therefore, SENP2 plays a role in maintenance of C/EBPβ proteins in clonal expansion of the early stage of adipogenesis [26].

Leptin-induced fatty acid oxidation by SENP2 in C2C12 myotubes (previous studies in our lab)

Our lab previously found that leptin increased the expression of SENP2 time and dose dependently over 24 hours in C2C12 myotubes (Fig. 2A). Leptin increased SENP2 expression via leptin receptor-STAT3 pathway in these cells (Fig. 2B). In addition, knockdown of SENP2 ameliorated leptin induced fatty acid oxidation and mRNA expression of FAO-associated enzyme (Acsl1 and Cpt1b) (Fig.2C and D). Knockdown study of AMPKα2 and SENP2 suggest that leptin increased FAO by activation of AMPK in early time period(6 hours) and SENP2 in delayed time period(24~48 hours) (Figure 3).

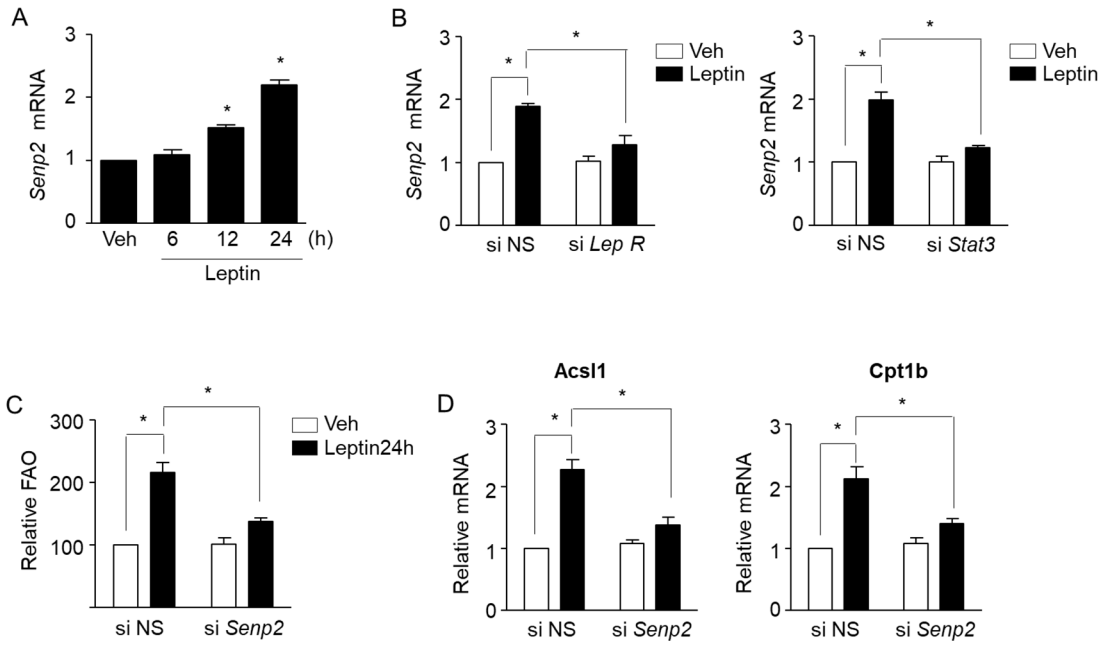

Figure 2. Leptin increased fatty acid oxidation via leptin receptor-STAT3-SENP2 pathway in C2C12 myotubes (previous studies in our lab)

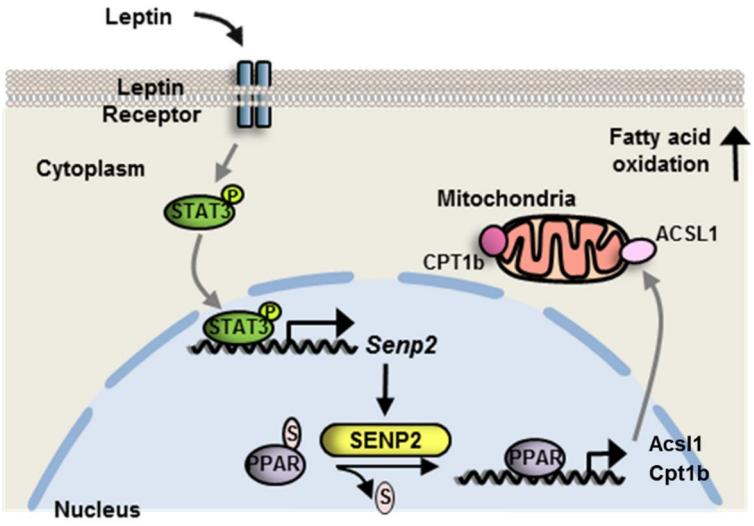

Figure 3. Scheme for leptin signaling pathway through leptin receptor, STAT3 and SENP2 in C2C12 myotubes

Aim of this study

Although our lab found the role of SENP2 in leptin-induced fatty acid oxidation in muscle cells, it is not clear whether leptin increases FAO in skeletal muscle in vivo since there have been debates for leptin's effect and possible mechanisms if any in skeletal muscle. Therefore, I aim to investigate whether leptin increases FAO and SENP2 plays a role in this process in skeletal muscle *in vivo*.

Materials and Methods

Animals

For animal experiment, Male C57BL/6J mice (8-12 weeks old) were performed in accordance with Institutional Animal Care and Use Committee of Seoul National University Hospital. And BKS.Cg-Dock7<m> +/+ Lepr<db>/J (dbdb) mice (8 weeks old) were purchased from Jackson laboratory in USA, and experiments were performed at 10 or 12 weeks of age. Leptin from mouse (recombinant expressed in *E. coli*, Sigma Aldrich) is dissolved in saline, and was injected in mice by intraperitoneal (i.p.) injection method (with 3mg/kg bodyweight concentration). Mice were sacrificed after 24 hours of leptin injection, and tissues were collected.

RNA extraction and real-time polymerase chain reaction (PCR)

For RNA isolation, total RNAs of soleus and gastrocnemius muscle tissues were extracted by Trizol reagent (Invitrogen, MA, USA) with manufacturer's instruction. And for cDNAs synthesis, 500ng or 1μg of total RNA, 5μl of reaction buffer, 2.5μl of 100mM DTT, 1.25μl of 10mM dNTP mix, 0.5μl of Oligo dT, 0.25μl of RNase inhibitor, 1μl of RTase (Invitrogen, MA, USA) and RNase free water for remnant amount were mixed for total 25μl, and processed in 1 hour of 37°C incubation and 10 min for 72°C. The cDNAs were mixed with SYBR Master Mix (Takara, Otsu, Shiga, Japan) and primers for measuring quantitative levels of genes by AB 7500 Real-time PCR system (Applied Biosystems, CA, USA). The primer sequence list is indicated in Table 1.

Western blot Analysis

Mice tissue after sacrifice, collected and powdered in liquid nitrogen. The powdered tissues were dissolved in RIPA lysis buffer (0.5M Tris-HCl pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) (Merck Millipore, Temecula, CA) containing protease inhibitors

(10 ug/ul aprotinin, 10 ug/ul leupeptin and 1mM PMSF) and protease and phosphatase inhibitor cocktail (Thermofisher) and rotated 1 hour in 4 °C. Concentrations of proteins were measured by using bicinchoninic-acid (BSA) assay. Tissue lysates were separated by centrifuge and 30ug of proteins was loaded in SDS-PAGE. And the separated proteins in gel were transferred to nitrocellulose membrane. For analysis, the membrane with proteins were incubated in ObR (Santa Cruz), STAT3 (cell signaling), phospho-STAT3 (cell signaling) and γ tubulin (Sigma-Aldrich) antibodies.

Ex vivo leptin treatment.

Soleus muscles were harvested from male mice 16 h after food removal and transferred to a 12-well plate containing 2 ml of Krebs-Henseleit buffer, pH 7.4 (Sigma-Aldrich, St. Louis, MO) with 2 mmol/L pyruvate, 8 mmol/L mannitol, and 0.1% BSA, as previously described [27]. Muscles were preincubated for 15 min in this buffer before replacing with the buffer containing leptin (300 ng/mL). Muscles were collected at the indicated time points and lysates were prepared as previously described. Briefly, muscles were homogenized in ice-cold RIPA buffer containing commercially available protease and phosphatase inhibitor cocktails (Roche) using glass teflon homogenizer. Protein content in lysates was measured by the bicinchoninic acid method (Pierce) and subject to immunostaining for detection of phospho- and total-STAT3.

Measurement of FAO

For assessing FAO rates in skeletal muscles, soleus and gastrocnemius muscles were homogenized in ice-cold mitochondria isolation buffer (250 mmol/L sucrose, 10 mmol/L Tris-HCl, and 1 mmol/L EDTA). And the homogenized lysates were with 0.2 mmol/L [1-^{14}C] palmitate for 2 hours in incubation of 37 °C. By a liquid scintillation counter, ^{14}CO2 and ^{14}C-labeled acid-soluble metabolites were measured. The radioactivity of each lysate was standardized by protein amount.

Serum leptin measurement

Commercial kit was used to assess serum leptin levels (EMD Millipore Corporation, Darmstadt, Germany), according to the manufacturer's procedure.

Statistical analyses

Data were expressed in mean ± standard error, and differences between groups were decided by Student's *t* test. $P < 0.05$ value indicated statistically significant between the groups.

Table 1. Sequences of primers for real time polymerase chain reaction.

	Forward primer sequences	Reverse primer sequences
SENP2	5' GCT GGC TAA GGT TCT CGG C 3'	5' CTG GGA TCT CAT CAG TGT CCA 3'
ACSL1	5' CTG GTT GCT GCC TGA GCT TG 3'	5' TTG CCC CTT TCA CAC ACA CC 3'
CPT1b	5' AAG TGT AGG ACC AGC CCC GA 3'	5' TGC GGA CTC GTT GGT ACA GG 3'
GAPDH	5' AGG TCG GTG TGA ACG GAT TTG 3'	5' TGT AGA CCA TGT AGT TGA GGT CA 3'
RPS2	5' CTG ACT CCC GAC CTC TGG AAA 3'	5' GAG CCT GGG TCC TCT GAA CA 3'

Results

I. Regulation of SENP2 expression by leptin in skeletal muscle through leptin receptor.

SENP2 expression was increased in soleus muscles but not in gastrocnemius muscles 24h after the injection of leptin *in vivo*.

According to the previous data in our lab, SENP2 expression was increased 12h after leptin treatment and sustained until 24h in C2C12 myotubes which was originated from soleus muscle. I tested whether SENP2 expression is regulated by leptin *in vivo*. After 24 hours of leptin injection, SENP2 mRNA levels were increased significantly in soleus muscles of mice (Figure 4a). However, SENP2 mRNA levels were not influenced by leptin in gastrocnemius muscles (GM) (Figure 4b).

Leptin receptor expression is greater in soleus muscles than other type of muscles.

To determine whether leptin increases SENP2 expression by leptin receptor, I looked for leptin receptor expression in different types of muscles in protein levels. As a result, leptin receptor expression is greater in soleus muscles than gastrocnemius and quadriceps muscles (Figure 5).

SENP2 expression by leptin in db/db mice.

To verify leptin effects on SENP2 expression are through leptin receptor, I investigated the SENP2 mRNA levels in db/db mice (leptin receptor long form deficient) after leptin treatment.

As a result, SENP2 mRNA levels of db/db mice injected with leptin after 24 hours were not significantly different from db/db mice with saline (Fig. 6). This result demonstrated that increase of SENP2 expression by leptin is through the leptin receptor long form.

Leptin effects in soleus muscles of mice through STAT3 phosphorylation.

It is well known that only the leptin receptor long form (LepRb) has STAT3 phosphorylation ability among several leptin receptor isoforms. I tested whether LepR/STAT3 signaling by leptin treatment occurs in soleus muscles. Soleus muscles of C57BL/6 mice were collected and treated with leptin (300ng/ml). By each time point (30min, 1 hour, 2 hour and 4 hour), the soleus muscles were lysed in RIPA lysis buffer with protease and phosphatase inhibitors. And protein levels of the soleus muscles were determined by western blot analysis. As a result, STAT3 phosphorylation levels were increased over 30 minutes until 1 hour of leptin treatment, and gradually decreased over 2 hours to 4 hours (Figure 7). These results indicated that leptin activates leptin receptor and STAT3 phosphorylation in earlier time (over 30 minutes to 1 hour). To summarize the first part, SENP2 expression is increased by leptin through the leptin receptor long form in soleus muscle. Leptin elevates SENP2 expression in soleus muscles but not in gastrocnemius muscles. And leptin receptor is expressed greater in soleus muscles than gastrocnemius muscles. For confirmation, SENP2 expression by leptin was measured in soleus muscles of db/db mice (leptin receptor long form deficient). As a result, SENP2 expression was not increased by leptin. Therefore, leptin elevates SENP2 expression through the leptin receptor in soleus muscles.

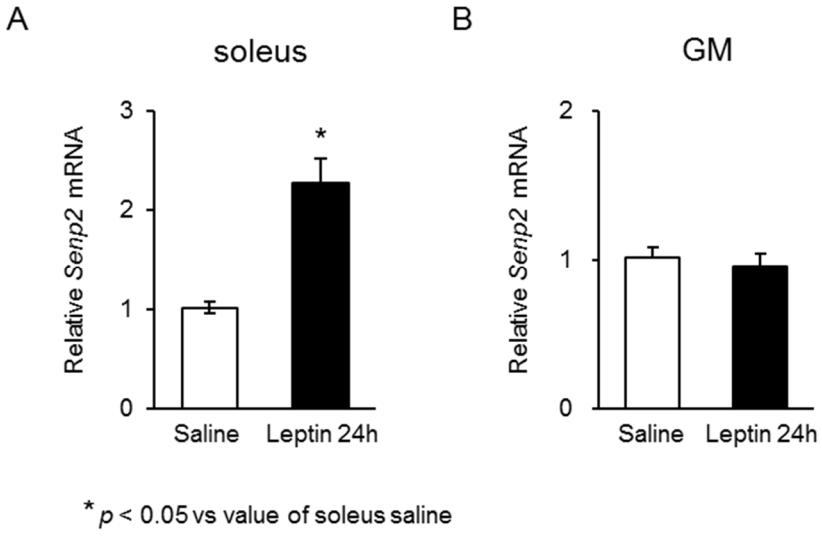

Figure 4. Leptin injection increased SENP2 mRNA levels in soleus but not in gastrocnemius muscle (GM) of C57BL/6 mice..

C57BL/6 mice were injected intraperitoneally with saline (n=7) and leptin (n=8). After 24 hours, RNAs in soleus and gastrocnemius muscle (GM) were extracted, and SENP2 mRNA levels were analyzed by real time PCR. Data represents saline as 1 (standard), and relative values were calculated in a compared group as mean ± standard errors (n=7-8 per group) *p<0.05 vs value of soleus saline

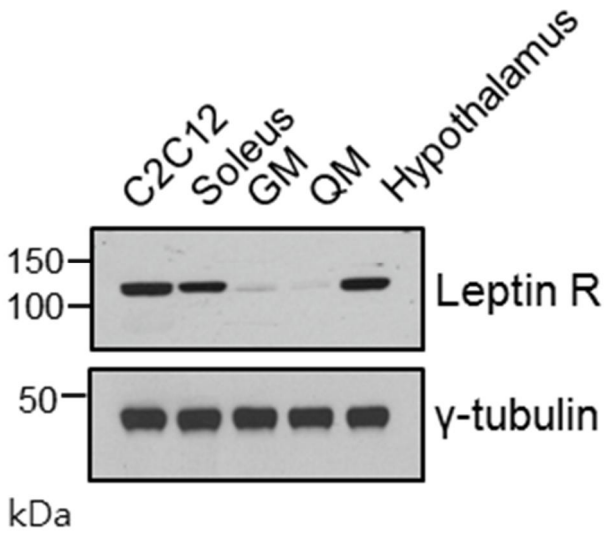

Figure 5. Leptin receptor protein levels in C2C12 myotubes, different types of muscles and hypothalamus.

Leptin receptor protein levels were determined by western blot analysis with leptin receptor antibody in C2C12 cell, soleus muscle, gastrocnemius muscle (GM), quadriceps muscle (QM) and hypothalamus, and standardized by γ-tubulin levels.

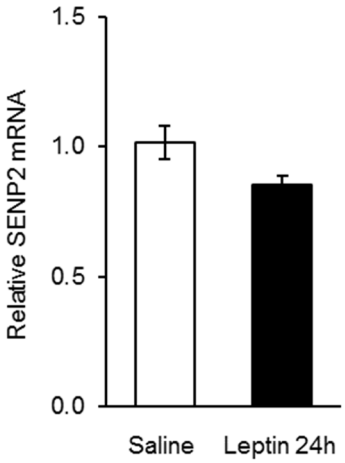

Figure 6. SENP2 expression by leptin in soleus muscles of db/db mice (leptin receptor long form deficient).

The diabetic mouse models, db/db mice with 10 and 12 weeks (n=20) were fasted overnight, and were injected leptin (n=10) in intraperitoneal part while other db/db mice (n=10) with saline. The soleus muscles were collected, and mRNA were measured by real time polymerase chain reaction (RT-PCR). Data represents saline as 1 (standard), and relative values were calculated in a compared group as mean ± standard errors (n= 10 per group)

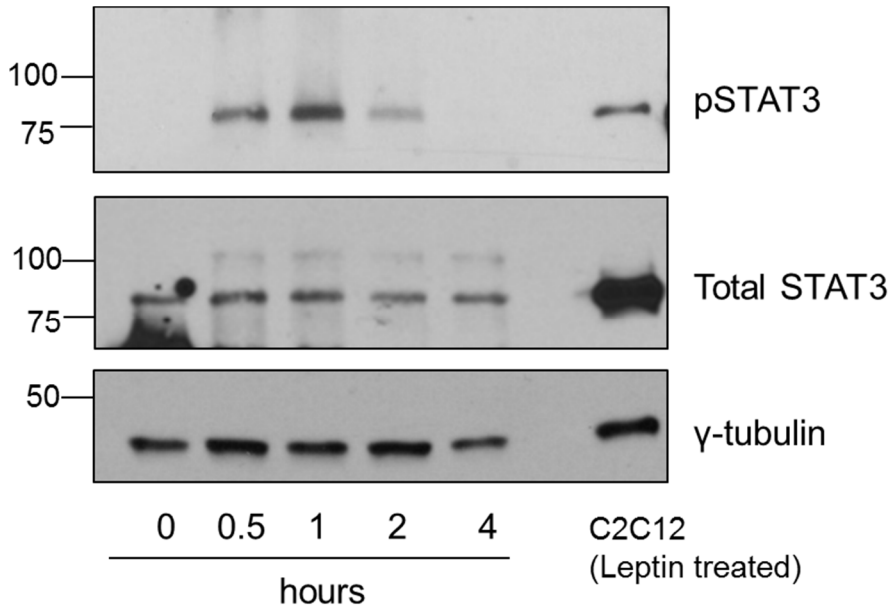

Figure 7. Regulation of STAT3 phosphorylation by leptin treatment in soleus muscles *ex vivo.*

Soleus muscles of C57BL/6 mice were collected, pre-incubated in Krebs Henseleit buffer with 2 mmol/l pyruvate, 8 mmol/l mannitol, and 0.1% BSA (in pH 7.4) for 15 min and incubated in the Krebs Henseleit buffer with leptin (300ng/ml) at time point of 30 min, 1 hour, 2 hour and 4 hour.

The lysates (30ug) were loaded, and standardized by γ-tubulin levels in western blot analysis.

II. Leptin-induced FAO-associated enzyme expression and fatty acid oxidation.

Leptin promoted FAO-associated enzyme expression and fatty acid oxidation in soleus muscles but not in gastrocnemius muscles.

In C2C12 myotubes, leptin increased expression of FAO-associated enzymes, such as *Acsl1* and *Cpt1b* through SENP2. I tested expression levels of FAO-associated enzymes in muscles 24h after leptin injection. Leptin injection enhanced mRNA levels of FAO-associated enzymes (*Acsl1* and *Cpt1b)* specifically in soleus muscles of mice, while there was no definite differences in gastrocnemius muscles (GM) (Figure 8a and 8b). And as FAO-associated enzymes levels were increased by leptin in soleus muscles, fatty acid oxidation was also elevated by leptin in soleus muscles but not in gastrocnemius muscles (Figure 9). Therefore, these results explain leptin only increases FAO-associated enzymes expression and fatty acid oxidation in soleus muscles where SENP2 expression is elevated by leptin.

Leptin does not increase FAO-associated enzymes and fatty acid oxidation in db/db mice.

To verify leptin increases FAO-associated enzymes expression and fatty acid oxidation through the leptin receptor, FAO-associated enzymes (*Acsl1* and *Cpt1b*) mRNA levels were measured in soleus muscles of db/db mice. mRNA levels of *Acs1* and *Cpt1b* were not changed by leptin in soleus muscles of db/db mice (Figure 10). And as FAO-associated enzymes expression was not increased by leptin, fatty acid oxidation was not changed in soleus muscles of db/db mice (Figure 11). According to the results, db/db mice, which are leptin receptor long form deficient, were not able to increase fatty acid oxidation and FAO-associated enzymes levels. Therefore, leptin receptor is very essential to leptin-induced fatty acid oxidation.

Serum leptin levels are increased after 1 hour of leptin injection and restored after 24 hours.

As leptin increased FAO-associated enzymes and fatty acid oxidation, I confirmed whether leptin is infused in body properly. The mice were same with measurement of fatty acid oxidation (in Figure 9 and 11). After 1 hour and 24 hours of the injection, blood was collected. Serum was collected by centrifuge, and serum leptin levels were calculated by commercial kit (EMD Millipore Corporation, Darmstadt, Germany). In C57B/6 (n=5) and dbdb (n=5) with leptin injection groups, serum leptin levels were significantly increased after 1 hour of the injection and restored at baseline (as 24 hours of PBS injection) after that of 24 hours. In addition, serum leptin levels were not changed in C57B/6 (n=5) and dbdb (n=5) with PBS injection after 1 hour and 24 hours (Table 2). Therefore, this result determined infusion of leptin

is correctly performed, and FAO-associated enzymes and fatty acid oxidation were increased by leptin.

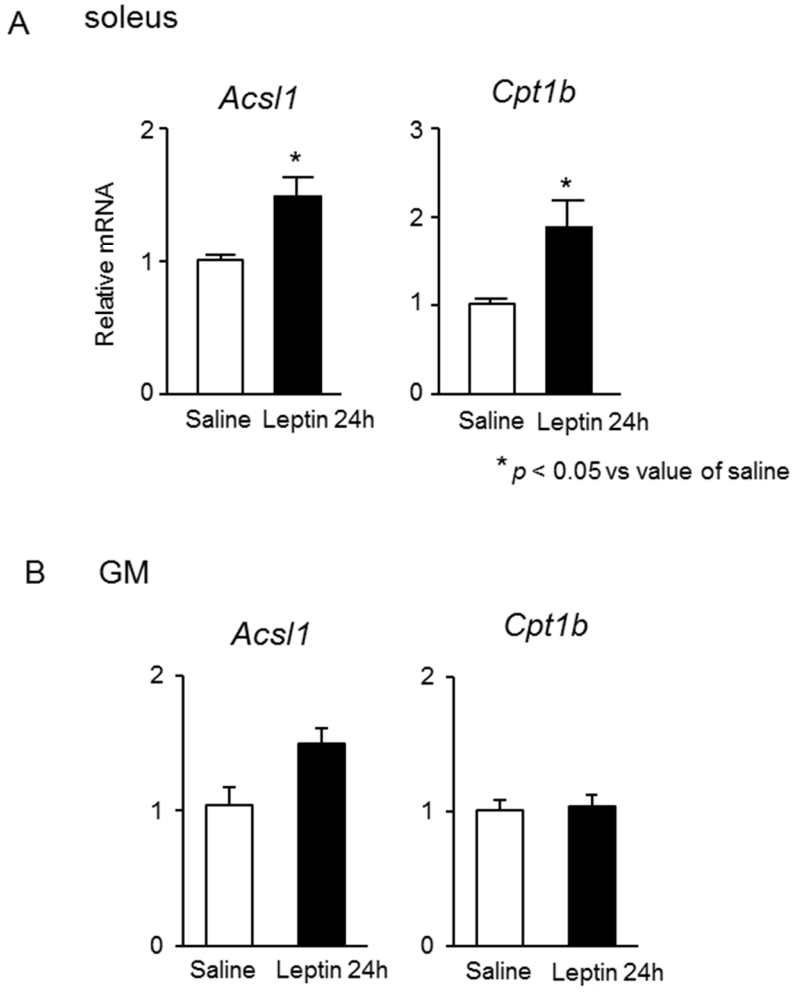

Figure 8. Leptin effect on FAO-associated enzyme expression in soleus and gastrocnemius muscles.

Leptin (3mg/kg, body weight) was infused to C57BL/6 mice by intraperitoneal injection, and soleus and gastrocnemius muscles were collected from mice after 24 hours of the leptin injection. FAO-associated enzyme (*Acsl1* and *Cpt1b*) mRNA levels were measured by real time

polymerase chain reaction (RT-PCR). Data represents saline as 1 (standard), and relative values were calculated in a compared group as mean ± standard errors of two or three independent experiments. *p<0.05 vs value of saline

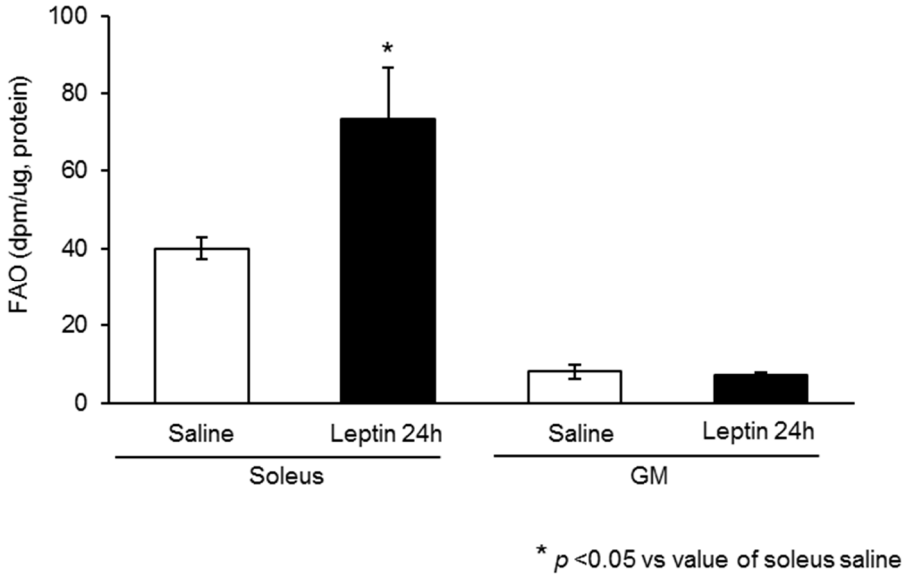

Figure 9. Fatty acid oxidation levels by leptin in soleus and gastrocnemius muscles.

C57BL/6 (12weeks) (n=5) mice were injected with leptin (3mg/kg, body weight) in intraperitoneal part and with saline (n=5). And soleus and gastrocnemius muscles were collected after 24 hours of the injection and homogenized in mitochondria isolation buffer. After homogenization, tissue lysates were with 0.2 mmol/L [1-^{14}C] palmitate for 2 hours in incubation

of 37 °C. And $^{14}CO2$ and ^{14}C-labeled acid-soluble metabolites were measured by a liquid scintillation counter. The fatty acid oxidation levels were standardized by protein amount. Data represents saline as 1 (standard), and relative values were calculated in a compared group as mean ± standard errors (n=5 per group) *p<0.05 vs value of soleus saline

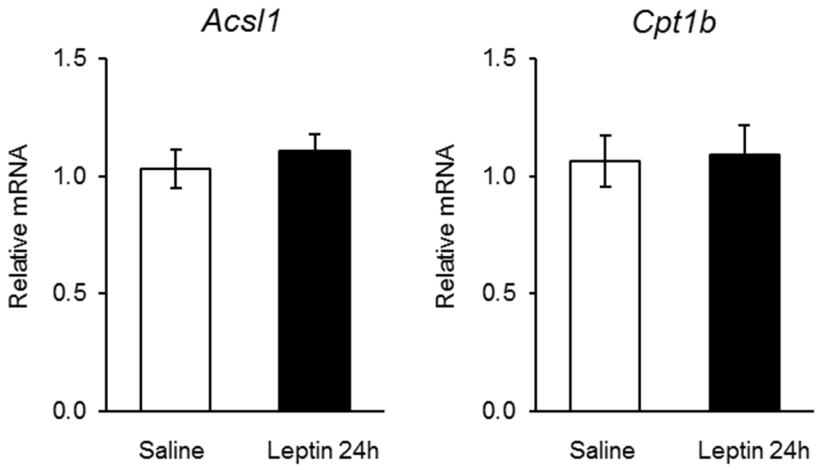

Figure 10. FAO-associated enzyme levels by leptin in soleus muscles of db/db mice.

Leptin was infused in db/db mice (10-12 weeks) by intraperitoneal injection (n=10) and saline was injected (n=10). Soleus muscles were collected after 24 hours of the injection, and FAO-associated enzymes (*Acsl1* and *Cpt1b*) mRNA levels were measured by quantitative polymerase chain reaction. Data represents saline as 1 (standard), and relative values were calculated in a compared group as mean ± standard errors (n= 10 per group)

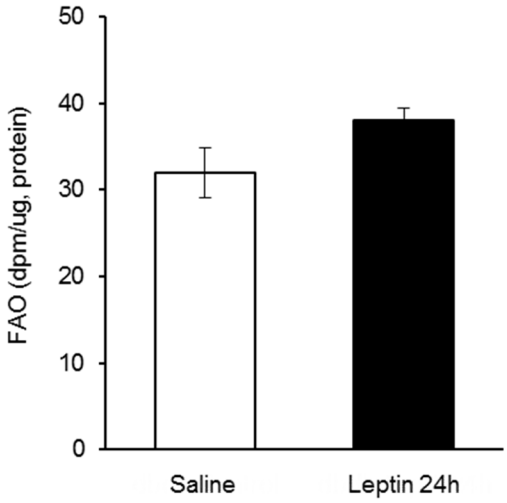

Figure 11. Fatty acid oxidation levels by leptin in soleus muscles of db/db mice.

db/db mice (12 weeks) were fasted overnight, and were injected with leptin (3mg/kg) (n=5) and with saline (n=5). After 24 hour, the soleus muscles of db/db mice were homogenized in MIB buffer (mitochondria isolation buffer) immediately. For measuring fatty acid oxidation, the tissue lysates were used with 0.2 mmol/L [1-^{14}C]palmitate, and incubated 2 hours in 37 °C. And ^{14}CO2 and ^{14}C-labeled were determined by liquid scintillation counter.

ng/mL	C57B/6		*db/db*	
	Vehicle (5)	Leptin (5)	Vehicle (5)	Leptin (5)
1 h	1.44 ± 0.14	1639.15 ± 227.85	48.84 ± 7.21	2205.34 ± 570.63
24 h	0.72 ± 0.13	1.75 ± 0.58	57.72 ± 8.86	65.08 ± 6.07

*16 h after food removal, male mice were injected intraperitoneally with vehicle (PBS) or leptin (3 mg/kg, body weight) and blood was withdrawn at 1 h and 24 h after injection.

*Data are means ± standard deviation of n (number in parenthesis).

TABLE 2. Comparison of serum leptin levels for vehicle- *vs.* leptin-injected male mice

Male C57B/6 (n=10) and dbdb (n=10) mice were fasted overnight (16 hours of food removal), and blood was collected after 1 hour and 24 hours of leptin (3mg/kg, body weight) or PBS injection intraperitoneally (n=5 in each group). By a centrifuge, serum were collected from the blood, and serum leptin levels were measured by commercial kit (EMD Millipore Corporation, Darmstadt, Germany).

Discussion

Increasing fatty acid oxidation by leptin through AMPK pathway is well reported in skeletal muscles [12]. However, our laboratory found another pathway for leptin-induced fatty acid oxidation through SENP2. The leptin-induced fatty acid oxidation through SENP2 is different from AMPK pathway. Leptin increases AMPK phosphorylation, and increases fatty acid oxidation in skeletal muscles before 6 hours while the leptin-induced fatty acid oxidation through SENP2 is occurred at 24 hours after leptin administration.

Leptin elevates fatty acid oxidation through AMPK pathway in C2C12 cells and skeletal muscles by two ways. In skeletal muscles, leptin-induced fatty acid oxidation through AMPK pathway is by direct effect or hypothalamus sympathetic nervous system. By 15 min and 6 hours leptin administration of intravenous (i.v.) injection, leptin activates α2 AMPK activity in soleus and red gastrocnemius muscle but this effect is not shown after 6 hours of i.v. leptin administration in denervation experiment. Therefore, i.v. 6 hours of leptin administration promotes fatty acid oxidation through hypothalamus sympathetic nervous system [12].

In C2C12 cells, α2 AMPK binds to β1 and γ1 subunits, and this complex phosphorylates ACC. In addition, the phosphorylated acetyl CoA carboxylase (ACC) promotes fatty acid oxidation in mitochondria after 6 hours of leptin treatment in cytoplasm. In the other hand, α2 AMPK binds to β2 and γ1 subunits, and fatty acid oxidation is elevated before 1 hour of leptin treatment. Moreover, activated α2 AMPK bound to β1 and γ1 subunits is translocated to nucleus through α2 nuclear localization signal and increases PPARα gene expression (which is one of genes related to fatty acid oxidation) after 6 hours and longer time of leptin treatment [15].

In contrast to the long time mechanism of leptin-induced fatty acid oxidation through α2 AMPK in soleus muscles *in vivo* was demonstrated at 6h, the leptin promotes fatty acid

oxidation through SENP2 after 24 hours of injection *in vivo*. Furthermore, late α2 AMPK mechanism for increasing fatty acid oxidation by 6 hours or longer time of leptin treatment in C2C12 cells *in vitro* occurs in cytoplasm but SENP2 transcription by leptin is processed in nucleus for leptin-induced fatty acid oxidation through SENP2 pathway after 24 hours in previous *in vitro* study of our laboratory.

Several studies are reported for direct or indirect leptin effect in skeletal muscle. As previously described, leptin increases fatty acid oxidation in soleus muscle through AMPK pathway. In addition, leptin also influences glucose metabolism. Leptin (5mg/kg, bodyweight; intraperitoneal injection) promotes glucose utilization and insulin sensitivity of whole body and skeletal muscle especially red type by melanocortin receptor (MCR) activation in ventromedial hypothalamus (VMH), and this signaling pathway is regulated by extracellular signal-regulated kinase (ERK) and its upstream MEK [28]. Leptin also influences skeletal muscle or muscle cells directly. Leptin (0.62nmol/10min) treatment in C2C12 myotubes activate glucose transport and glycogen synthesis through janus kinase 2 (JAK2)/insulin receptor substrate 2 (IRS-2) dependent mechanism. In addition, this study demonstrated JAK2 and IRS-2 tyrosine phosphorylation by leptin in C2C12 myotubes, and suggested tyrosine phosphorylation of JAK2 by leptin may promote IRS-2 tyrosine phosphorylation, and this mechanism may activate phosphatidylinositol-3 (PI) kinase [29]. Although leptin signaling and its effect in skeletal muscle are reported in several studies, leptin effect on skeletal muscle is controversial. Some studies explained that leptin increases glucose uptake and insulin sensitivity while other studies found no changed or contrast results [30]. Therefore, the leptin effect in skeletal muscles is still uncertain, and further studies are required. Leptin receptors are expressed in skeletal muscles but the intensity of leptin receptor expression is different in types of skeletal muscles. Leptin receptor long form is expressed in soleus muscles but not in white gastrocnemius muscles [31]. In this study, I found a different kind of white muscles such as quadriceps muscle also has lower leptin receptor expression compared to soleus muscles.

Leptin binds to leptin receptor and STAT3 phosphorylation is increased [5]. STAT3 phosphorylation by leptin is increased from 30 min to 6 h in hypothalamus (female FVB mice)

[32]. But studies for leptin-induced STAT3 phosphorylation in skeletal muscles is unclear. In this study, increase of STAT3 phosphorylation was determined *ex vivo* because it is difficult to demonstrate STAT3 phosphorylation in skeletal muscle after leptin injection *in vivo*. It may be due to lower (long form) leptin receptor expression in skeletal muscle or dilution and delayed effect of leptin after injection *in vivo* while isolated muscle tissue can be exposed to higher enough concentration of leptin in *ex vivo* study. I expect that increase of STAT3 phosphorylation by leptin can be shown *in vivo* with specific conditions such as exact time points for tissue collection, direct injection into skeletal muscles.

This study has several limitations. Previous studies in our laboratory demonstrated that SENP2 is involved directly in leptin-induced fatty acid oxidation in C2C12 myotubes, however, direct evidence for SENP2 involvement in this signaling pathway was not elucidated in skeletal muscles *in vivo*. In addition, this study did not provide clear distinction between AMPK pathway and SENP2 pathway which can contribute to leptin's effect on FAO in skeletal muscle. Studies of SENP2 muscle-specific knockout mice model might help to provide the answer to these questions.

In summary, leptin increases SENP2 expression through leptin receptor in soleus muscle *in vivo* but not in gastrocnemius muscles (lower leptin receptor expression) of C57BL/6 mice and soleus muscles of db/db mice (leptin long form receptor deficient). In addition, leptin promotes STAT3 phosphorylation in soleus muscles from 30 min to 2 hours *ex vivo*. Leptin elevates FAO-associated enzyme expression such as ACSL1 and CPT1b and fatty acid oxidation in soleus muscles but not in gastrocnemius muscles of C57BL/6 mice and soleus muscles of db/db mice. Therefore, SENP2 may contribute to leptin-induced fatty acid oxidation in soleus muscles *in vivo* through leptin receptor and STAT3.

Conclusion

SENP2 expression was increased by leptin through leptin receptor in soleus muscles *in vivo*. In addition, leptin increased STAT3 phosphorylation in soleus muscles *ex vivo*. FAO-associated enzyme (Acsl1 and Cpt1b) expression and fatty acid oxidation were elevated by leptin through leptin receptor in soleus muscles. Therefore, as leptin-induced fatty acid oxidation through SENP2 in C2C12 myotubes *in vitro*, SENP2 may be involved in delayed effect of leptin-induced fatty acid oxidation in soleus muscles *in vivo*.

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국문초록

렙틴은 지방세포에서 분비되는 아디포카인으로 먹이섭취와 에너지 소비량을 조절한다. 렙틴은 렙틴수용체와 결합하면 JAK2/ signal transducer and activator of transcription 3 (STAT3) 신호전달을 통해 작용한다. 뇌의 시상하부 arcuate 부분에서 pro-opiomelanocortin (POMC) 뇌세포들은 식욕을 없애는 펩타이드인 호르몬을 활성을 하고, POMC/ cocaine and amphetamine-regulated transcript (CART)는 음식섭취를 줄이는데 작용한다고 알려져 있다.

한편 렙틴은 골격근에서 AMP-kinase 활성화를 통해 지방산 산화를 증가시킨다고 알려져 있다. 본 실험실에서는 최근 렙틴이 골격근에서 지방산 산화를 증가시키는데 sumo specific protease 2(SENP2)가 관여하는 것을 근육세포에서 확인한 바 있다. SENP2는 탈수모화에 관여하는 단백질로서 peroxisome proliferator activated receptor gamma와 delta 를 탈수모화시켜 이들의 전사활성을 증가시켜 미토콘드리아에서 지방산 산화를 증가시킨다. 그러나 골격근에 대한 렙틴의 in vivo 효과 대해서 논란이 많아 동물실험을 통해 렙틴의 골격근에서의 지방산 산화에 대한 작용에 SENP2가 관여하는지 연구하고자 하였다.

C57BL/6J 생쥐에게 렙틴 (3mg/kg, 체중)을 복강주사를 통해 주입시키고 24 시간 뒤에 비장근 근육에서 SENP2 메신저 RNA 양이 증가하였고, 렙틴 수용체가 결합된장딴지근 근육에서는 변화가 없는 것을 확인하였다. 또한, 렙틴 긴 형태 수용체

가 결합된 db/db 생쥐 비장근 근육에서도 SENP2 메신저 발현이 차이가 없는 것을 확인하였다. 더 나아가, ex vivo 방법으로 비장근 근육을 렙틴 에 배양할때 STAT3 인산화반응이 30분부터 2시간 사이에 나타나는 것을 확인하였다. 이 결과들을 통하여, 골격근에서 렙틴에 의한 SENP2의 증가가 렙틴 수용체와 STAT3를 통한다고 예측할 수 있다. 더 나아가, 렙틴에 의해 비장근 근육에서 지방산산화에 관여하는 유전자들과 지방산산화가 증가하였지만 장딴지 근육과 db/db 생쥐 비장근 근육에서는 차이가 나지 않는 것을 확인하였다. 이상의 결과를 통하여, 렙틴이 골격근에서 지방산 산화를 증가시키며, 이 과정에 렙틴에 의해 증가되는 SENP2가 중요한 역할을 한다는 것을 동물실험에서도 확인할 수 있었다.

Keywords: SUMO-specific protease 2, 렙틴, 렙틴 수용체, 지방산 산화, 비만

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