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メタデータ	言語: eng
	出版者:
	公開日: 2017-10-03
	キーワード (Ja):
	キーワード (En):
	作成者:
	メールアドレス:
	所属:
URL	http://hdl.handle.net/2297/37595

LECT2 functions as a hepatokine that links obesity to skeletal muscle insulin resistance

Fei Lan[1, 13], Hirofumi Misu[1, 13], Keita Chikamoto[1,2], Hiroaki Takayama[1],Akihiro Kikuchi[1] ,

Kensuke Mohri[1], Noboru Takata[1], Hiroto Hayashi[1], Naoto Matsuzawa-Nagata[1] , Yumie

Takeshita[1], Hiroyo Noda[1], Yukako Matsumoto[1], Tsuguhito Ota[1], Toru Nagano[3], Masatoshi

Nakagen[3], Ken-ichi Miyamoto[4, 5] ,Kanako Takatsuki[6], Toru Seo[6], Kaito Iwayama[7], Kunpei

Tokuyama[7], Seiichi Matsugo[8, 9], Hong Tang[10], Yoshiro Saito[11], Satoshi Yamagoe[12], Shuichi

Kaneko[1], and Toshinari Takamura[1*]

[1]Department of Disease Control and Homeostasis, Kanazawa University Graduate School of

Medical Sciences, Kanazawa, Ishikawa, Japan

[2]Division of Natural System, Graduate School of Natural Science and Technology, Kanazawa

University, Kanazawa, Ishikawa, Japan

[3]Public Central Hospital of Matto Ishikawa, Hakusan, Ishikawa, Japan

[4]Department of Hospital Pharmacy, Kanazawa University Graduate School of Medical

Sciences, Kanazawa, Ishikawa, Japan

[5]Department of Medicinal Informatics, Kanazawa University Graduate School of Medical

Sciences, Kanazawa, Ishikawa, Japan

[6]Merck & Co.Inc.,126E.Lincoln Ave. P.O.Box 2000, Rahway, NJ 07065-0900, USA

[7]Division of Sports Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan

[8]Division of Material Engineering, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan

[9]Institute of Science and Engineering, Faculty of Natural System, Kanazawa University, Kanazawa, Japan

[10]Center of Infectious Diseases, West China Hospital of Sichuan University, Chengdu, China

[11]Department of Medical Life Systems, Faculty of Medical and Life Sciences, Doshisha University, Kyotanabe, Kyoto, Japan

[12]Department of Bioactive Molecules, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan

[13]These authors contributed equally to this work.

ABSTRACT

Recent papers have reported an association between fatty liver disease and systemic insulin resistance in humans, but the causal relationship remains unclear. The liver may contribute to muscle insulin resistance by releasing secretory proteins, termed hepatokines. Here, we demonstrate that leukocyte cell-derived chemotaxin 2 (LECT2), as an energy-sensing hepatokine, is a link between obesity and skeletal muscle insulin resistance. Circulating

LECT2 positively correlated with the severity of both obesity and insulin resistance in humans. LECT2 expression was negatively regulated by starvation-sensing kinase adenosine monophosphate-activated protein kinase (AMPK) in H4IIEC hepatocytes. Genetic deletion of LECT2 in mice increased insulin sensitivity in the skeletal muscle. Treatment with recombinant LECT2 protein impaired insulin signaling via phosphorylation of JNK in C2C12 myocytes. These results demonstrate the involvement of LECT2 in glucose metabolism, and suggest that LECT2 may be a therapeutic target for obesity-associated insulin resistance.

INTRODUCTION

Insulin resistance is a characteristic feature of people with type 2 diabetes (1) and plays a major role in the development of various diseases such as cardiovascular diseases (2) and nonalcoholic steatohepatitis (3; 4). In an insulin-resistant state, impaired insulin action promotes hepatic glucose production and reduces glucose uptake by peripheral tissues. Insulin resistance is commonly observed in obese and overweight people, suggesting a potential role of ectopic fat accumulation in each insulin-target tissue for mediating insulin resistance (5). However, the molecular mechanisms underlying insulin resistance are now known to be influenced by the abnormal secretion of tissue-derived factors, traditionally considered separate from the endocrine system, such as adipokines (6-9), myokines (10; 11), and hepatokines (12-14).

Leukocyte cell-derived chemotaxin 2 (LECT2) is a secretory protein originally identified in the process of screening for a novel neutrophil chemotactic protein (15). LECT2 (in humans encoded by the *LECT2* gene) is expressed preferentially by human adult and fetal liver cells and is secreted into the blood stream (16). The early study using *Lect2*-deficient mice showed that LECT2 negatively regulates the homeostasis of natural killer T cells in the liver (17). More recently, Anson et al. reported that LECT2 exerts anti-inflammatory and tumor-suppressive actions in β-catenin-induced liver tumorigenesis (18). To date, however, the role of LECT2 in the development of obesity and insulin resistance induced by over-nutrition has not yet been established.

We have previously demonstrated that overproduction of liver-derived secretory protein selenoprotein P (SeP) contributes to hyperglycemia in type 2 diabetes by inducing insulin resistance in the liver and skeletal muscle (12). SeP has emerged from comprehensive liver screenings for secretory proteins whose expression levels are correlated with the severity of insulin resistance in patients with type 2 diabetes (12; 19; 20). Based on these findings, we have proposed that, analogous to adipose tissue, the liver may participate in the pathology of type 2 diabetes and insulin resistance, through the production of secretory proteins, termed hepatokines (12). Recently, the other liver-secreted proteins such as fetuin-A (21), angiopoietin-related protein 6 (22), fibroblast growth factor 21 (23), insulin-like growth factors (24), and sex hormone-binding globulin (25) have been reported as hepatokines

involved in glucose metabolism and insulin sensitivity. However, identification of hepatokines involved in fat accumulation was not adequate. In the present study, we have identified LECT2 as a hepatokine whose expression levels were positively correlated with the severity of obesity in humans. Blood levels of LECT2 were also elevated in animal models with obesity. *Lect2*-deficient mice showed an increase of insulin signaling in skeletal muscle. Conversely, treatment with recombinant LECT2 protein impaired insulin signaling in C2C12 myotubes. Our data demonstrate that LECT2 functions as a hepatokine that links obesity to insulin resistance in the skeletal muscle.

RESEARCH DESIGN AND METHODS

Human clinical studies. Liver samples to be analyzed by SAGE were obtained from five patients with type 2 diabetes and five non-diabetic subjects who underwent surgical procedures for malignant tumors, including gastric cancer, gall bladder cancer, and colon cancer. Liver samples to be subjected to DNA chip analysis were obtained from 22 patients with type 2 diabetes and 11 subjects with normal glucose tolerance using ultrasonography-guided biopsy needles. Detailed clinical information about these subjects is presented elsewhere (12; 19).

Serum samples were obtained from 200 participants who went to the Public Central Hospital of Matto, Ishikawa, for a complete physical examination. Following an overnight fast, venous blood samples were taken from each patient. Serum levels of LECT2 were measured by an

Ab-Match ASSEMBLY Human LECT2 kit (MBL) (26; 27).

The HOMA-IR was calculated using the following formula: HOMA-IR = [fasting insulin (µU/ml) x fasting plasma glucose (mmol/L)] / 22.5 (28). All patients provided written informed consent for participation in this study. All experimental protocols were approved by the relevant ethics committees in our institution and Matto Ishikawa Central Hospital, and were conducted in accordance with the Declaration of Helsinki.

Animals. Eight-week-old c57BL/6J mice were obtained from Sankyo Lab Service (Tokyo, Japan). All animals were housed in a 12-h light/dark cycle and allowed free access to food and water. The 60% high fat diet (D12492) was purchased from Research Diets (New Brunswick, NJ).

Purification of LECT2. Murine LECT2 was expressed and purified as previously described (29) with minor modifications. Briefly, LECT2 was stably expressed in CHO cells. The protein was purified from the cultured medium by ion exchange chromatography. Subsequently, the fractions containing LECT2 were applied to a mono S column (GE Healthcare) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) and eluted with a linear gradient of 150-350 mM NaCl.

***Lect2* knockout mice.** *Lect2* knockout mice were produced by homologous recombination using genomic DNA cloned from an Sv-129 P1 library, as described previously (17). All experimental mice were generated from intercross between heterozygous mice, and groups

were divided from littermates. As female *Lect2* knockout mice had inconsistent phenotypes, only male mice were used in all the experiments except the experiment of the starvation.

Materials. H4IIEC and C2C12 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Human recombinant insulin was purchased from Sigma Aldrich (St. Louis, MO). Rabbit anti-phospho-Akt (Ser473) monoclonal antibody, rabbit anti-total Akt polyclonal antibody, rabbit anti-phospho-AMPK (Thr172) monoclonal antibody, rabbit anti- AMPKα antibody, rabbit anti-phospho-JNK (Thr183/Try185), rabbit anti-JNK, rabbit anti-BiP antibody, rabbit anti-phospho-eIF2α (Ser51) antibody, rabbit anti-NF-κB p65 antibody, rabbit anti-phospho-IKKαβ (Ser176/180) antibody, rabbit anti-IKKα antibody, and rabbit anti-phospho-IκBα(Ser32) antibody were purchased from Cell Signaling (Danvers, MA). Rabbit anti-leukocyte cell-derived chemotaxin 2 polyclonal antibody (sc-99036) and rabbit anti-GAPDH polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Transient transfection experiment. C2C12 myoblasts were grown in 12-well multi-plates When 30-50% confluency was reached, cells were transfected with the Fugene 6 Transfection Reagent (Roche) with a 1 μg control or with mLect2 expression plasmid DNA per well. After 24 h of transfection, the medium was replaced with DMEM containing 10% FBS. 24 h later, when the cells reached to 100% confluency, the cells were differentiated into myotubes with DMEM containing 2% horse serum for 24-48 h. Then, the cells were stimulated with 100

ng/ml human recombinant insulin for 15 min.

siRNA transfection in C2C12 myoblasts. C2C12 myoblasts were transiently transfected with a total of 15 nM of siRNA duplex oligonucleotides using Lipofectamine™ RNAiMAX (Invitrogen), using the reverse-transfection method according to the manufacturer's instructions. A JNK1-specific siRNA with the following sequence was synthesized by Thermo Scientific: 5'- GGAAAGAACUGAUAUACAA -3' (sense). A JNK2-specific siRNA with the following sequence was synthesized by Thermo Scientific: 5'- GGAAAGAGCUAAUUUACAA -3' (sense). Negative control siRNA was purchased from Thermo Scientific. Two days after transfection, cells were pretreated with LECT2 protein, followed by stimulation with 100 ng/ml of human recombinant insulin for 15 min.

RNA isolation, cDNA synthesis, and real-time PCR analysis. Total RNA was isolated from cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich). Total RNA was isolated from mouse skeletal muscle and heart using RNeasy® Fibrous Tissue Mini Kit (Qiagen). Total RNA was isolated from white adipose tissue using RNeasy® Lipid Tissue Mini Kit (Qiagen). RNA concentrations were measured by a NanoDropR ND-1000 spectrophotometer (NanoDrop Technology). cDNA was synthesized from 100 ng of total RNA using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed by using TaqMan® Gene Expression Assays (Applied Biosystems). Primer sets and TaqMan® probes were proprietary to Applied Biosystems

(Assays-on-Demand™ Gene Expression Products). To control for variation in the amount of

DNA available for PCR, target gene expression in each sample was normalized relative to the

expression of an endogenous control (18srRNA or GAPDH) (TaqMan® Control Reagent Kit;

Applied Biosystems).

Treatment with recombinant LECT2 protein in C2C12 myotubes. C2C12 myoblasts were

grown in 24-well multi-plates; after 100% confluency was reached, cells were differentiated

into myotubes by culturing in DMEM supplemented with 2% horse serum for 42 h. C2C12

myotubes were serum-starved and incubated in DMEM for 6 h and then treated with LECT2

recombinant protein for various times in the absence of serum. Following treatment with

LECT2 recombinant protein, cells were stimulated with 100 ng/ml human recombinant

insulin for 15 min.

Western blot studies in C2C12 myotubes. After the inulin stimulation, the cells were then

washed in ice-cold PBS, frozen in liquid nitrogen and lysed at 4°C in 1X RIPA lysis buffer

(Upstat Biotechnology) containing Complete Mini EDTA-free cocktail tablet

(Roche-diagnostics) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets

(Roche-diagnostics). Lysates were then centrifuged to remove insoluble material. Samples

were sonicated with BIORUPTOR® (Cosmo Bio, Tokyo, Japan). Whole-cell lysates were

then separated by 5–20% SDS-PAGE gels and were transferred to polyvinylidiene (PVDF)

fluoride membranes, using an iBlot® gel transfer system (Invitrogen). Membranes were

blocked in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat milk (pH 7.5) or 5% PhosphoBLOCKER™ reagent (Cell Biolabs, Inc) for 1 h at 24°C. They were then probed with antibodies for 16 h at 4°C. Afterward, membranes were washed in a buffer containing 50 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5 and then incubated with anti-rabbit IgG HRP-linked antibody (Cell Signaling) for 1 h at 24°C. Protein signals were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd.). Densitometric analysis of blotted membranes was performed using ImageJ software (NIH).

Glucose or insulin tolerance tests in mice. In preparation for glucose tolerance testing, mice were fasted for 12 h. After fasting, glucose was administered intraperitoneally, and blood glucose levels were measured at 0, 30, 60, 90 and 120 min. For insulin tolerance testing, mice were fasted for 4 h. After fasting, insulin was administered intraperitoneally, and blood glucose levels were measured. Blood glucose levels were determined by the glucose-oxidase method using Glucocard (Aventis Pharma, Tokyo, Japan). The measurable levels of blood glucose by Glucocard range from 20 to 600 mg/dl. Because HFD-fed mice are much insulin resistant compared with standard diet-fed mice, lower doses of glucose were injected in glucose tolerance testing, as indicated in the figure legend, to avoid the elevation of blood glucose levels to more than 600 mg/dl. Additionally, more doses of insulin were injected to HFD-fed mice in insulin tolerance testing, as indicated in the figure legend, to decrease blood

glucose levels sufficiently.

Western blot studies in mice. After 12 h fasting, mice were anesthetized by intraperitoneal administration of sodium pentobarbital. Then, insulin (1 units/kg body weight) or PBS (vehicle) was injected through vena cava. 10 minutes later, hind limb muscles, liver and epididymal white adipose tissue were removed and immediately frozen in liquid nitrogen. Tissue samples were homogenized using a Polytron homogenizer running at half-maximal speed (15,000 rpm) for 1 min on ice in 1 mL of 1X RIPA lysis buffer (Upstat Biotechnology) containing a Complete Mini EDTA-free cocktail tablet (Roche-diagnostics) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche-diagnostics). Tissue lysates were solubilized by continuous stirring for 1 h at 4°C and centrifuged for 15 min at 14,000 rpm. Protein samples were separated by 5–20% SDS-PAGE gels and were transferred to PVDF membranes. Serine and tyrosine phosphorylation of specific target proteins was analyzed by Western blotting.

Hyperinsulinemic–euglycemic clamp studies in mice. Clamp studies were performed, as described previously (12; 30), with slight modifications. Briefly, 2 days before the study, an infusion catheter was inserted into the right jugular vein of 13-week-old male C57BL6J wild type and *Lect2*-deficient mice under general anesthesia induced using sodium pentobarbital. Before insulin infusion, mice were fasted for 6 h. Clamp studies were performed on conscious and unrestrained animals. Insulin (Novolin R; Novo Nordisk, Denmark) was

continuously infused at a rate of 5.0 mU/kg/min, and the blood glucose concentration (monitored every 5 min) was maintained at 100 mg/dl through the administration of glucose (50%, enriched to approximately 20% with [6,6-2H2]glucose; Sigma) for 120 min. Blood was sampled through tail-tip bleeds at 0, 90, 105, and 120 min for the purpose of determining the rate of glucose disappearance (Rd). Rd values were calculated according to non-steady-state equations, and endogenous glucose production (EGP) was calculated as the difference between the Rd and the exogenous glucose infusion rates (GIR) (30).

Exercise tolerance test in mice. Ten-week-old male C57BL 6/J wild type and *Lect2*-deficient mice were set in a running machine. After 5 min warming up running and 5 min rest, mice started running at 11.2 m/min on 0% incline. Running speed was increased every 5 min till the mice reached exhaustion, defined as when the mouse stopped running for 10 seconds on the electric tubes.

Acute exercise experiment in mice. Eight week-old male C57BL 6/J mice were randomly divided into 2 groups, the exercise group and the rest group. All the mice in each group were warm up for 10 min at 12.6 m/min on 5% incline. After 3 hours fasting, blood was sampled through tail-tip bleeds. Mice in the exercise group were set in a running machine and started running at 12.6 m/min on 5% incline. Mice were allowed to have a 5 min rest for every 30 min running. Meanwhile, the mice in the rest group were continually fasted. After 3 hours running or resting, blood was sampled again through tail-tip bleeds. Then, the mice were

anesthetized and sacrificed to allow isolation of the liver tissue.

Starvation experiment in mice. 20 weeks old female C57BL 6/J wild type and *Lect2*-deficient mice were starved for totally 60 hours with water supplied. Body weight was measured and blood was sampled through tail-tip bleeds at 12hour, 24hour and 36 hour after starvation. 60 hours later, mice were injected with insulin intraperitoneally at the concentration of 10 units/kg body weight. Fifteen minutes later, mice were anesthetized and sacrificed to allow isolation of femoral muscle.

Blood samples assays in mice. Serum levels of Lect2 were measured by Ab-Match ASSEMBLY Mouse LECT2 kit (MBL). Serum levels of insulin were determined using a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan), according to the manufacturers' instructions.

Adenovirus-mediated gene transfer in H4IIEC hepatocytes. H4IIEC hepatocytes were grown to 90% confluence in 24-well multi-plates. Cells were infected with adenoviruses encoding dominant negative α1 and α2 AMPK, constitutively-active AMPK, or LacZ for 4 h (8.9 X 106 PFU/well) (31). We expressed α1 and α2 dominant negative AMPK simultaneously to maximize the effect on AMPK activity. After removing the adenoviruses, the cells were incubated with DMEM for 24 h. Then, RNA was isolated from the cells by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich).

Indirect calorimetry. Mice were housed in standard metabolic cages for 24 h. An indirect

calorimetry system (Oxymax Equal Flow System, Columbus Instruments, Columbus, U.S.A.) was used, in conjunction with the computer-assisted data acquisition program Chart5.2 (AD Instruments, Sydney, Australia), to measure and record oxygen consumption and carbon dioxide production at 5-min intervals. Heat generation was calculated per weight (kcal/kg/h).

Measurement of hepatic triglyceride content in mice.

Frozen liver tissue was homogenized in 2 ml ice-cold isopropanol after weight measurement. After 10min incubation with shaking at room temperature, the samples were centrifuged at 3000 rpm for 10 min and supernatant 1 ml was transferred. Triglyceride content in each sample was measured by using commercial Triglyceride E-test WAKO kit (Wako Pure Chemical Industries, Osaka, Japan). Results were normalized to weight of each liver sample.

Statistical analyses. All data were analyzed using the Japanese Windows Edition of the Statistical Package for Social Science (SPSS) Version 21.0. Numeric values are reported as the mean ± SEM. Differences between two groups were assessed using unpaired two-tailed t-tests. Data involving more than two groups were assessed by analysis of variance (ANOVA). Glucose and insulin tolerance tests were examined using repeated measures ANOVA.

RESULTS

Identification of a hepatic secretory protein involved in obesity

To identify hepatokines involved in the pathophysiology observed in obesity, we performed liver biopsies in humans and conducted a comprehensive analysis of gene expression profile, as we previously described (12; 19; 32; 33). We obtained ultrasonography-guided percutaneous needle liver biopsies from 10 people with type 2 diabetes and seven normal subjects. We subjected them to DNA chip analysis to identify genes whose hepatic expression was significantly correlated with body mass index (BMI) (Table S1). As a result, we found a positive correlation between hepatic *LECT2* messenger RNA (mRNA) levels and BMI, indicating that elevated hepatic *LECT2* mRNA levels were associated with obesity.

Circulating LECT2 levels correlate with adiposity and insulin resistance in humans.

To characterize the role of LECT2 in humans, we measured serum LECT2 levels in participants who visited the hospital for a complete physical examination. (Table S2), using enzyme-linked immunosorbent assays (26; 27). We found a significant positive correlation between serum LECT2 levels and BMI and waist circumference (Fig. 1A and 1B). LECT2 levels also showed a significant correlation with the homeostasis model assessment of insulin resistance (HOMA-R) positively and with insulin sensitivity indices (Matsuda index) negatively (Fig. 1C and 1D). In addition, serum levels of LECT2 positively correlated with those of selenoprotein P, an already-reported hepatokine that induces insulin resistance (Fig. 1E) (12). Moreover, LECT2 showed a correlation with levels of both hemoglobin A1c (HbA1c) and systolic blood pressure (Fig. 1F and 1G), both of which were reported to be

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associated with insulin resistance (34; 35). These results indicate that serum levels of LECT2 are positively associated with both adiposity and the severity of insulin resistance in humans.

AMPK negatively regulates LECT2 expression in hepatocytes

To confirm the elevation of LECT2 in animal models with obesity, we fed C57BL6J mice with a high fat diet (HFD) for 8 weeks (Fig. 2A-2F). HFD increased body weight time-dependently (Fig. 2A), and tended to increase triglyceride contents in the liver (Fig. 2B). Hematoxylin and Eosin staining showed mild steatosis in the mice fed HFD (Fig.2C). Gene expression for *Lect2* was elevated in the livers of the mice fed HFD, in accordance with the steatosis-associated genes such as *Fasn* and *Srebp1c* (Fig. 2D). Serum levels of LECT2 showed a sustained increase since a week after the beginning of HFD (Fig. 2E). Additionally, we confirmed that HFD even for a week resulted in an increase of serum levels of insulin and a decrease of insulin-stimulated Akt phosphorylation in the skeletal muscle in C57BL6J mice (Supplementary Fig. 1). Importantly, the livers from mice fed a HFD for 8 weeks showed a decrease of phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) (Fig. 2F), the energy-depletion-sensing kinase that phosphorylates a variety of energy-associated enzymes and functions as a metabolic regulator that promotes insulin sensitivity (36). Since a HFD for a short period increased LECT2 concentrations, we then examined the effects of feeding on blood LECT2 levels. LECT2 levels were elevated in blood obtained from fed C57BL6J mice, compared with samples from the fasting mice (Fig.

2G). Moreover, AMPK phosphorylation decreased in the livers from the mice that had been

fed (Fig. 2H). Since LECT2 expression was inversely correlated with AMPK

phosphorylation in the liver, we hypothesized that AMPK negatively regulates LECT2

production in the hepatocytes. Exercise is reported to increase phosphorylation and activity of

AMPK not only in the skeletal muscle but also in the liver in relation to the intrahepatic

elevation of AMP levels (37; 38). Thus, we examined the actions of aerobic exercise on

LECT2 expression in the liver. C57BL6J mice were loaded onto a running treadmill for a

total of 3 h. Exercise decreased levels of gene expression and protein for LECT2 in the liver

(Fig. 2I and 2J). Aerobic exercise for 3 h, but not resting, significantly reduced serum levels

of LECT2 (Fig. 2K). Percent changes from baseline showed that the reduction of serum

LECT2 in the exercise group was significantly larger than that in the rest group (FIG. 2K). In

addition, aerobic exercise increased AMPK phosphorylation in the liver (Fig. 2L). To

determine whether AMPK suppresses LECT2 expression, we transfected H4IIEC hepatocytes

with adenoviruses either encoding constitutively active (CA-) or dominant-negative (DN-)

AMPK. First, we found that transfection with CA-AMPK significantly decreased mRNA

levels for *Lect2* in H4IIEC hepatocytes, similarly to those for *G6Pc* that encodes key

gluconeogenic enzyme glucose-6 phosphatase already-known to be suppressed by AMPK

(39) (Fig. 2M). In contrast, transfection with DN-AMPK increased *Lect2* gene expression

(Fig. 2N). These results indicate that AMPK negatively regulates LECT2 production in the hepatocytes.

Lect2 deletion increases muscle insulin sensitivity in mice

Next, we examined the role of LECT2 in the development of insulin resistance in mice. We found that gene expression for _Lect2_ in the liver was overwhelmingly dominant compared with that in the other tissues in mice (Fig. 3A). This result suggests that the contribution of the other tissues except the liver on the circulating levels of LECT2 is very small or negligible in mice. Hence, we used systemic knockout mice of LECT2 in the following experiments, although the animal models of liver-specific down-regulation for LECT2 might be more suitable. We confirmed that serum LECT2 was undetectable in _Lect2_-deficient mice by using ELISA (Fig. 3B). Body weight, food intake, and resting heat production were unaffected by _Lect2_ knockout (Fig. 3C-3E). However, the treadmill running challenge revealed that physical-exercise-assessed muscle endurance was significantly higher in _Lect2$^{-/-}$_ mice (Fig. 3F and 3G). A glucose or insulin loading test revealed that _Lect2$^{-/-}$_ mice showed lower blood glucose levels after glucose or insulin injection (Fig. 3H and 3I). _Lect2$^{-/-}$_ mice exhibited an increase in insulin-stimulated Akt phosphorylation in skeletal muscle (Fig. 3J and 3K), but not in the liver or adipose tissue (Supplementary Fig. 2A and 2B). Furthermore, JNK phosphorylation was unchanged in the liver and adipose tissue of these knockout mice (Supplementary Fig. 2C and 2D). Consistent with the results of insulin signaling,

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hyperinsulinemic-euglycemic clamp studies showed that the glucose infusion rate and peripheral glucose disposal were increased, whereas endogenous glucose production was unaffected by *Lect2* deletion (Fig. 3L, and Supplementary Fig. 3). In addition, expression of the genes involved in mitochondria and myogenesis such as *UCP3*, *Myh*1, *Myh2*, and *Ppard*, were upregulated in the muscle of *Lect2*$^{-/-}$ mice (Fig. 3M). These results indicate that a *Lect2* deletion increases insulin sensitivity in the skeletal muscle in mice.

***Lect2* deletion attenuates muscle insulin resistance in dietary obese mice**

To further elucidate the role of LECT2 in the development of obesity-associated insulin resistance, we fed *Lect2*-deficient mice a HFD. HFD-induced body weight gain was smaller in *Lect2*-deficient mice compared with wild-type animals (Fig. 4A). To examine the mechanism by which *Lect2*-deficient mice were less obese following HFD feeding, we measured food intake and heat production in *Lect2*-deficient mice fed a HFD for only a week, when the body weight was comparable between the wild-type and knockout mice (Supplementary Fig. 4A). Food intake was unaffected (Supplementary Fig. 4B), but heat production as measured by O_2 consumption was significantly increased in *Lect2*-deficient mice fed HFD (Supplementary Fig. 4C) in both light and dark phases (Supplementary Fig. 4D and 4E). 11 weeks later after HDF feeding, serum levels of insulin and blood levels of glucose decreased in these knockout mice (Fig. 4B and 4C). A glucose or insulin loading test revealed that *Lect2*-knockout mice showed lower blood glucose levels after glucose or insulin

injection (Fig. 4D and 4E). Consistent with the result of the insulin loading test, Western

blotting revealed that insulin-stimulated Akt phosphorylation increased in skeletal muscle of

these knockout animals (Fig. 4F and 4G). In contrast, JNK phosphorylation significantly

decreased in skeletal muscle of *Lect2*-deficient mice (Fig. 4H and 4I). Furthermore, we

examined muscle insulin signaling in *Lect2*-deficient mice fed **a** HFD for only 2 weeks, when

the body weight was comparable between wild-type and the knockout mice (Supplementary

Fig. 5A, 5B, and 5C). Insulin-stimulated Akt phosphorylation was significantly increased in

the muscle of *Lect2*-deficient mice under conditions of HFD for 2 weeks (Supplementary Fig.

5D and 5E). These results indicate that *Lect2* deletion reduces muscle insulin resistance in

dietary obese mice.

Starvation abolishes the insulin sensitive phenotype in *Lect2*-deficient mice

Next, to elucidate the role of LECT2 in a condition of severe undernutrition, we starved

Lect2-deficient mice for 60 hours. Starvation decreased body weight and blood glucose levels

time-dependently, whereas there was no significance between wild-type and *Lect2*-deficient

mice (Fig. 5A and 5B). Consistent with the changes of body weight, serum levels of LECT2

in wild-type animals significantly decreased during the starvation (Fig. 5C). Before the

starvation, serum levels of insulin in LECT2 knockout mice were lower compared with

wild-type mice (Fig. 5D). However, the starvation reduced insulin levels to the extent to

which the difference abolished between the two groups (Fig. 5D). Insulin-stimulated Akt

phosphorylation in the skeletal muscle also showed no difference between the two groups after the starvation for 60 hours (Fig. 5E). These results indicate that starvation abolishes the insulin-sensitive phenotype in *Lect2*-deficient mice.

LECT2 impairs insulin signaling by activating JNK in C2C12 myotubes

Firstly, to examine the effect of LECT2 on insulin signaling *in vitro*, we transfected C2C12 myocytes with a plasmid expression vector encoding mouse LECT2. Expression of endogenous LECT2 was negligible in C2C12 myocytes transfected with a negative control vector (Fig. 6A and 6B). We confirmed that C2C12 myotubes transfected with the LECT2 expression vector expressed *Lect2* mRNA and released LECT2 protein into culture medium (Fig. 6A and 6B). LECT2 transfection suppressed myotube differentiation in C2C12 cells (Fig. 6C). The cells transfected with the LECT2 vector showed a decrease in insulin-stimulated Akt phosphorylation (Fig. 6D) and an increase in basal c-Jun-N-terminal kinase (JNK) phosphorylation (Fig. 6E).

To further confirm the acute action of LECT2 on insulin signaling, we treated C2C12 myotubes with recombinant LECT2 protein at nearly physiological concentrations. Treatment with 400 ng/ml of LECT2 protein for 3 h decreased insulin-stimulated Akt phosphorylation (Fig. 6F). In addition, treatment with LECT2 protein for 30-60 min increased JNK phosphorylation transiently in C2C12 myotubes (Fig. 6G). LECT2-induced JNK phosphorylation was observed to occur in a concentration-dependent manner (Fig. 6H). To

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determine whether JNK pathway mediates LECT2-induced insulin resistance, we transfected

C2C12 myoblasts with siRNAs specific for JNK1 and JNK2. Because knockdown of JNK is

known to alter the myotube-differentiation in C2C12 myotubes (40), we used undifferentiated

C2C12 myoblasts to purely assess the action of LECT2 on insulin signal transduction in the

following experiments. Double knockdown of JNK1 and JNK2 rescued the cells from the

inhibitory effects of LECT2 on insulin signaling (Fig. 6I). Inflammatory signals and

endoplasmic reticulum stress are known to be the powerful inducers of JNK (41). However,

the markers of neither inflammation nor endoplasmic reticulum stress were changed in

C2C12 myotubes overexpressed with LECT2 and in the skeletal muscle of LECT2 knockout

mice (Supplementary FIG. 4). These *in vitro* experiments indicate that, at nearly

physiological concentrations, LECT2 impairs insulin signal transduction by activating JNK in

C2C12 myocytes.

DISCUSSION

Our research reveals that the overproduction of hepatokine LECT2 contributes to the

development of muscle insulin resistance in obesity (Fig. 7). Recently, growing evidence

suggests a central role for fatty liver disease in the development of insulin resistance in

obesity (4; 42). Kotronen et al. have reported that intrahepatocellular rather than

intramyocellular fat associates with hyperinsulinemia independently of obesity in

non-diabetic men (43). Fabbrini et al. have revealed that intrahepatic triglyceride, but not visceral adipose tissue, is a better marker of multiorgan insulin resistance associated with obesity (44). D'Adamo et al. have shown that obese adolescents with high hepatic fat content show lower whole-body insulin sensitivity independently of visceral fat and intramyocellular lipid content (45). These papers indicate a strong correlation between fatty liver and muscle insulin resistance in humans, but it was still unknown whether fatty liver disease directly causes muscle insulin resistance in obesity. The liver is a major site for the production of bioactive secretory proteins, termed hepatokines (12; 19). Many lines of evidence have reported that the dysregulation of hepatokine production such as selenoprotein P or fetuin A is involved in the development of systemic insulin resistance (12; 13; 46; 47). The current study demonstrates a previously-unrecognized role for LECT2 in glucose metabolism and suggests that LECT2 is a strong candidate to explain a mechanism by which the fatty liver leads to whole-body insulin resistance in obesity.

The energy-depletion-sensing kinase AMPK functions as a metabolic sensor that promotes insulin sensitivity (36). Exercise is known to increase phosphorylation and activity of AMPK in skeletal muscle. Early reports have shown that exercise-induced AMPK phosphorylation is also observed in the liver (37; 38). On the other hand, a high fat diet is reported to decrease AMPK phosphorylation in the liver, probably due to excessive accumulation of energy (48; 49). Negative regulation of LECT2 by energy-depletion-sensing kinase AMPK supports the

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concept that LECT2 functions as an over-nutrition-sensing hepatokine. One limitation of this

study is that the molecular mechanism by which AMPK reduces *LECT2* expression is still

unknown. Additional studies are needed to determine the transcriptional factors that

negatively regulate *LECT2* expression downstream of AMPK pathway.

JNK is a mitogen-activated protein kinase that is activated by various stimuli, including

cytokines, reactive oxygen species, endoplasmic reticulum stress, and metabolic changes (41).

JNK plays a major role in the development of insulin resistance induced by high fat diet, by

phosphorylating insulin receptor substrates at specific serine and threonine residues (50; 51).

More recently, several studies suggest a role for JNK in the development of insulin resistance

in skeletal muscle, as well as in the liver or adipose tissue. Ferreira et al. reported an increase

of JNK phosphorylation and a decrease of insulin-stimulated Akt phosphorylation in the

skeletal muscle from patients with non-alcoholic steatohepatitis (52). Henstridge et al.

showed that muscle-specific overproduction of constitutively active JNK induces muscle

insulin resistance in mice (53). Conversely, Sabio et al. revealed that muscle-specific JNK

knockout mice exhibit improved insulin sensitivity in skeletal muscle (54). Hence,

overproduction of LECT2 in the liver may contribute, at least partly, to JNK phosphorylation

and the subsequent insulin resistance observed in the skeletal muscle of patients with obesity.

However, the mechanism by which LECT2 increases JNK phosphorylation remains

unresolved. Our results suggest that LECT2-induced JNK activation in cultured myocytes is

independent of inflammation or endoplasmic reticulum stress (Supplementary Fig. 4). Identification of the LECT2 receptor and characterization of its downstream signaling will provide insights into the involvement of LECT2 in JNK phosphorylation.

We show that overexpression of LECT2 does not alter the inflammatory response in cultured myotubes. Early reports suggest that LECT2 exerts different effects on inflammation depending on various pathological conditions. Inflammation observed in autoimmune disorders such as collagen antibody-induced arthritis or concanavalin A-induced hepatitis is reported to be suppressed by LECT2 (17; 55). LECT2 also attenuates β-catenin-induced inflammation associated with hepatocellular carcinoma in mouse models (18). On the other hand, a more recent report showed that LECT2 activates lipopolysaccharide-stimulated macrophages via the CD209a receptor, resulting in an improvement of survival prognosis in mice with bacterial sepsis (56). Because we found no gene expression levels of CD209a in C2C12 myotubes in Realtime PCR experiments (data not shown), LECT2-induced insulin resistance in cultured myocytes is likely to be independent of inflammatory response via the CD209a receptor. However, it is unknown whether LECT2 affects macrophages observed in the adipose tissue of obesity. The actions of LECT2 on low grade inflammation seen in obesity are now under investigation.

Interestingly, although LECT2 knockout mice showed an increase of insulin signaling in the skeletal muscle when fed HFD or regular chow, this increase was abolished after starvation

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for 60 hours. Serum levels of LECT2 were increased by HFD (FIG. 2E), whereas they were decreased by starvation in wild-type mice (FIG. 5C). Hence, it seems most likely that the difference in serum LECT2 levels between wild-type and the knockout mice was enhanced by HFD, whereas it was reduced by starvation. The abolishment of the insulin-sensitive phenotypes in LECT2 knockout mice after starvation may be explained by reduction of the difference in serum LECT2 levels. These results suggest that LECT2 plays a major role in the regulation of insulin sensitivity in the over-nutritional conditions, but not in the under-nutritional ones.

Our data reveal that 60% HFD for 1 week resulted in a decrease of insulin signaling of the skeletal muscle and an increase of circulating levels of LECT2 in C57BL6 mice concurrently. A previous clinical report showed that overfeeding and inactivity for only 3 days impaired insulin sensitivity in healthy young men (57). Impairment of insulin sensitivity occurred before changes in body composition such as total fat mass and visceral fat area. However, additional clinical studies are required to determine whether high fat diet for several days indeed induces simultaneous alterations of circulating LECT2 and muscle insulin sensitivity in humans.

C2C12 myocytes transfected with pLect2 showed an impairment of myotube differentiation and insulin signal transduction (FIG. 6C and 6D). The presence of LECT2 protein in the culture medium (FIG. 6B) suggests that LECT2 derived from the pLect2 acted on the cells in

an autocrine or paracrine manner. Because the half-life of LECT2 protein was predicted to be short due to the low molecular weight of LECT2 (16 kDa), we initially overexpressed LECT2 in the cultured myocytes to examine the chronic actions of LECT2. In the next experiments, we directly treated well-differentiated C2C12 myoblasts with recombinant LECT2 protein for 3 hrs (FIG. 6F) to exclude the possibility that LECT2-induced suppression of myotube differentiation secondarily causes insulin resistance in pLect2 experiments. The results obtained from the experiments using the recombinant LECT2 protein suggest that LECT2 directly induces insulin resistance in C2C12 cells independently of its action on myotube differentiation.

Okumura et al. reported that treatment with LECT2 ameliorated collagen antibody-induced arthritis in mice (55). This report suggests that LECT2 suppresses the inflammatory response that progresses after the development of autoantibodies. Several clinical studies showed that the onset of inflammatory polyarthritis such as Rheumatoid arthritis is accelerated by obesity (58; 59). Because our current data reveal the positive correlation between body mass index and serum LECT2 levels in humans, it appears that overproduction of LECT2 fails to exert sufficient suppressive action on inflammatory polyarthritis in people with obesity. However, it is still unknown whether LECT2 acts on the process of autoantibody production by B lymphocytes in the acquired immune system. Further basic and clinical studies are needed to investigate the relationship between LECT2 and obesity-associated arthritis.

Our current cross-sectional data show that serum levels of LECT2 positively correlate with the severity of insulin resistance in human subjects. However, many lines of evidence demonstrated that various circulating proteins whose expression is altered in obesity, such as adiponectin and resistin, participate in the development of insulin resistance (60). Hence, our study does not necessarily place LECT2 as only a single causal factor of insulin resistance. Additionally, further prospective studies are needed to confirm the causal relationship between LECT2 and insulin resistance in people with obesity.

In summary, our experiments have identified LECT2 as an obesity-upregulated hepatokine that induces skeletal muscle insulin resistance. LECT2 may be a potential target for the treatment of obesity-associated insulin resistance.

ACKNOWLEDGMENTS

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Figure legends

FIG.1. Circulating LECT2 correlates with adiposity and insulin resistance. *A-F*: Individual correlations between serum levels of LECT2 and body mass index (*A*) waist circumference (*B*), HOMA R index (*C*), insulin sensitivity (Matsuda index) (*D*), selenoprotein P (SeP) (*E*), HbA1c (*F*), and systolic blood pressure (*G*) in humans (*n* = 200).

FIG.2. AMPK negatively regulates Lect2 expression in the liver. *A*: Body weight of C57BL6J mice fed a high fat diet (HFD; *n* = 15) or regular diet (RD; *n* = 16). Five-week-old male mice were fed a HFD for 8 weeks. *B*: Triglyceride contents in the livers of C57BL6J mice fed a HFD or a RD for 8 weeks (*n* = 7-8). *C*: Hematoxylin and Eosin staining of livers from C57BL6J mice fed a HFD or a RD for 8 weeks. *D*: mRNA levels for *Lect2*, *Fasn*, and *Srebp1c* in the livers of C57BL6J mice fed a HFD or a RD for 8 weeks (*n* = 7-8). *E*: Changes

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of blood levels of LECT2 in C57BL6J mice fed HFD ($n = 8$) or RD ($n = 8$). Blood samples

were obtained after fasting for 12 hr. *F*: Phosphorylation of AMPK in the livers of C57BL6J

mice fed a HFD or a RD after a 12-h fast ($n = 4$). *G*: Blood levels of LECT2 from C57BL6J

mice following fasting for 12 h and subsequent feeding for 12 h ($n = 7$-8). *H*:

Phosphorylation of AMPK in the livers of C57BL6J mice following fasting for 12 h and

subsequent feeding for 12 h ($n = 3$). *I*: mRNA levels for *Lect2* in the livers of C57BL6J mice

following running exercise for 3 h ($n = 7$-8). *J*: Protein levels of LECT2 in the livers of

C57BL6J mice following running exercise for 3 h ($n = 7$-8). *K*: Serum levels of LECT2 in

C57BL6J mice before and after running exercise for 3 h ($n = 8$, Paired t-test). Right panel:

Percentage changes of serum LECT2 after running exercise for 3h (unpaired t-test). *L*:

Phosphorylation of AMPK in the livers of C57BL6J mice following running exercise for 3 h

($n = 3$-4). *M*: Effects of constitutively active (CA-) AMPK on mRNA levels for *Lect2* and

G6pc in H4IIEC hepatocytes ($n = 4$). *N*: Effects of dominant-negative (DN-) AMPK on

mRNA levels for *Lect2* and *G6pc* in H4IIEC hepatocytes ($n = 4$).

Data in (*A*-*B*), and (*D*-*N*) represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fasn, fatty acid synthase; *Srebp1c,* sterol regulatory-element binding protein-1c; *G6pc,*

glucose-6 phosphatase.

FIG.3. *Lect2* deletion increases muscle insulin sensitivity in mice. *A*: *Lect2* mRNA levels

in various tissues of C57BL6J mice ($n = 4$-8). *B:* Serum levels of LECT2 in *Lect2*-deficient

and wild-type mice fed a HFD for 10 weeks (n = 9-13). Blood samples were obtained in fed condition. *C*: Body weight of *Lect2*-deficient and wild-type mice fed a regular diet (n = 6-8). *D*: Food intake of *Lect2*-deficient and wild-type mice (n = 6-8). *E*: Heat production of *Lect2*-deficient and wild-type mice (n = 6-8). *F* and *G*: Running endurance was tested in *Lect2*-deficient and wild-type mice (n = 7-8). Running endurance is depicted as distance (*F*) and time (*G*). *H* and *I*: Intraperitoneal glucose (*H*) and insulin (*I*) tolerance tests in *Lect2*-deficient and wild-type mice (n = 7-8). Glucose and insulin were administered at doses of 2.0 g/kg body weight and 1.0 units/kg body weight, respectively. *J* and *K*: Western blot analysis and quantification of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice (n = 5). 19-week-old female mice were stimulated with insulin (administered through vena cava) at doses of 1 unit/kg body weight. At 10 min after insulin administration, the hind-limb muscles were removed. *L*: Glucose infusion rate (GIR), endogenous glucose production (EGP), and rate of glucose disposal (Rd) during hyperinsulinemic-euglycemic clamp in *Lect2*-deficient and wild-type mice (n = 6-7). *M*: mRNA levels of genes involved in myogenesis and mitochondria in skeletal muscle of *Lect2*-deficient and wild-type mice (n = 4-5).

Data in (*A-I*), and (*K-M*), represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (*Lect2*-deficient mice *versus* wild-type mice)

FIG. 4. *Lect2* deletion attenuates muscle insulin resistance in diet-induced obesity in mice. *A*: Changes of bodyweight of *Lect2*-deficient mice and wild-type mice fed with high fat diet (HFD; *n* = 9-13). *B*: Serum insulin levels of *Lect2*-deficient and wild-type mice fed with HFD for 11 weeks in a fed condition and after 12 hours fasting. (*n* = 9-13). *C*: Blood glucose levels of *Lect2*-deficient and wild-type mice fed with HFD for 11 weeks in a fed condition and after 12 hours fasting. (*n* = 9-13). *D*: Intraperitoneal glucose tolerance tests in *Lect2*-deficient and wild-type mice fed with HFD for 9 weeks (*n* = 9-13). Glucose was administered at doses of 0.5 g/kg body weight. *E*: Intraperitoneal insulin tolerance tests in *Lect2*-deficient and wild-type mice fed with HFD for 10 weeks (*n* = 9-13). Insulin was administered at doses 1.2 units/kg body weight. *F-G*: Western blot analysis and quantification of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice (*n* = 3-4). *H-I*: Western blot analysis and quantification of phosphorylated JNK in skeletal muscle of *Lect2*-deficient and wild-type mice (*n* = 3-4). Mice were stimulated with insulin (administered through vena cava) at doses of 1 unit/kg body weight. At 2 min after insulin administration, the hind-limb muscles were removed.

Data in (*A-E*) and (*G*) represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (*Lect2*-deficient mice *versus* wild-type mice)

FIG. 5. Starvation abolishes the insulin sensitive phenotype in *Lect2*-deficient mice.

A: Body weight of female *Lect2*-deficient and wild-type mice (*n* = 5) during starvation at the

age of 20 weeks. *B*: Blood glucose levels of *Lect2*-deficient and wild-type mice ($n = 5$) during starvation. *C:* Serum levels of insulin of *Lect2*-deficient and wild-type mice ($n = 4$-5) in a fed condition or after starvation for 36 hours (paired t-test). *D*: Serum levels of LECT2 of wild-type mice ($n = 4$-5) in a fed condition or after starvation for 36 hours. *E*: Western blot analysis of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice after 60 hours starvation. Mice were stimulated with insulin (administered intraperitoneally). At 15 min after insulin administration, mice were anesthetized and hind-limb muscle samples were removed for analysis.

Data in (*A*-*D*) represent the means ± SEM. Data in (D) were assessed by paired t-tests. *$P <$ 0.05.

FIG. 6. LECT2 impairs insulin signaling by activating JNK in C2C12 myotubes.

C2C12 myoblasts in 30-50% confluency were transfected with negative control or mLect2 expression plasmid in (*A*)-(*E*). When the cells reached to 100% confluency, the cells were differentiated into myotubes with DMEM containing 2% horse serum for 24-48 h. *A*: *Lect2* mRNA levels in C2C12 myotubes transfected with control or *Lect2* expression vector ($n = 6$). mRNA was obtained from the cells differentiated into myotubes for 24 h. *B*: LECT2 protein levels in culture medium of C2C12 myotubes transfected with control or *Lect2* expression vector for 24h or 72h ($n = 3$). LECT2 production was measured in by ELISA. *C*: Representative images of C2C12 myotubes transfected with control or *Lect2* expression

vector. The cells were differentiated into myotubes for 48 h. *D*: Western blot analysis of phosphorylated Akt in C2C12 myotubes transfected with control or *Lect2* expression vector (*n* = 4). The cells were stimulated by 100 ng/ml of insulin for 15 min. *E*: Western blot analysis of phosphorylated JNK in C2C12 myotubes transfected with control or *Lect2* expression vector (*n* = 3). *F*: Western blot analysis of phosphorylated Akt in C2C12 myotubes pretreated with recombinant LECT2 protein (*n* = 4). The cells were pretreated with LECT2 protein. Three hours later, the cells were stimulated with insulin. *G*: Effects of recombinant LECT2 protein on JNK phosphorylation in C2C12 myotubes (*n* = 3). The cells were treated with 400 ng/ml of recombinant LECT2 protein for the indicated times. *H*: Concentration-dependent effects of recombinant LECT2 protein on JNK phosphorylation in C2C12 myotubes (*n* = 3). The cells were treated with LECT2 protein for 1 h. *I*: Effects of JNK-knockdown on LECT2 protein-induced insulin resistance in C2C12 myoblasts (*n* = 4).

Data in (*A)*, (*B)* and (*D-I)* represent the means ± SEM. **P* < 0.05, **P < 0.01, ***P< 0.001 *versus* cells transfected with control vector or cells treated with vehicle.

FIG. 7. The hepatokine LECT2 links obesity to insulin resistance in the skeletal muscle.

Table S1, relative to Fig. 1. Candidate hepatokine genes involved in obesity.

Unigene	Symbol	Name	DM/NGT, by SAGE	p value vs. BMI	Correlation coefficient
Hs.73853	BMP2	Bone morphogenetic protein 2	4.54	0.0001	0.63
Hs.512580	LECT2	Leukocyte cell-derived chemotaxin2	2.11	0.0001	0.61
Hs.3416	ADFP	adipose differentiation-rclate4 protein	0.15	0.0020	0.54
Hs.443518	BPAG1	Homo sapiens dystonin (DST), transcript variant leA, mRNA	1.51	0.01	0.49
Hs.173594	SERPIN F1	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	2.02	0.01	0.47
Hs.157307	GNAS	GNAS complex locus	1.99	0.01	0.47
Hs.324746	AHSG	AHSG alpha2 HS glycoprotein	0.02	0.02	0.42
Hs.10458	CCL 16	chemokine (C-C motif) ligand 16	6.82	0.02	0.42

Hs.168718	AFM	afamin	0.57	0.02	0.42
Hs.241257	LTBP1	LTBP1 latent transforming growth factor beta binding protein 1	0.05	0.02	0.42
Hs.1012	C4BPA	complement component 4 binding protein, alpha	0.29	0.03	0.41
Hs.234734	LYZ	lysozyme (renal amyloidosis)	0.45	0.03	0.4
Hs.2 12581	MMP24	MMP24 matrix metalloproteinase 24 (membrane inserted)	0.05	0.03	0.4
Hs.4 06455	PSAP	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	2.47	0.03	0.4
Hs. 1498	ERG	histidine-rich glycoprotein	8.49	0.04	0.39
Hs.9914	FST	FST follistatin	0.02	0.04	0.38
Hs.119651	GPC3	glypican 3	67.75	0.04	-0.38
Hs.512001	GPLD1	glycosylphosphatidy linositol specific phospholipase D1	2.27	0.04	0.38
Hs.75615	APOC2	Apolipoprotein C-II	2.02	0.04	0.37
Hs.515258	GDF15	GDF15 growth differentiation factor 15	0.03	0.04	0.37
Hs. 31439	SPINT2	SPINT2 serine protease inhibitor Kunitz type 2	0.02	0.04	0.37
Hs.159	TNFRSF 1A	TNFRSF1A tumor necrosis factor receptor superfamily member 1A	0.05	0.05	0.36

BMI, body mass index.

Table S2, relative to Fig.1. Clinical characteristics of the human subjects whose blood was sampled.

N	200
Age (years)	55±11
Sex (M/F)	118/82
Body mass index (kg/m^2)	22.9±3.1
Waist circumstance (cm)	81.0±9.0
Fasting plasma glucose (mg/dl)	97±11
HOMA-IR	1.4±0.9
HbA1c (%)	5.7±0.4
Systolic blood pressure (mmHg)	124±18
Selenoprotein P (μg/ml)	6.0±0.8

HOMA-IR, homeostasis model assessment for insulin resistance; HbA$_{1c}$, hemoglobin A$_{1c}$.

Blood was sampled following overnight fasting.

Supplementary Figure 1. Insulin resistance in mice fed a 60% high fat diet for 1 week.

(**A**) Body weight of C57BL6J mice fed a high fat diet (HFD; $n = 8$) or regular diet (RD; $n = 8$). Five-week-old male mice were fed a HFD for 1 week. (**B**) Blood glucose levels of C57BL6J mice fed a HFD or a RD for 1 week ($n = 8$). (**C**) Serum levels of insulin in C57BL6J mice fed a HFD or a RD for 1 week ($n = 8$). (**D**) Serum levels of LECT2 in C57BL6J mice fed HFD ($n = 8$) or RD ($n = 8$). (**E**) and (**F**) Western blot analysis and quantification of the bands of phosphorylated Akt in skeletal muscle of C57BL6J mice fed a HFD or a RD after a 12-h fast ($n = 3$). Mice were injected with insulin intraperitoneally (10 units/kg body weight). At 15 min after insulin administration, mice were anesthetized and hind-limb muscle samples were removed for analysis. Data in (**A-D**) and (**F**) represent the means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$
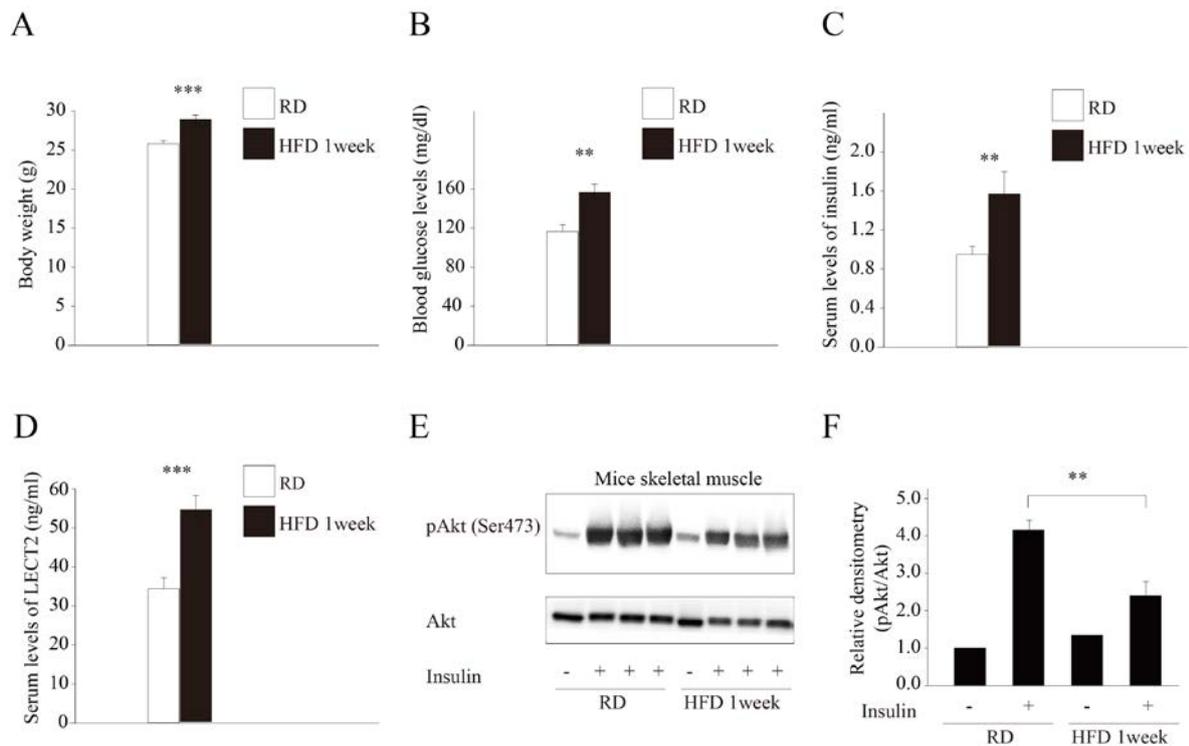
Supplementary Figure 2. Insulin signalling and JNK phosphorylation in the liver and adipose tissue of *Lect2*-deficinet and wild-type mice. (**A**) and (**B**) Western blot analysis of phosphorylated Akt in liver and white adipose tissue of *Lect2*-deficient and wild-type mice. Mice were stimulated with insulin intraperitoneally (10 units/kg body weight). At 15 min after insulin administration, mice were sacrificed and liver and epididymal white adipose tissue samples were removed for analysis. (**C**) and (**D**) Western blot analysis of phosphorylated JNK in liver and white adipose tissue of *Lect2*-deficient and wild-type mice fed a normal chow.
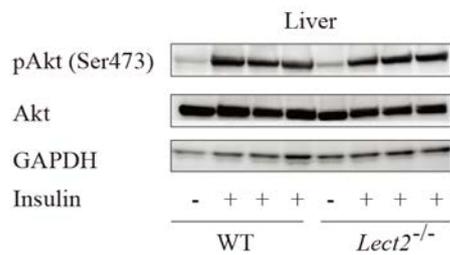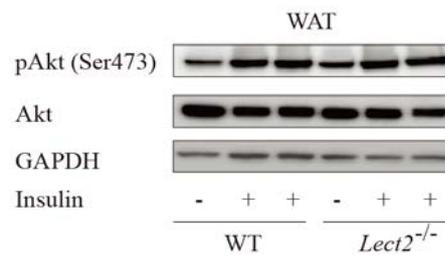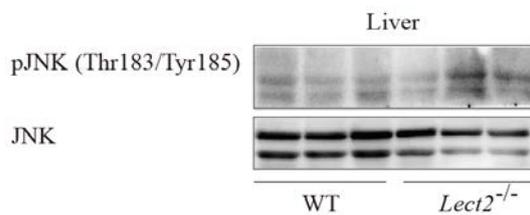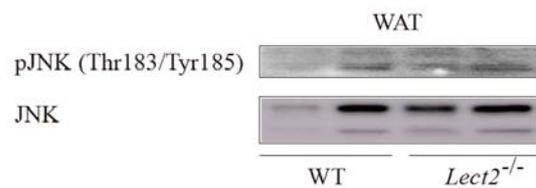
Supplementary Figure 3. Glucose levels during hyperinsulinemic-euglycemic clamp. (**A**) Time course of blood glucose levels during hyperinsulinemic-euglycemic clamp in wild-type and *Lect2*-deficient mice (*n* = 6-7). (**B**) Time course of glucose infusion rate during hyperinsulinemic-euglycemic clamp in wild-type and *Lect2*-deficient mice (*n* = 6-7). (**C**) Average of blood glucose levels at the last 30 min (90min, 105min and 120min) in wild-type and *Lect2*-deficient mice (*n* = 6-7). Data in (**A**)- (**C**) represent the means ± SEM. *P < 0.05 *versus* the wild-type mice.
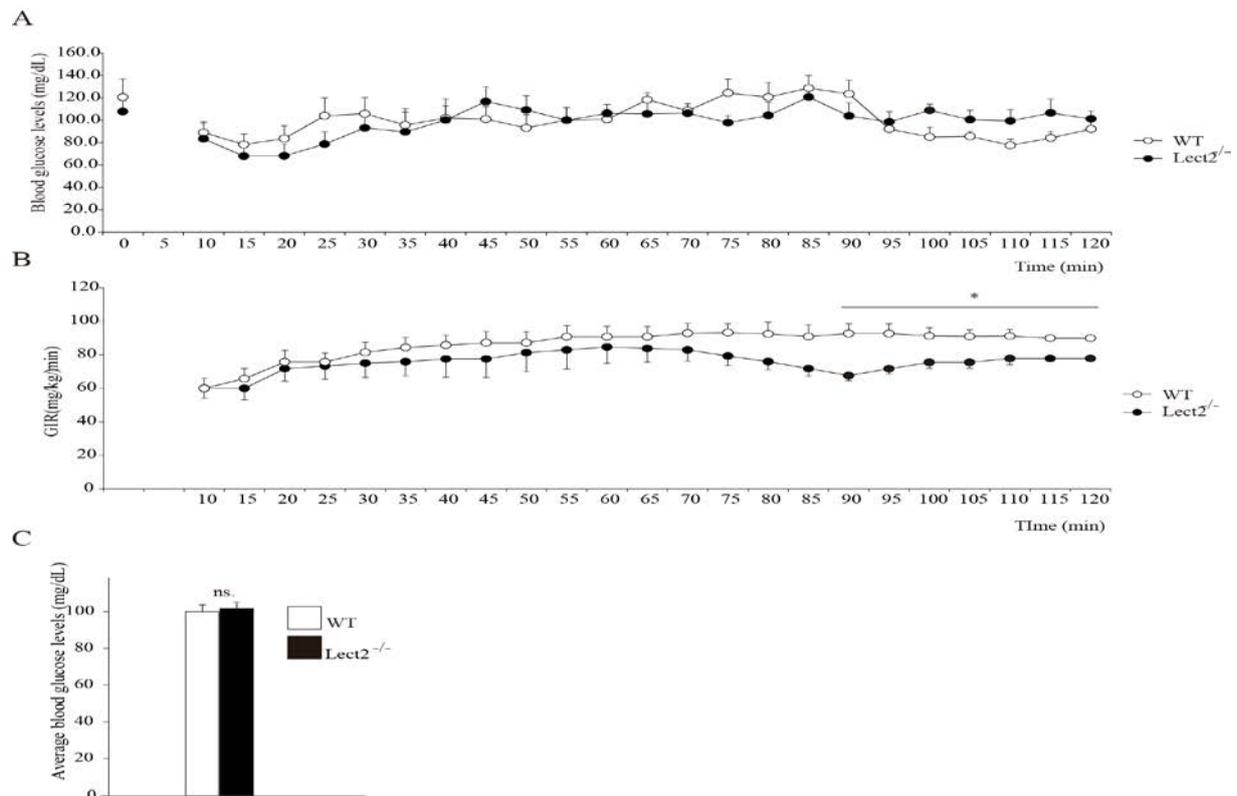
Supplementary Figure 4. Food intake and heat production in wild-type and *Lect2*-deficient mice fed a HFD for a week **(A)** Body weight of *Lect2*-deficient and wild-type mice fed a HFD for a week (*n* = 7-8). **(B)** Food intake of of *Lect2*-deficient and wild-type mice fed a HFD for a week (*n* = 7-8). **(C)** Heat production of of *Lect2*-deficient and wild-type mice fed a HFD for a week (*n* = 7-8). **(D)** Heat production in light phase of *Lect2*-deficient and wild-type mice fed a HFD for a week (*n* = 7-8). **(E)** Heat production in light phase of *Lect2*-deficient and wild-type mice fed a HFD for a week (*n* = 7-8). Data represent the means ± SEM. **$P < 0.01$
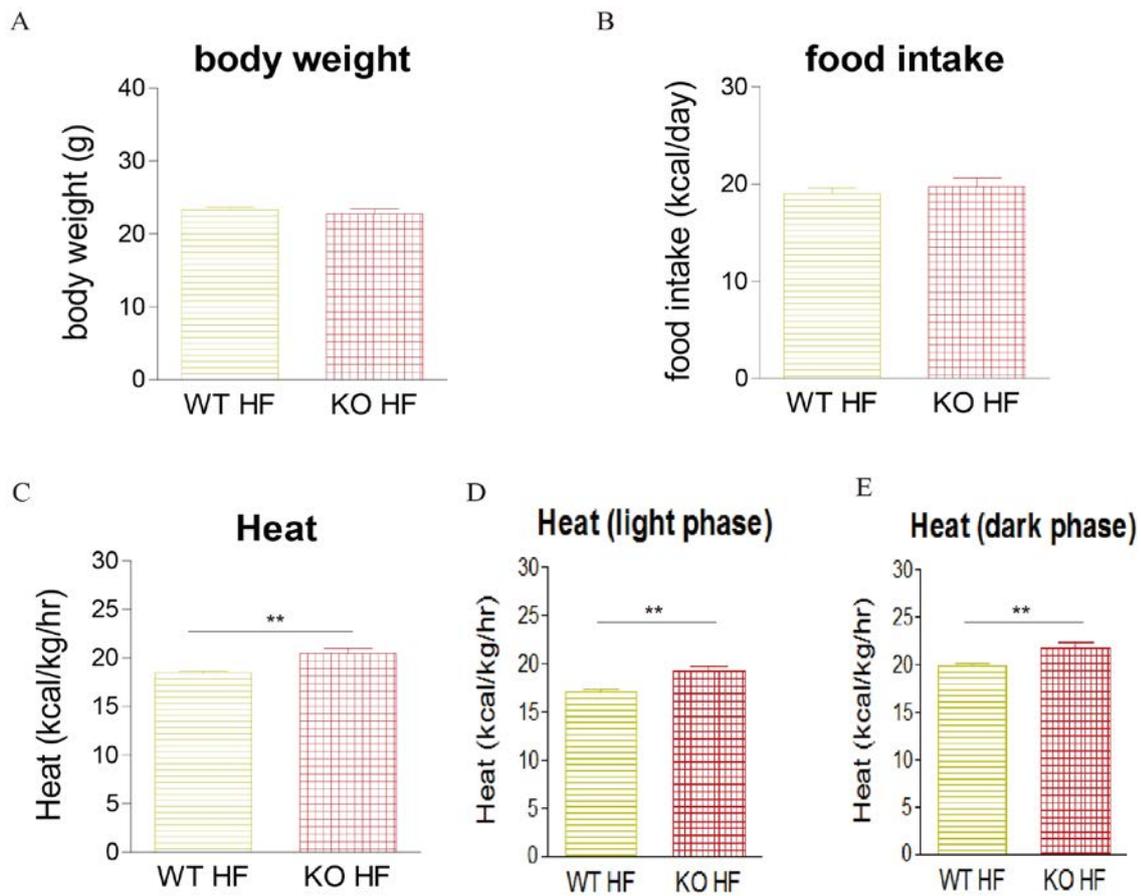
Supplementary Figure 5. *Lect2* deletion increases muscle insulin sensitivity in mice fed a HFD for 2 weeks. (**A**) Changes of bodyweight of 22 weeks-old *Lect2*-deficient and wild-type mice fed a HFD for 2 weeks (n=7). (**B**) The bodyweight of 22 weeks-old *Lect2*-defeicient and wild-type mice before sacrifice. (n=7). (**C**) The weight of left epididymal adipose tissue of *Lect2*-deficient and wild-type mice (n=7). (**D**) Western blot analysis of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice. Mice were stimulated with insulin (administered through vena cava) at doses of 1 units/kg body weight. At 10 min after insulin administration, hind-limb muscles were removed. (**E**) Quantification of phosphorylated Akt in **D** (n=3). *$P<0.05$ (student's *t*-test). Data represent the means ± SEM.
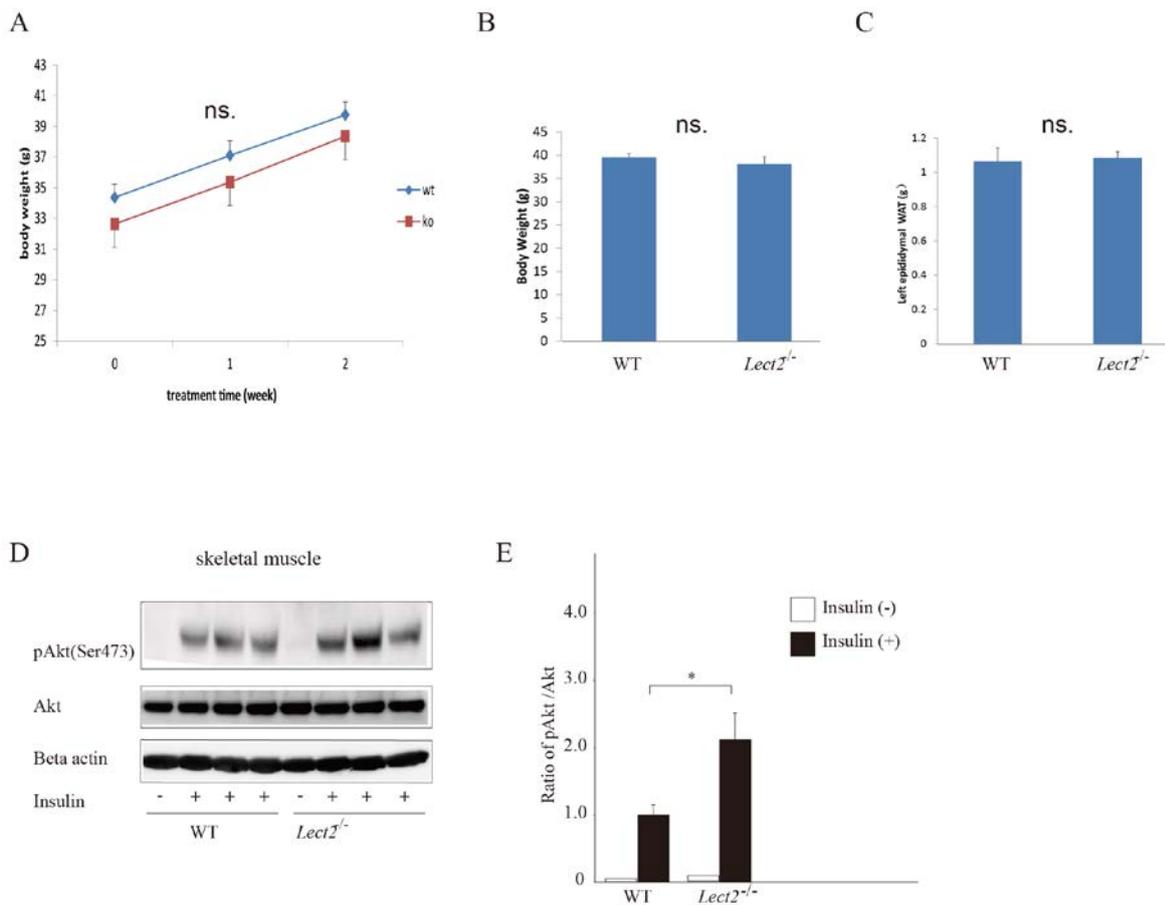
Supplementary Figure 6. Markers of ER stress or inflammation in C2C12 myocytes transfected with pLect2 or in the muscles of *Lect2*-deficient mice. (**A**) *CHOP* mRNA levels in skeletal muscle of *Lect2*-deficient and wild-type mice fed with normal chow ($n = 8$-9). (**B**) *CHOP* mRNA levels in C2C12 myotubes transfected with control or *Lect2* expression vector ($n = 6$), mRNA was obtained from the cells differentiated into myotubes for 48 h. (**C**) Western blot analysis of BiP in in skeletal muscle of *Lect2*-deficient and wild-type mice fed with 60% high fat diet for 15 weeks. (**D**) Western blot analysis of p-eIF2α in C2C12 myotubes transfected with control or *Lect2* expression vector. (**E**) *Tnf-α* mRNA levels in skeletal muscle of *Lect2*-deficient and wild-type mice fed a regular diet ($n = 7$-8). (**F**) *Tnf-α* mRNA levels in C2C12 myotubes transfected with control or *Lect2* expression vector ($n = 6$). mRNA was obtained from the cells differentiated into myotubes for 2 days. (**G**) Western blot analysis of inflammation-associated proteins in C2C12 myotubes transfected with control or *Lect2* expression vector. Data in (**A-B**) and (**E-F**) represent the means ± SEM.
CHOP, CCAAT/-enhancer-binding protein homologous protein; BiP, Binding immunoglobulin protein; eIF2, Eukaryotic Initiation Factor 2; Tnfa, Tumor necrosis factor α; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IKK, IκB kinase; IκBα, inhibitor of kappa B.

Fig.1

A

p < 0.0001 r = 0.372

B

p < 0.0001 r = 0.379

C

p = 0.001 r = 0.237

D

p = 0.002 r = -0.222

E

p = 0.002 r = 0.223

F

p = 0.025 r = 0.159

G

p < 0.0001 r = 0.265

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Fig.7