Lactate dehydrogenase in adipocyte regulates glucose metabolism

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Abstract

Plasma glucose concentrations are homeostatically regulated and maintained within strict boundaries. During fasting, liver produces glucose via gluconeogenesis stimulated by hormonal factors, however, little has been known about the mechanism of adipose tissue to regulate glucose uptake during fasting.

Here, I revealed that expression levels of 8 glycolytic enzymes were significantly reduced in adipose tissue under fasting conditions using public microarray database. Among them, I focused on lactate dehydrogenase A, which is responsible for conversion of pyruvate into lactate, the last step of glycolysis pathway. In adipose tissues of WT mice, LDHA expression decreased in parallel with reduced Glut1 protein, a major glucose transporter during fasting.

To elucidate the significance of LDHA in adipocytes, I generated adipocyte-specific LDHA knockout mice (AdLDHAKO). AdLDHAKO mice showed no apparent differences in body weight and tissue weight. Under fasting conditions, ADLDHAKO mice exhibited significantly decreased Glut1 protein. Subsequently, adipose tissue of AdLDHAKO mice exhibited reduced glucose uptake than that of control mice.

These results indicate that LDHA-deficiency causes reduction of Glut1 protein and glucose uptake, suggesting that there is a new mechanism to reduce glucose uptake. Considering that both LDHA and Glut1 expressions are decreased during fasting, LDHA might regulate glucose uptake in adipose tissues to supply it to other demanding organs. It

could lead to a new insight of adipose tissue as a metabolic organ to maintain glucose homeostasis by reducing glucose uptake during fasting, in addition to taking up excess glucose during feeding.

Table of contents

Abbreviations

AdLDHAKO	Adipocyte-specific LDHA knockout
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
Glut1	Glucose transporter 1/solute carrier family 2, facilitated glucose transporter member 1
Glut4	Glucose transporter 4/solute carrier family 2, facilitated glucose transporter member 4
GTEx	The Genotype-Tissue Expression
LC/CE-MS	Liquid Chromatography/capillary electrophoresis -mass spectrometry
LDHA	Lactate dehydrogenase A
MAF	Mature adipocyte fraction
NAD	Nicotinamide adenine dinucleotide
NMN	Nicotinamide mononucleotide
SVF	Stromal vascular fraction
TCA cycle	tricarboxylic acid cycle
TG	triglyceride
WAT	White adipose tissue
2DG	2-deoxyglucose
2DG6P	2-deoxyglucose 6-phosphate

1. Significance statement

This study shows the contribution of adipose tissue related to glucose homeostasis especially under fasting conditions. There have been no reports regarding the importance and the mechanism of glucose uptake into adipose tissue under fasting conditions.

In the current study, I revealed that adipocytes might regulate glucose utilization through preventing glucose uptake under fasting conditions. In addition, the expression of glycolytic enzyme, especially lactate dehydrogenase A (LDHA) could offer this regulation by influencing one of glucose transporter, Glut1.

This study revealed the new regulatory mechanism of glucose uptake into adipocyte under fasting conditions. This finding could offer a new insight of adipose tissue related to glucose utilization.

2. General introduction

Living organisms are defined as the ones with a cell membrane, function of reproduction, and metabolic system. Especially, metabolism is essential for all organisms. In order to react and adapt to environmental changes, organisms are required to keep metabolizing external nutrients, and obtain energy constantly for themselves.

Glucose is one of the vital energy sources for living things. And the brain cannot synthesize glucose although it requires a lot. Therefore, there are an important mechanism to keep blood glucose levels constantly within normal limits, called glucose homeostasis. Nowadays, we live in “the age of plenty”, however, humans had been struggling to get energies from outside and keep their lives for a long time. Therefore, we have a system to keep blood glucose levels even under energy shortage conditions. On the other hand, under overnutrition conditions, it is necessary to store all the energy we get efficiently by all means to prepare for energy shortage. Metabolic organs are regulated and collaborate with each other to maintain glucose homeostasis in a normal condition. Major metabolic organs are the liver, the skeletal muscle, and the adipose tissue. Whole-body metabolism is regulated precisely since all organs require energy supply constantly to play each role properly. Regarding mammals, insulin is a key hormone for regulating blood glucose levels, it was discovered in 1921, just a hundred years ago (Banting et al. 1922). Insulin secretion from pancreatic beta cell is enhanced by high glucose levels in blood after feeding. Insulin upregulates glucose incorporation into the tissues, such as skeletal muscle,

adipose tissue, whereas it suppresses gluconeogenesis in the liver (Haeusler, McGraw, and Accili 2018; Gastaldelli et al. 2001). On the other hand, under fasting state, various hormones, such as glucagon, glucocorticoids, and catecholamines, suppress glucose uptake into the tissues and enhance gluconeogenesis so as to maintain blood glucose levels (Ramnanan et al. 2011; Facchi et al. 2020; Barth et al. 2007). In this way, blood glucose levels are regulated continuously and precisely under dynamic nutritional changes like fasting-feeding cycle (Aronoff et al. 2004).

At the first step of glucose utilization, glucose transporters are responsible for glucose incorporation into the cells. There are two types of glucose transporters, sodium glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (Gluts). There are 14 members of Glut protein known as Glut1-Glut14. These isoforms have distinct expression patterns among tissues and have different functions and characteristics (Navale and Paranjape 2016). Regarding metabolic tissues, it has been mainly reported that the liver expresses Glut1 and 2, the skeletal muscle does Glut1, 4, and 12, and adipose tissue expresses Glut1 and Glut4. Since adipose tissue expresses both insulin-dependent, and independent Gluts, adipose tissue can contribute to glucose incorporation under basal and postprandial conditions. (Gastaldelli et al. 2001).

Adipose tissue consists of mainly adipocytes, that is characterized by accumulation of lipids within the cell. Basically, adipose tissue is categorized into two separated groups, white adipose tissue and brown

adipose tissue (Cinti 2012). Here, focusing on white adipose tissue that is classically regarded as the “fat tissue”. It localizes the whole body, especially in humans, identified subcutaneous white adipose tissue and visceral white adipose tissue according to the location. Adipose tissues have four main functions; glucose uptake, lipogenesis, lipolysis, and adipocytokine secretion. All these functions make a great contribution for the maintenance of systemic energy homeostasis.

Regarding contribution for glucose homeostasis, the most important role of adipose tissue is to take up glucose as much as possible. It has been well studied about the glucose incorporation into adipose tissue under feeding conditions. Adipose tissue promotes glucose incorporation by insulin and stores it as triglyceride in the lipid droplet through conversion to glycerol. As mentioned above, it is critical to accumulate energy as much as possible under feeding condition to prepare for the energy shortage. When blood glucose levels increase acutely, secreted insulin stimulates translocation of Glut4 from cytosol to plasma membrane, followed by promotion of glucose uptake into the cell. On the other hand, little has been known about the regulation of glucose uptake under energy shortage conditions.

Here, I focused on the mechanism of glucose uptake especially under fasting conditions and revealed a novel regulatory mechanism of glucose uptake through the glycolytic enzyme, lactate dehydrogenase A (LDHA). This finding could offer a new insight of adipose tissue related to glucose homeostasis under fasting conditions.

3. Introduction

Adipose tissue has a crucial role to regulate blood glucose levels constantly and contributes for glucose homeostasis. Adipocytes express two types of glucose transporter, Glut1 and Glut4, these proteins are encoded by the genes slc2a1 and slc2a4 respectively. Basically, Glut1 is responsible for basal glucose incorporation in a manner independent from insulin, while Glut4 is for enhanced glucose uptake by insulin. Glut1 is constitutively targeted to the plasma membrane, whereas insulin triggers translocation of Glut4 vesicle to the plasma membrane (Kanzaki and Pessin 2001; Cheatham 2000). In 3T3-L1 adipocytes, high glucose concentration increases Glut1 protein levels especially in low density microsomes, but not Glut4 (Hosaka, Yaga, and Oka 1999). Moreover, troglitazone, one of activator of peroxisome proliferator-activated receptors (PPARs), increases Glut1, but not Glut4 protein levels in 3T3-L1 adipocyte (Tafuri 1996). These studies suggest that the nutritional conditions can regulate the expression of Glut1.

In addition, adipocyte and muscle specific Glut4 deficient mice shows impaired glucose tolerance and insulin resistance along with decreased glucose uptake into both tissues (Kotani et al. 2004). Furthermore, adipocyte-specific Glut4 deficient mice show systemic defects on glucose tolerance and insulin sensitivity through impaired glucose incorporation into adipose tissue (Abel et al. 2001). Some reports suggest the vital role of Glut4 in adipose tissue for the whole-body energy metabolism, however, the significance of Glut1 has been unrevealed.

Considering that fasting is totally different from feeding condition, there should be a unique mechanism to regulate Glut1 and glucose uptake under fasting condition.

Glycolysis is the major pathway to utilize glucose in adipocytes. This process is efficient to produce adenosine triphosphate (ATP), that is often called “molecular unit of currency” of intracellular energy transfer. Lately, global transcriptome analysis has reported the changes of metabolic pathways and expressions of numerous genes under fasting conditions in adipose tissue (Defour et al. 2020). However, little has been known about the detailed mechanism underlying these drastic changes during fasting conditions.

At the final step of anaerobic glycolysis, Lactate dehydrogenase A (LDHA) is responsible for converting pyruvate into lactate. LDHA is a glycolytic enzyme catalyzing the conversion of pyruvate and Nicotinamide adenine dinucleotide (NADH) to lactate and Nicotinamide adenine dinucleotide^{+} (NAD^{+}). There have been no reports about the significance of LDHA in adipose tissue.

4. Materials and Methods

Mice

Adipoq-cre mice were kindly provided by Dr. Rosen (Eguchi et al. 2011). Adipoq-cre mice were crossed with LDHA floxed mice (Wang et al. 2014) (Jackson 030112) to generate AdLDHAKO mice. The following primers were used to distinguish the floxed allele: 5'-CTCGCTTGCCTTATGGGTTC-3', and 5'-TCGTGGTATCGTTATGCGCC-3'. Furthermore, the following primers to distinguish the deleted alleles: 5'- CTCGCTTGCCTTATGGGTTC-3', and 5'-TGGCAGTCAAGTCTCCAAGAAG-3'. Mice were maintained in a room under controlled temperature (23 ± 1.5 ℃) and humidity (45 ± 15 %) on a 12-h dark/12-h light cycle and had free access to water and chow (MF; Oriental Yeast). C57BL/6J mice were purchased from Charles River Japan. The procedures were approved by the Gene Modification Experiments Safety Committee of Osaka University (approval ID; 03883-002), and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University, Graduate School of Medicine (approval ID; 27-023-004). All animal experiments were carried out in accordance with the Institutional Animal Care Use Committee Guidelines of Osaka University.

Database study

The data discussed in this research have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO

Series accession number GSE46495 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46495). The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from dbGaP (accession number phs000424.v8.p2).

White Adipose tissue fractionation

Subcutaneous fat pads from male mice were excised and minced in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (PS). Tissue suspension were centrifuged at 1,000 rpm for 5 min to remove erythrocytes and free leukocytes. The floating fraction was subjected to the subsequent procedures. Collagenase (1 mg/ml) and DNase (0.1 mg/ml) was added, and incubated at 37 ℃ for 40 min with shaking. The cell suspension was filtered through a 110 μm cell strainer and then centrifuged at 1,000 rpm for 5 min to separate the stromal vascular fraction (SVF) pellet from the floating mature adipocytes fraction (MAF). The separated MAF and SVF cells were resuspended in different tubes and centrifuged at 1,000 rpm for 5 min for washing out the cell debris. Each fraction was used for isolated fraction analysis of white adipose tissue. In addition, the SVF was filtered through a 40 μm cell strainer. After centrifugation at 1,000 rpm for 5 min, the pellet was re-suspended in DMEM containing 10 % FBS and 1% PS. The isolated SVF were seeded in an appropriate culture dish and 4-6

hours later, the cells were washed with DMEM so as to remove cell debris. The cells were used as primary preadipocytes for *in vitro* study.

Cell culture

3T3-L1 cells were purchased from ATCC (American Type Culture Collection, ATCC, Manassas, VA). 3T3-L1 preadipocytes were seeded until confluence. After 2 days in culture, the medium was replaced with the differentiation medium; DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 1 μM insulin. Only for the mouse primary adipocytes, 10 μM pioglitazone were supplemented additionally. 3 days later, the cell differentiation medium was removed and the culture medium was replaced with the maintenance medium; DMEM supplemented with 10% FBS and 1% PS. The maintenance medium was renewed every 2 days until day 7. In all experimental procedures, the medium was replaced with DMEM without serum to avoid any effects of the serum.

Gene expression analysis

Total RNA was isolated from 3T3-L1 adipocytes and mouse primary adipocytes, and subcutaneous adipose tissue using TRI reagent (Sigma-aldrich). For *in vitro* study, the culture medium was removed completely and the cells were washed with cold D-PBS (Nacalai Tescue). The cells were suspended with cold TRI reagent by pipetting. The tissue samples were freshly frozen in liquid N2 and resolved in TRI reagent. The tissues

were homogenized using homogenizer, and the suspension were centrifuged at 14,500 x *g* for 20 minutes and the lipids and cell debris were removed. The supernatants were subjected to RNA isolation according to the manufacturer's instructions. The total RNA amount of RNA was quantified by optical density (OD) at 260 nm. RNA purity was determined by measuring the 260/280 and 260/230 ratios with the Nanodrop instrument. 500 ng RNA was used to synthesize complementary DNA (cDNA) with a Reverse Transcription kit (Takara-bio). Real-time PCR was performed on the LightCycler using THUNDERBIRD SYBR qPCR Mix (Toyobo) or FastStart Essential DNA Green Master (Roche) according to the protocol provided by the manufacturer. mRNA levels were normalized to the level of mRNA for 36B4. The specific primers listed in Supplemental Table1 were purchased from sigma-aldrich.

Transfection with small interfering RNA

On the Day 5 during differentiation, the culture medium was replaced in DMEM with 10% FBS and incubated for a few hours. Fully differentiated adipocytes were treated with trypsin-EDTA and incubated at 37℃ for 2 minutes. The cells were collected in 50ml of conical centrifuge tube and centrifuge at 1,000 rpm for 5 min. The siRNA mixture was prepared according to the manufacturer's instructions. Briefly, Opti-MEM, siRNA solution (Qiagen, AllStars Negative as control siRNA and Flexitube siRNA for the target genes), and Lipofectamine RNAiMAX (Invitrogen) were mixed and incubated for 15 min at room temperature. For reverse

transfection protocol, the cell pellets were harvested in 12 or 24 well dishes after the siRNA mixture was divided to each well. The cells were subjected to the subsequent experiments.

Western blot analysis

The protein was extracted from the tissue samples and the cells in lysis buffer (20 mM Tris/HCl pH 7.4, 1.0% Triton X100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with Protease Inhibitor Cocktail (Nacalai Tescue). The cell medium was completely removed and the culture dishes were washed with ice-cold D-PBS, and then freshly frozen in liquid N2. The cells were suspended in an ice-cold lysis buffer and centrifuged at 14,500 x *g* for 15 min to remove the debris. Tissue samples were homogenized in an ice-cold lysis buffer using homogenizer and the homogenates were centrifuged at 14,500 x *g* for 15 min. The supernatants were subjected to the following procedures. Protein concentration was measured by the bicinchoninic acid method using the BCA protein assay reagent (Pierce). The samples were added Sample Buffer Solution (FUJIFILM Wako Chemicals) and heated at 95°C for 5 min. Equal amounts of each sample were separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) using e-PAGEL (ATTO). The samples were transferred electrophoretically to the polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.05% Tween-20 (TBS-T) and 5% skim milk for 1 hour at room temperature. The membranes were washed with

TBS-T and incubated with primary antibody diluted in TBS-T or Can Get Signal Immunoreaction Enhancer Solution 1 (Can Get Signal Solution 1) (TOYOBO) at 4 ℃ overnight. All of the primary antibodies were listed in Supplemental Table 2. After washing in TBS-T, the membranes were incubated with Amersham ECL Rabbit or mouse IgG, HRP-linked whole antibody (cytiva) diluted 1/10,000 in TBS-T or Can Get Signal Solution 2. After washing with TBS-T, the detection was performed using ChemiDoc Touch (Biorad) with enhanced chemiluminescence by ECL Western Blotting Substrate (Pierce). Equal loadings were verified by ponceau S staining. Densitometry analysis was conducted using Image-J Software.

Fasting and refeeding experiments

C57BL/6J mice fasted for 24 hours and then subjected to refeeding for 24 hours. At the beginning of fasting (ad libitum), 24 hours of fasting, and 24 hours of refeeding, the mice were sacrificed, and the tissue samples were harvested.

Glucose uptake assay

In vivo study, mice were fasted for 4h and injected 10 μmol 2DG via i.p injection. 30 minutes after injection, mice were sacrificed and the tissues were weighed and frozen in LN2 immediately. Approximately 50 mg tissue samples were homogenized in Tris-HCl buffer and heated at 80℃ for 15 min. Then samples were centrifuged at 15,000 x *g* for 15 min, the supernatants were used for the 2DG measurements using 2DG

measurement kit (Cosmo Bio) according to the manufacture's instruction. As a positive control, 15 minutes before the 2DG injection, mice were treated 0.5U/kg body weight insulin by i.p injection.

In vitro study, cells were treated with 1 mM 2DG for 30 min, and then washed in ice-cold PBS for three times and collected in the Tris-HCl buffer. The cells were homogenized by ultrasonic homogenizer and heated at 80℃ for 15 min, centrifuged at 15,000 x *g* for 15 min. The supernatants were diluted in 5 folds, to use the subsequent measurements.

NAD/NADH measurement

Differentiated 3T3-L1 adipocytes were treated w/o 1mM NMN for 20 hours and frozen in LN2 immediately. The tissue samples were harvested and weighted, then snapped frozen in LN2. The samples were homogenized in the extraction buffer offered by NAD/NADH assay kit (ab65348, abcam). The measurement was performed following the manufacturer's instruction.

RNA-seq analysis

Total RNA was extracted using RNeasy Plus Universal Midi Kit (Qiagen) according to the manufacturer's instructions. RNA quality was measured with the Ribogreen method using Victor X2 fluorometry. Therefore, mRNA libraries were generated from these samples with the TruSeq Stranded mRNA Library Prep Kit, and paired end reads were sequenced on NovaSeq6000. RNA sequencing and data analysis were conducted by

Macrogen and CoMIT omics center (Osaka University).

Metabolomics

Approximately 200-300 mg of frozen subcutaneous white adipose tissue was milled by multi beads shocker, and then extracted in water/methanol/chloroform. Samples were centrifuged at 1,000 x *g* for 15 min, the supernatants were dried by Turbovap LV (Biotage), re-diluted in 10 % acetonitrile to subsequent analysis. LC/CE-MS were performed and analyzed by QTOF-MS (Agilent). Metabolome measurements were carried out at LSI Medience.

Statistical analysis

All data are presented as mean ± SEM. Differences between two groups were examined for statistical significance by the Student t test. A P value <0.05 denoted the presence of a statistically significant difference.

5. Results

5-1. Fasting glycolytic gene expressions

At the first step of glucose utilization, glucose is metabolized by glycolysis. To reveal the changes in the expression levels of glycolytic enzyme under fasting conditions, I used the open database obtained from NCBI's Gene Expression Omnibus through GEO Series accession number GSE46495. Figure.1 showed the expression levels of glycolytic enzyme. The 8 of these enzymes, hexokinase-2 (hk2), phosphofructokinase (pfkp), phosphoglycerate kinase-1 (pgk1), phosphoglycerate mutase-1 (pgam1), enolase-1 (eno1), pyruvate kinase (pkm), lactate dehydrogenase A (ldha) were significantly decreased in fasted group (Figure.1). It indicates that the expression levels of glycolytic genes, especially downstream of the pathway, were decreased under fasting conditions.

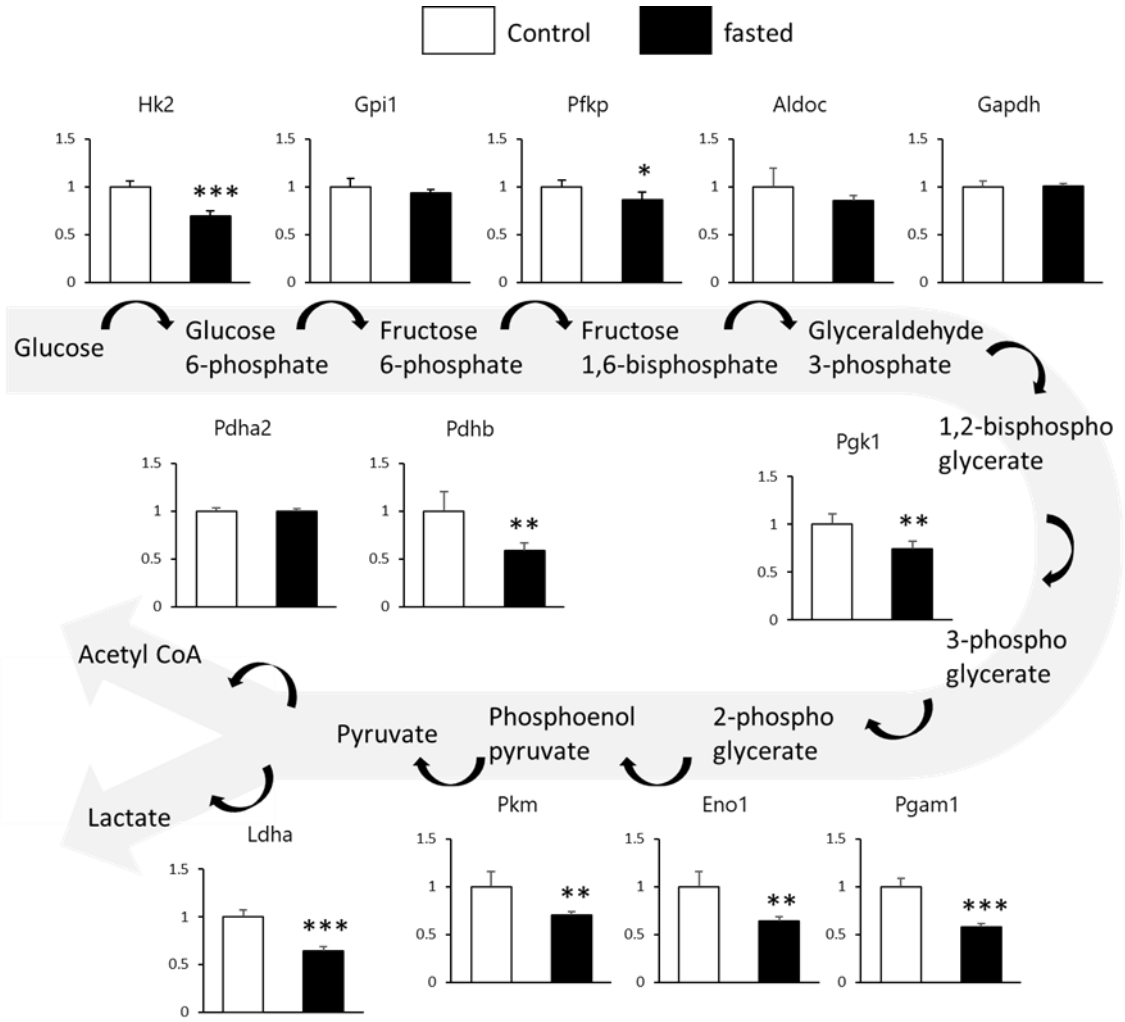


Figure.1 The expression levels of glycolytic enzymes decreased under fasting conditions

Relative mRNA levels associated with glycolytic pathway under fasting experiments (datasets series: GSE46495). Data are presented as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001.

5-2. LDHA expression

2-1. LDHA tissue distribution in mice

Under fasting conditions, expressions of 8 glycolytic enzymes were lower than adlib conditions in adipose tissue. I focused on Lactate dehydrogenase A (LDHA) as an enzyme responsible for the final step of the glycolysis.

I used the GTEx dataset to see the tissue distribution of LDHA gene expression levels in mice. The GTEx project is a comprehensive public resource to study tissue-specific gene expression and regulation collected from 54 non-diseased tissue sites across nearly 1,000 individuals, primarily for molecular assays including whole-genome sequencing, whole-exome sequencing, and RNA-Seq (Carithers et al. 2015). LDHA expression in adipose tissue was the third highest of all organs following skin and skeletal muscle (Supplemental Figure.1A). In addition, I measured the protein levels of LDHA in C57BL/6J mice. Western blot analysis revealed that the protein level of LDHA in skeletal muscle was the highest among all the tissues. And also, each white adipose tissue (subcutaneous, mesenteric, and epididymal) and brown adipose tissue showed high expressions followed by the skeletal muscle (Supplemental Figure.1B).

2-2. LDHA expression in the adipose tissue and adipocytes

Adipose tissue consists of the stromal vascular fraction (SVF) including macrophages and hematopoietic cells as well as the mature adipocyte fraction (MAF). Thus, to examine the abundance of LDHA gene

expression in adipose tissue, I separated murine subcutaneous adipose tissue into the SVF and the MAF fraction. To confirm the cross contamination, I measured the gene expression of adiponectin and dipeptidyl peptidase 4 (DPP4), which mainly expressed in the MAF and the SVF fraction respectively. I confirmed the successful fractionation of adipose tissues (Supplemental Figure.2A). In this condition, LDHA gene expression was significantly higher in the MAF than in the SVF (Supplemental Figure.2B).

In addition, I examined the LDHA expression before and after adipose differentiation to reveal the significance of LDHA in mature adipocytes. To this end, I isolated mouse primary preadipocytes from subcutaneous white adipose tissue of C57BL/6J mice, and differentiated them to mature adipocytes *in vitro*. mRNA expression of LDHA was significantly elevated in differentiated primary adipocytes (Day 7) compared with undifferentiated fibroblast (Day 0) (Supplemental Figure.2C left). Similar results were observed in differentiated (Day 7) and undifferentiated (Day 0) 3T3-L1 cells (Supplemental Figure.2C right). This suggests that LDHA expressions are promoted along with adipose differentiation. These results suggested that mature differentiated adipocytes expressed LDHA higher than preadipocytes.

2-3. The regulation of LDHA during drastic nutritional changes

In order to confirm the database results that LDHA expression reduced under fasting conditions, mice were subjected to fasting and

refeeding cycle each for 24 hours, and I compared the fated group with the refed group. Twenty-four hours fasting significantly reduced blood glucose levels, body weight (Figure.2A). In this condition, mRNA expressions of LDHA were reduced by fasting significantly especially in subcutaneous adipose tissue (Figure.2B). And also, western blot analysis demonstrated that LDHA expression were decreased under fasting conditions (Figure.2C). Furthermore, I found that Glut1 expression were reduced under fasting conditions (Figure.2C). Densitometry analysis showed that Glut1 protein levels in fasted group were reduced (Figure.2D). This suggests that glucose incorporation via Glut1 might decrease under fasting conditions.

2-4. Identification of regulatory factors for LDHA expression

I found that drastic nutritional changes, fasting-refeeding treatment significantly changed LDHA expression in adipose tissue, I wondered which factors were responsible for regulating LDHA expression. For that purpose, I measured mRNA expression of LDHA in 3T3-L1 adipocytes subjected to mimetic conditions. I found that mRNA expression of LDHA was upregulated treated with 10 nM insulin for 6 hours (Supplemental Figure.3A). However, gene expressions did not show significant differences between culturing in low (5.5 mM) and high (25 mM) glucose concentration for 20 hours (Supplemental Figure.3B). These results might indicate that reduction of LDHA expression under fasting conditions was partly due to the lack of insulin.

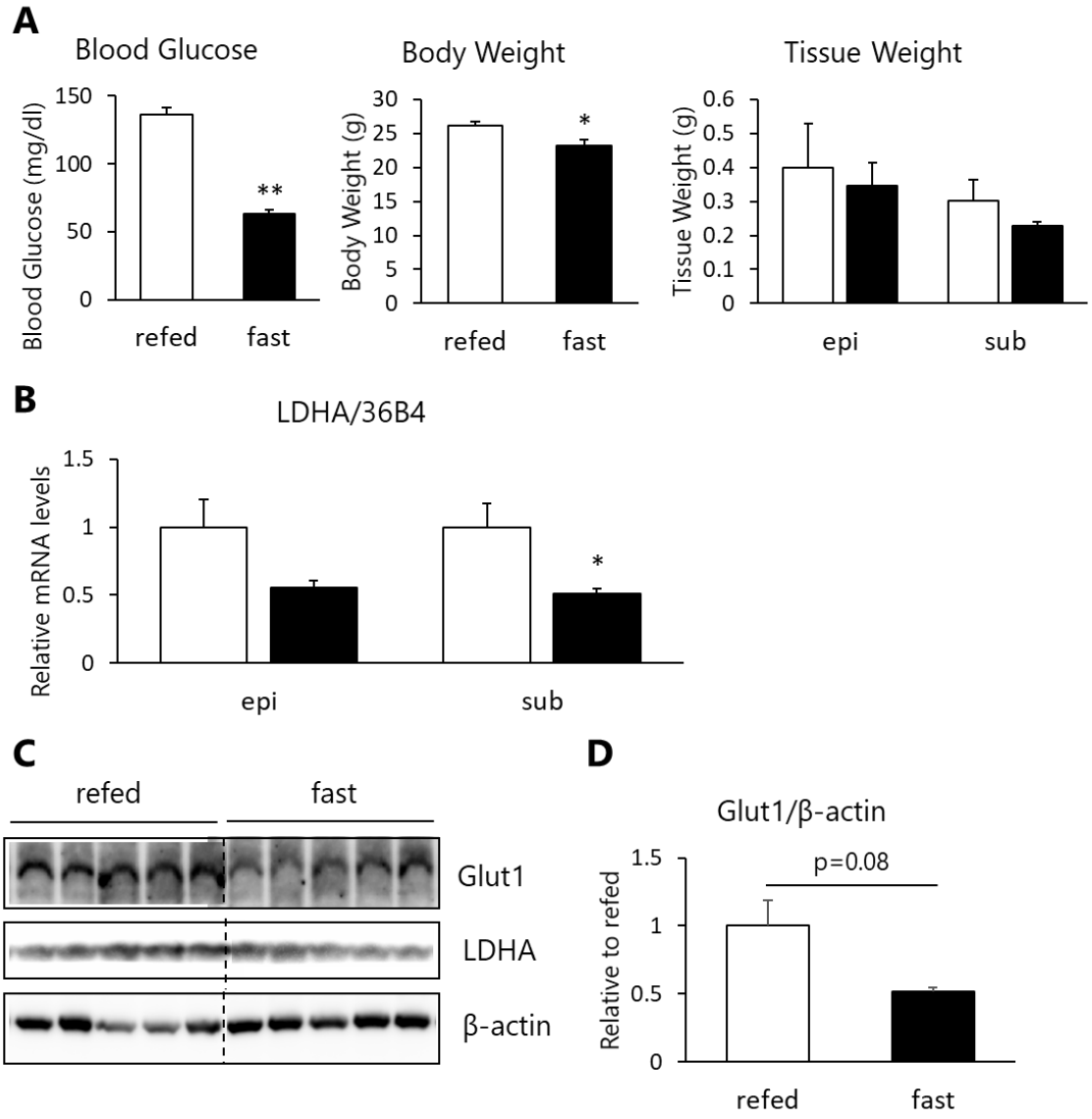


Figure.2 fasting feeding cycle affect the expression levels of LDHA and Glut1.

(A) Blood glucose levels of C57BL/6J mice during the fasting-refeeding cycle. Fasted group were compared with refed group. Body weight and tissue weight (epididymal and subcutaneous WAT) of C57BL/6J. n=5. (B) Gene expression levels of LDHA in epi WAT and sub WAT of C57BL/6J mice during fasting-refeeding cycle. n=5. (C) Protein expression levels of Glut1, LDHA and β-actin in subcutaneous WAT from C57BL/6J mice during the fasting-refeeding cycle. n=5. (D) densitometry analysis of Glut1 expression in western blotting. Data are presented as mean ± SEM. *p<0.05, **p<0.01.

5-3. Generation of adipocyte-specific LDHA knockout (AdLDHAKO) mice

3-1. Adipocyte specific LDHA-deficient mice were successfully generated

I found that the reduction of LDHA gene in adipose tissue under fasting conditions. Thus, in order to reveal the relationship between downregulation of LDHA and Glut1 under fasting conditions, I generated adipocyte-specific LDHAKO mice (AdLDHAKO) by crossing LDHA flox/flox mice (Wang et al. 2014) with adiponectin-Cre mice (Eguchi et al. 2011). In AdLDHAKO mice, Cre-mediated recombination occurred specifically in gonadal (gWAT), subcutaneous (subWAT), and brown adipose tissues (BAT), but not in the liver or the skeletal muscle (Figure.3A). The expression levels of LDHA gene in AdLDHAKO was decreased to 10% of that in control (flox/flox) mice in WAT and BAT, while there are no significant differences in the liver and the skeletal muscle (Figure.3B). The similar results were observed in the protein levels of LDHA (Figure.3C). These results indicated that AdLDHAKO mice were generated successfully with reduced LDHA expression only in adipose tissues.

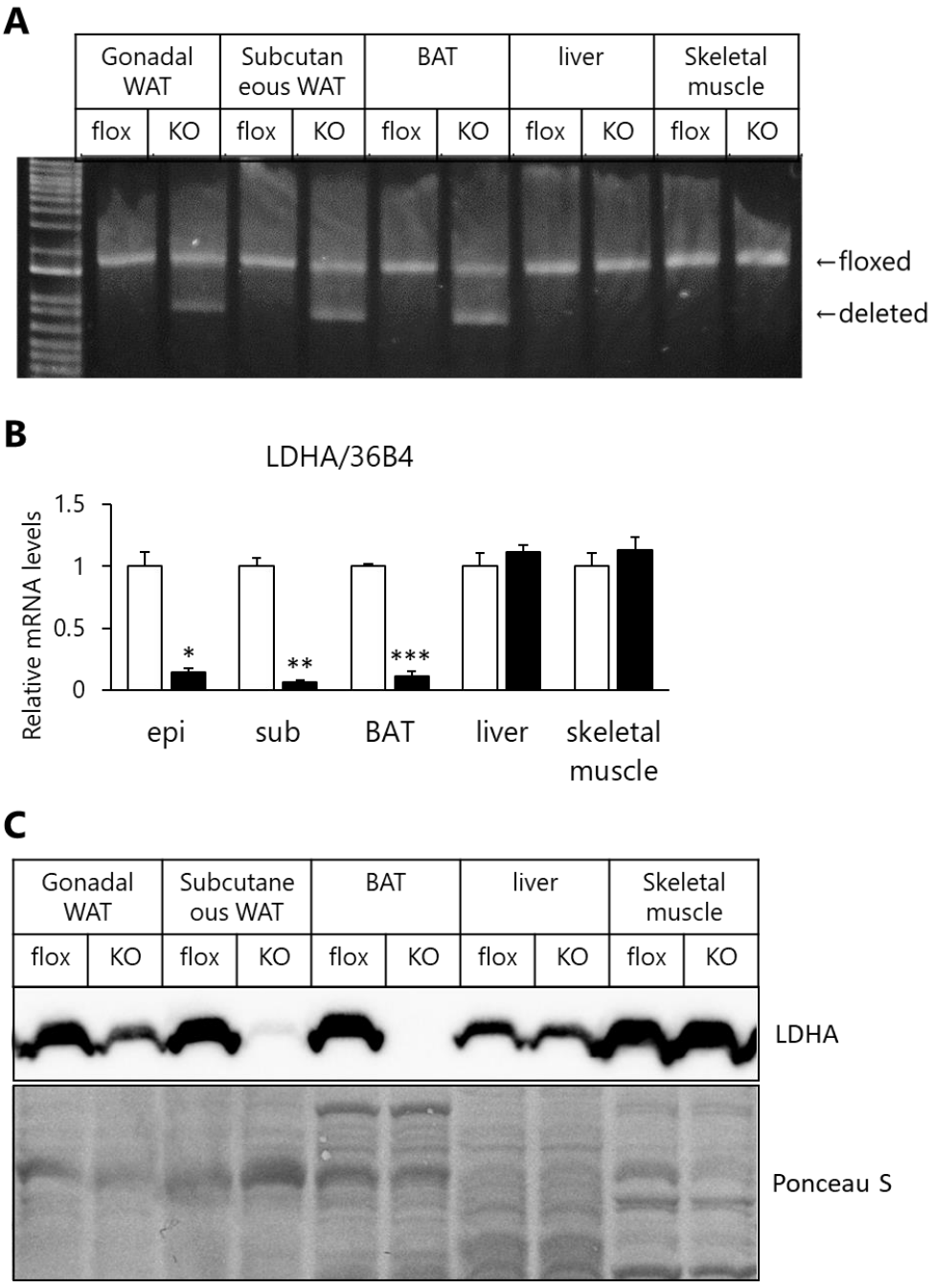


Figure.3 Generation of Adipocyte-specific LDHA knockout mice
(A) Cre-mediated recombination assessed by PCR. The upper bands are floxed alleles, and the lower bands are null alleles. (B) LDHA gene expression levels in each tissue from LDHA floxed and AdLDHAKO mice. n=3. (C) LDHA protein levels in tissues from floxed and AdLDHAKO mice. ponceau S staining confirms loading same amounts of samples. Data are presented as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001.

3-2. AdLDHAKO mice showed normal phenotypes.

AdLDHAKO mice were born at the expected Mendelian ratio, and appeared to be grossly normal, with no apparent differences in body weight (Supplemental Figure.4A), tissue weight (Supplemental Figure.4B), compared with the control mice. Next, I performed global gene expression analysis by RNA-sequencing to find genetic changes without any bias. Supplemental Figure.4C shows the representative gene expressions related to adipocyte functions, such as adipogenesis, adipocytokine, lipogenesis, lipolysis, and glucose transporters. It displays that LDHA deficiency in adipocytes had no obvious effects on gene expressions regarding adipocyte functions (Supplemental Figure.4C).

In addition, since the LDHA is a glycolytic enzyme, I speculated that glucose metabolism might be affected in AdLDHAKO mice. I conducted a comprehensive metabolomic analysis to measure the metabolites that are involved in glycolysis, TCA cycles, and the pentose phosphate pathway using LC/CE-MS. Surprisingly, all the metabolites in these pathways, including lactic acid, did not change in subcutaneous adipose tissue from AdLDHAKO mice compared to control mice (Supplemental Figure.4D). This result suggested that LDHA deficient in adipose tissue caused no effects on metabolic pathway itself.

5-4. Glucose metabolism in AdLDHAKO mice

4-1. LDHA deficiency caused decrease of Glut1 protein

I measured expression of glucose transporters, especially Glut1, which was responsible for insulin-independent glucose uptake. RNA-seq analysis demonstrated that there were no differences in the levels of Glut1 (Slc2a1) and Glut4 (Slc2a4) expression in adipose tissue between AdLDHAKO and LDHA flox (Supplemental Figure.4C). However, western blot analysis showed the reduction of Glut1 protein significantly in adipose tissues of AdLDHAKO mice compared with control in normal conditions (Figure.4A). Densitometry analysis demonstrated the expression of Glut1 decreased to 50 % in AdLDHAKO compared to control mice (Figure.4B). These results suggest that Glut1 is regulated by LDHA at the protein level, but not mRNA level.

To further confirm the relationship between LDHA and Glut1 protein, I performed siRNA-mediate gene silencing of LDHA in 3T3-L1 adipocytes. The expression of LDHA was significantly reduced by siRNA (Supplemental Figure.5A). LDHA knockdown in 3T3-L1 adipocytes also exhibited reduced Glut1 protein levels (Figure.4C). In addition, to further elucidate the mechanism regulating Glut1 protein levels independent of mRNA levels, I examined protein degradation of Glut1. Treatment with Bafilomycin A1, an inhibitor of protein degradation in lysosomes, rescued Glut1 protein levels reduced by transfected with siRNA of LDHA in 3T3-L1 adipocytes (Figure.4D). On the other hand, MG132, an inhibitor of proteasomal protein degradation, did not restore Glut1 protein levels

(Supplemental Figure 5B). These results suggest that LDHA deficiency induces lysosomal degradation of the Glut1 protein. This suggests that LDHA deficiency could upregulate lysosomal degradation of Glut1.

LDHA produces lactate and NAD, therefore I speculated that these factors might be responsible for Glut1 degradation in LDHA-deficient cells. Interestingly, the protein levels of Glut1 protein were not changed by lactate treatment in 3T3-L1 adipocytes (Supplemental Figure.5C). It indicates that LDHA deficiency promoted Glut1 degradation independent of lactate levels in adipose tissue. LDHA deficiency regulates Glut1 protein possibly independent of lactate signaling pathway, such as cell surface receptor.

Furthermore, I found that the amount of nicotinamide adenine dinucleotide (NAD) was significantly lower in adipose tissue from AdLDHAKO mice during metabolomic analysis (Supplemental Figure.5D). Furthermore, I found that the amount of nicotinamide adenine dinucleotide (NAD) and NAD/NADH ratio was significantly lower in adipose tissue from AdLDHAKO mice than in adipose tissue from control mice (Supplemental Figure.5E). LDHA knockdown in 3T3-L1 adipocytes also showed the same reduction in NAD content and NAD/NADH ratio (Supplemental Figure 5F). I used nicotinamide mononucleotide (NMN) treatment in 3T3-L1 adipocytes to rescue the amount of intracellular NAD and to determine the effects of NAD reduction on Glut1 degradation. NMN increased the NAD content (Supplemental Figure.5G). Even under this condition, the reduction in Glut1 protein was not rescued (Supplemental

Figure.5H). These results suggest that Glut1 protein levels were not regulated by NAD content or redox state in adipose tissue.

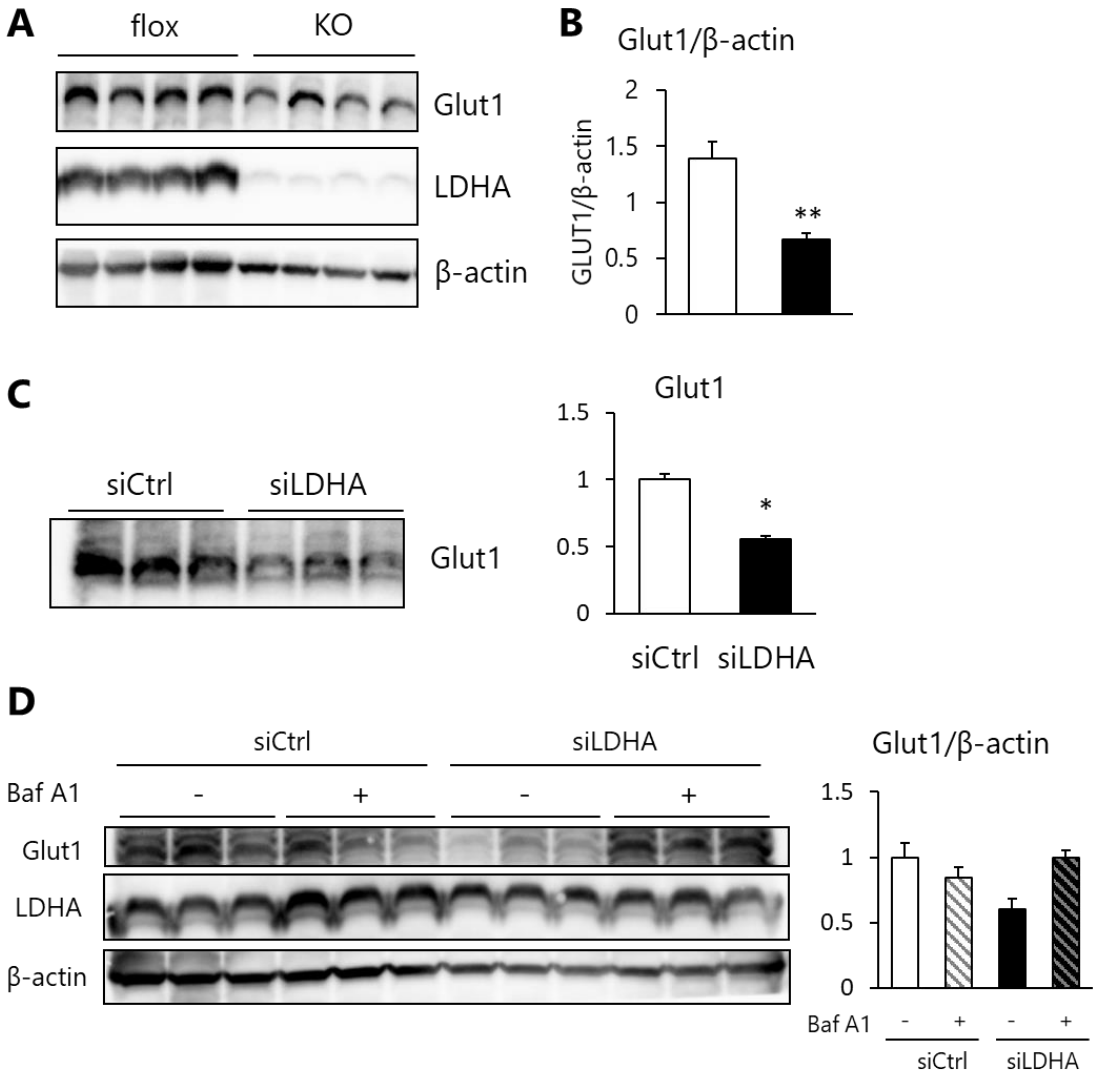


Figure.4 Glut1 protein reduced in LDHA deficient adipocytes

(A) Protein expression levels of Glut1, LDHA and β-actin in subcutaneous WAT from LDHA floxed and AdLDHAKO mice. n=7-8. (B) densitometry analysis of Glut1 expression in western blotting. (C) Glut1 expressions in 3T3-L1 adipocytes treated with siRNA(left), and densitometry analysis of the western blotting (right). n=4. (D) western blotting analysis in 3T3-L1 adipocytes treated w/o Bafilomycin A1 after LDHA gene silenced by siRNA (left), and the densitometry analysis of the western blotting (right). n=4. Data are presented as mean ± SEM. *p<0.05, **p<0.01.

4-2. AdLDHAKO mice showed decreased glucose uptake into adipose tissue

As Glut1 protein expression decreased in adipose tissue from AdLDHAKO mice, I wonder the amount of glucose incorporation into adipose tissue decrease in AdLDHAKO mice. Thus, I established the *in vivo* glucose uptake assay using 2-deoxyglucose (2DG) so as to measure glucose incorporation into adipose tissue *in vivo*. Briefly explained, mice were fasted for 4 hours, and 1, 5, 25 μmol 2DG were injected by ip injection. 30 minutes later, tissue samples were collected and the amount of 2-deoxy glucose-6-phosphate (2DG6P), that were not metabolized and accumulated in the cells, were measured. As positive control, 0.5 U/kg body weight insulin was injected 30 minutes in advance to 2DG administration. Both in epididymal and subcutaneous adipose tissue, the amount of incorporated 2DG increased in a dose-dependent manner, and insulin stimulation dramatically upregulated the 2DG uptake (Figure.5A). I concluded that this method works properly for 2DG uptake assay *in vivo*. I used this method for the analysis of AdLDHAKO mice. AdLDHAKO mice exhibited decreased glucose uptake compared to control mice especially in subcutaneous adipose tissue (Figure.5B). In addition, I measured the effects of LDHA deficiency on glucose uptake *in vitro*. Basal glucose uptake was also significantly reduced in siLDHA compared with siContorl cells (Figure.5C). These results indicated that LDHA deficiency results in reduced glucose uptake in basal conditions.

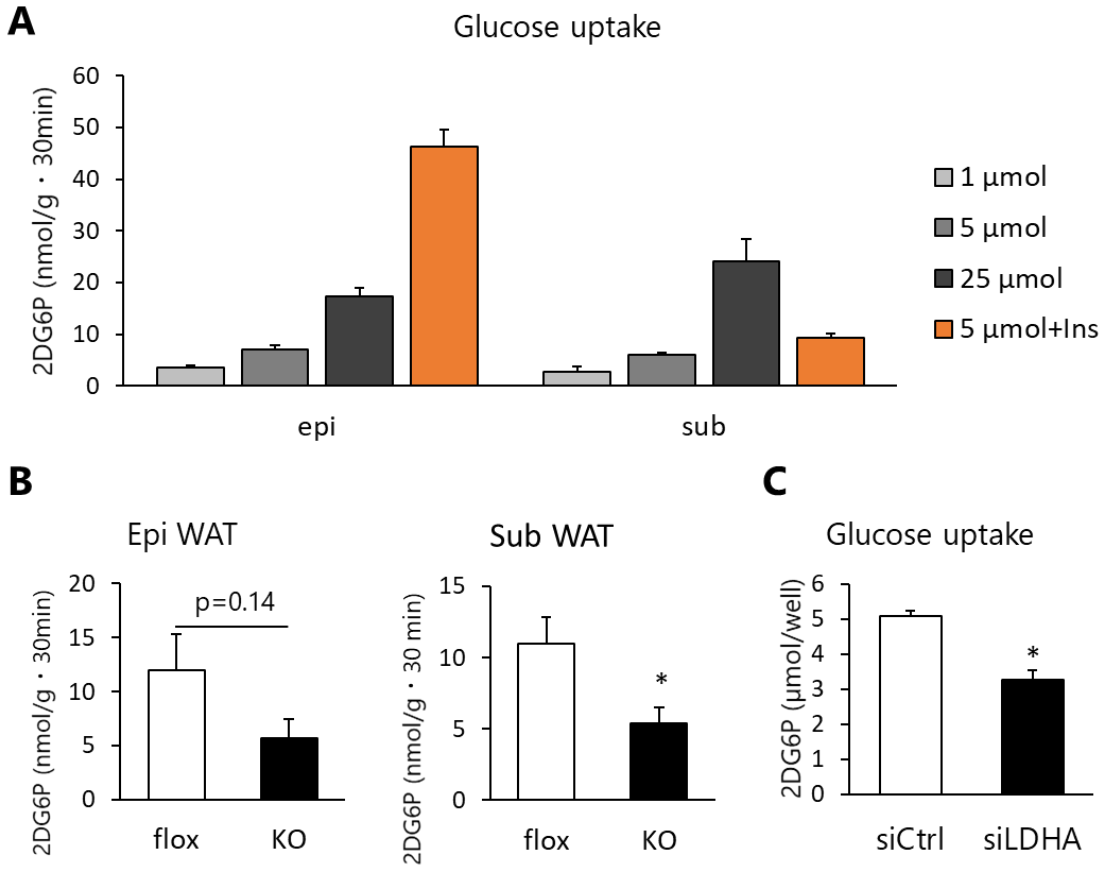


Figure.5 Glucose incorporation into adipocytes in LDHA deficient
(A) 2DG incorporation into adipose tissue. n=3. (B and C) 2DG incorporation into (B) WAT (epididymal and subcutaneous) in floxed and AdLDHAKO mice, and (C) 3T3-L1 adipocytes the gene LDHA was silenced. n=8, and 4. Data are presented as mean ± SEM. *p<0.05.

6. Discussion

Here, I revealed that the expression of enzymes associated with glycolysis pathway decreased in mice adipose tissue under fasting conditions. Especially, LDHA protein levels reduces in parallel with Glut1 protein in adipose tissue under fasting conditions. Furthermore, by using gene modified mice, I found that LDHA-deficiency caused decrease of Glut1 protein and glucose incorporation into adipose tissue.

Changes in adipose tissue under fasting conditions.

The adaptive responses to fasting is necessary to survive during fasting periods. Each metabolic organ switches to keep maintaining blood glucose level in a coordinated manner. Nowadays, some global transcriptome analysis demonstrates the dramatical changes in adipose tissue under fasting condition both in human and rodents. Many biological pathways are influenced by fasting conditions (Fazeli et al. 2020; Defour et al. 2020). In the current study, I found that the glycolysis pathway was downregulated under fasting conditions. Since the downregulated genes included the rate-limiting enzymes, hk2, pfkp, and pkm, the flow might reduce as a whole. In this study, we focused on LDHA as the final step of glycolysis, and revealed that LDHA-deficiency itself caused reduction of glucose uptake. The metabolomic analysis showed no differences by LDHA deficiency, thus this phenomenon was not caused by the disruption of glycolysis.

LDHA regulation

In the current study, I found that fasting caused reduced expression of LDHA in adipocytes. There had been no reports regarding the regulation of LDHA in adipocytes. Many studies have shown the regulation of LDHA, especially in myocytes and cancer cells.

During hypoxic conditions, cell metabolism undergoes a shift from oxidative phosphorylation to glycolysis. Hypoxia Inducible Factor (HIF) 1α is a master regulator of hypoxia-induced gene responses, regulating LDHA expression through two consensus sequences in promoter regions (Firth, Ebert, and Ratcliffe 1995; Semenza et al. 1996). In cancer cells, Myc contributes the metabolic shift to glycolysis by inducing LDHA expression through two consensus sequences in the promoter region (Shim et al. 1997). Peroxisome proliferator-activated receptor-gamma coactivator -1alpha (PGC-1α) also controls lactate production via regulating LDHA/LDHB expression in myocytes, and regulates muscle contraction (Summermatter et al. 2013). Regarding physiological regulation of LDHA, exercise induces LDHB, but not LDHA (Liang et al. 2016).

In the current study, I found that insulin stimulation upregulated the LDHA expression *in vitro*. Previously reported, in colorectal cancer, inhibition of Akt/mTOR/HIF1α signaling downregulates expressions of glycolytic enzymes including LDHA (Weng et al. 2020). I suppose there is a similar mechanism associated with downregulation of LDHA through insulin signaling in adipocytes.

LDHA itself regulates Glut1 protein.

It has been well reported that Glut1 and LDHA have common regulatory mechanisms. Under hypoxia, metabolic switch shifts glucose metabolism from the mitochondria to anaerobic glycolysis to maintain ATP, and both LDHA and Glut1 are induced by HIF1α (Semenza et al. 1996; Nagao et al. 2019). Moreover, as positive feedback of Warburg effect, pyruvate kinase M2 (PKM2) induces c-Myc resulting in the upregulation of both LDHA and Glut1 (Yang et al. 2012). These reports indicate the transcriptional regulation of LDHA and Glut1. In addition to these reports, lactate regulates translocation of Glut1 to the plasma membrane via a non-PI3K-mediated pathway in the rat heart (Medina et al. 2002). In the current study, I revealed for the first time that LDHA itself regulates Glut1 protein expression directly by regulating protein degradation. Moreover, I demonstrated that LDHA influences glucose uptake in adipocytes. Taken all things together, LDHA might control glucose homeostasis through regulating Glut1 protein.

In refeeding conditions, Glut1 synthesis is upregulated, and glucose deprivation causes the decrease of protein synthesis on the contrary (McMahon and Frost 1995) . On the other hand, Glut4 protein levels in the epididymal adipose tissue were dramatically decreased after 48 hours fasting and rescued by refeeding in the rat study (Im et al. 2006). In the current study, I demonstrated the decrease of Glut1 protein under fasted state in C57BL/6J mice. Glut1 might be regulated not in mRNA levels but in protein levels through the nutritional changes.

In this study, bafilomycin A1 treatment rescued the decrease of Glut1 protein in LDHA-deficient adipocytes, thus I concluded that LDHA-deficiency promoted the lysosomal degradation of Glut1. As previously reported, LDHB overexpression in cancer cells supports lysosomal acidifications and it plays essential role for lysosomal activity and autophagy by offering protons via pyruvate-lactate conversion (Brisson et al. 2016). Considered together, I supposed that LDHA-deficiency also promotes lysosomal activity by its acidification.

Furthermore, considering there were no changes in Glut1 expression in siControl cells w/o bafilomycinA1 treatment, LDHA might regulate the fate of Glut1 affecting its translocation into lysosome.

The enzyme involved in glycolysis regulates glucose homeostasis.

LDHA is an important glycolytic enzyme in adipocytes (Digirolamo, Newby, and Lovejoy 1992), and I revealed for the first time that its deficiency results in reduced glucose uptake in adipocytes. As previously reported, one of the LDHA inhibitor, Oxamate administration improves glucose metabolism of a diabetic model mouse in a dose-dependent manner (Ye et al. 2016). They concluded that oxamate treatment improves insulin secretion along with the changes on islet morphology. Muñoz et al. reported the effect of glucokinase, a key glycolytic enzyme catalyzing the first step of glycolysis, on glucose uptake in adipocytes (Muñoz et al. 2010). Adipocyte-specific glucokinase overexpression showed higher glucose uptake with or without insulin stimulation in

adipocytes. These mice exhibited improved glucose tolerance and insulin resistance. On the other hand, I measured the amount of Glucose 6-phosphate in adipocytes whether LDHA deficiency affects on the glucose flux. Interestingly, there were no changes in intracellular G6P contents between siCtrl and siLDHA treated adipocytes (data not shown). This indicated that glucose flux did not alter even when glucose conversion into lactate were reduced.

In AdLDHAKO mice, Glut1 protein levels were decreased with reduced basal glucose uptake. In adipocytes, Glut1 and Glut4 facilitates diffusion of glucose into the cell. Glut1 is responsible for basal glucose uptake, and insulin stimulates glucose uptake for 20-30-folds by recruiting Glut4 from an intracellular compartment to the cell surface (Bogan and Lodish 1999). In RNA-seq analysis, we found that Glut4 expresses 20 folds (flox mice), and 5-folds (3T3-L1 adipocytes) comparable to Glut1 respectively (data not shown). It suggested that the contribution of Glut1 were less than Glut4 especially in mice. Considering these things together, reduction of basal glucose uptake by Glut1 might be compensated by insulin-stimulated glucose uptake by Glut4 in AdLDHAKO mice and it could explain for no changes in adipose tissue weight.

7. Conclusion

In the current study, I demonstrated the regulation of glucose incorporation into adipose tissue under fasting conditions. The expression levels of several glycolytic genes reduced in adipose tissue under fasting conditions. Furthermore, I elucidated that deficiency of LDHA caused reduction of Glut1 protein and subsequent glucose uptake by using gene modified mice.

Further research will be needed for revealing the detailed regulation of LDHA under fasting conditions. It will offer a new discovery for understanding the regulation of LDHA independent from hypoxia.

Moreover, it is also necessary to find the mechanism underlying degradation of Glut1. Using the labeling tool of surface Glucose transporter in living cells (Hirayama et al. 2016), I hope to reveal the mechanism of LDHA to regulate expression levels of Glut1.

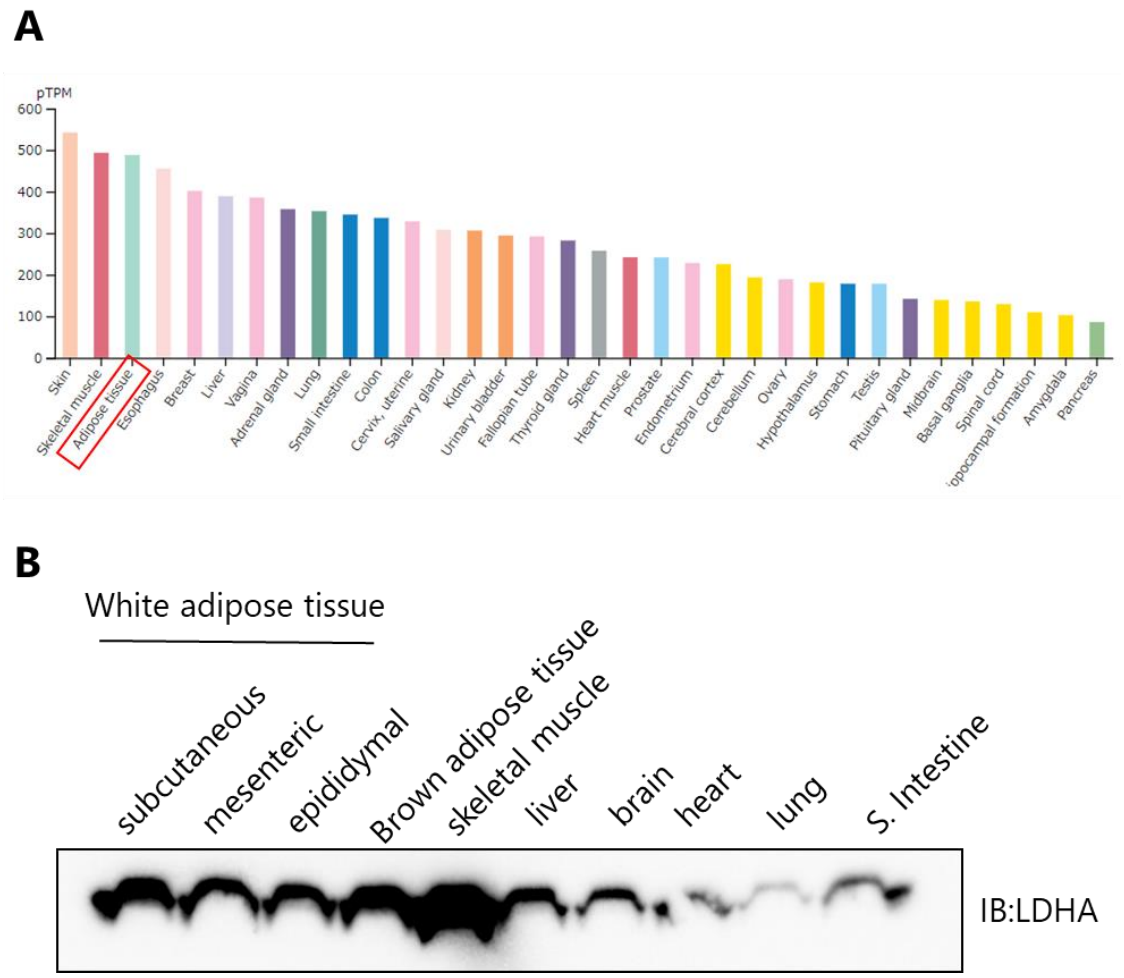
8. Supplemental figures

Supplemental Table1. Primers for real-time qPCR

Adiponectin	Forward	GTTCTACTGCAACATTCCGG
	Reverse	TACACCTGGAGCCAGACTTG
DPP4	Forward	GTGGCAAGAGGGGATCACTA
	Reverse	CCCAGCCTGTGGTACTCATT
LDHA	Forward	AATGAAGGACTTGGCGGATGAG
	Reverse	CAGCTTGCAGTGTGGACTGT
36B4	Forward	GCTCCAAGCAGATGCAGCA
	Reverse	CCGGATGTGAGGCAGCAG

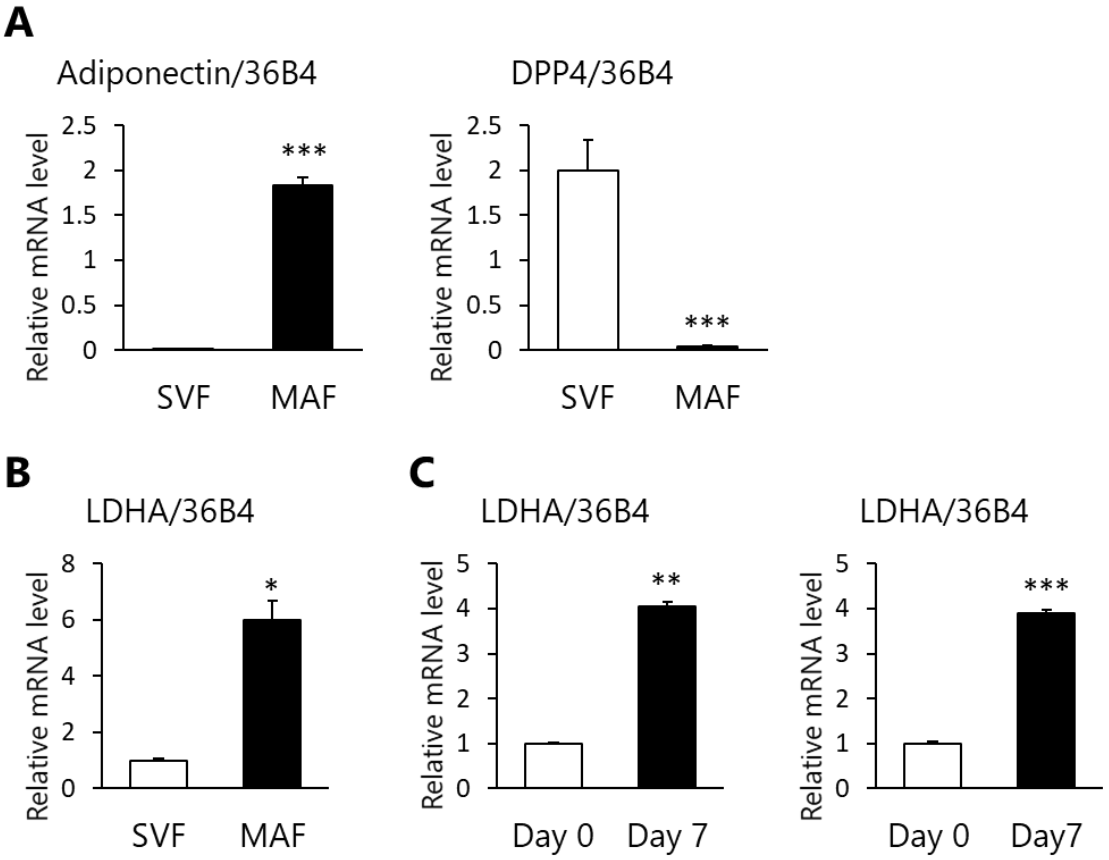
Supplemental Table2. Primary antibody for western blotting.

Product	Supplier	Code	Host	Dilution
Monoclonal Anti-β-Actin antibody produced in mouse	Sigma-Aldrich	A5441	Mouse	1:5000
Anti-Glucose Transporter GLUT1 antibody [EPR3915]	Abcam	ab115730	Rabbit	1:1000
LDHA-Specific Antibody	Proteintech	19987-1-AP	Rabbit	1:1000



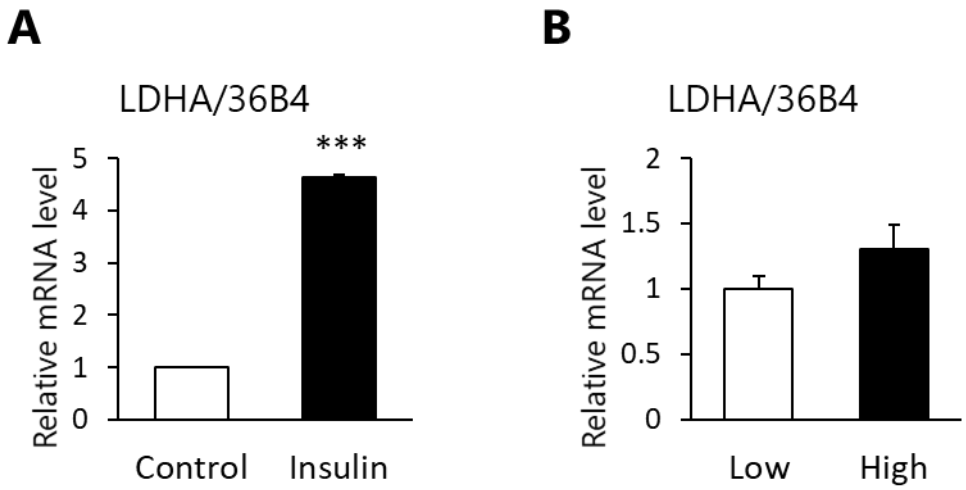
Supplemental Figure.1 Tissue distribution of LDHA

(A) Gene expression of LDHA data from the GTEx. (B) Protein expression levels of LDHA in each tissue (subcutaneous WAT, mesenteric WAT, epididymal WAT, brown adipose tissue, skeletal muscle, liver, brain, heart, lung, and small intestine) of male C57BL/6J mice.



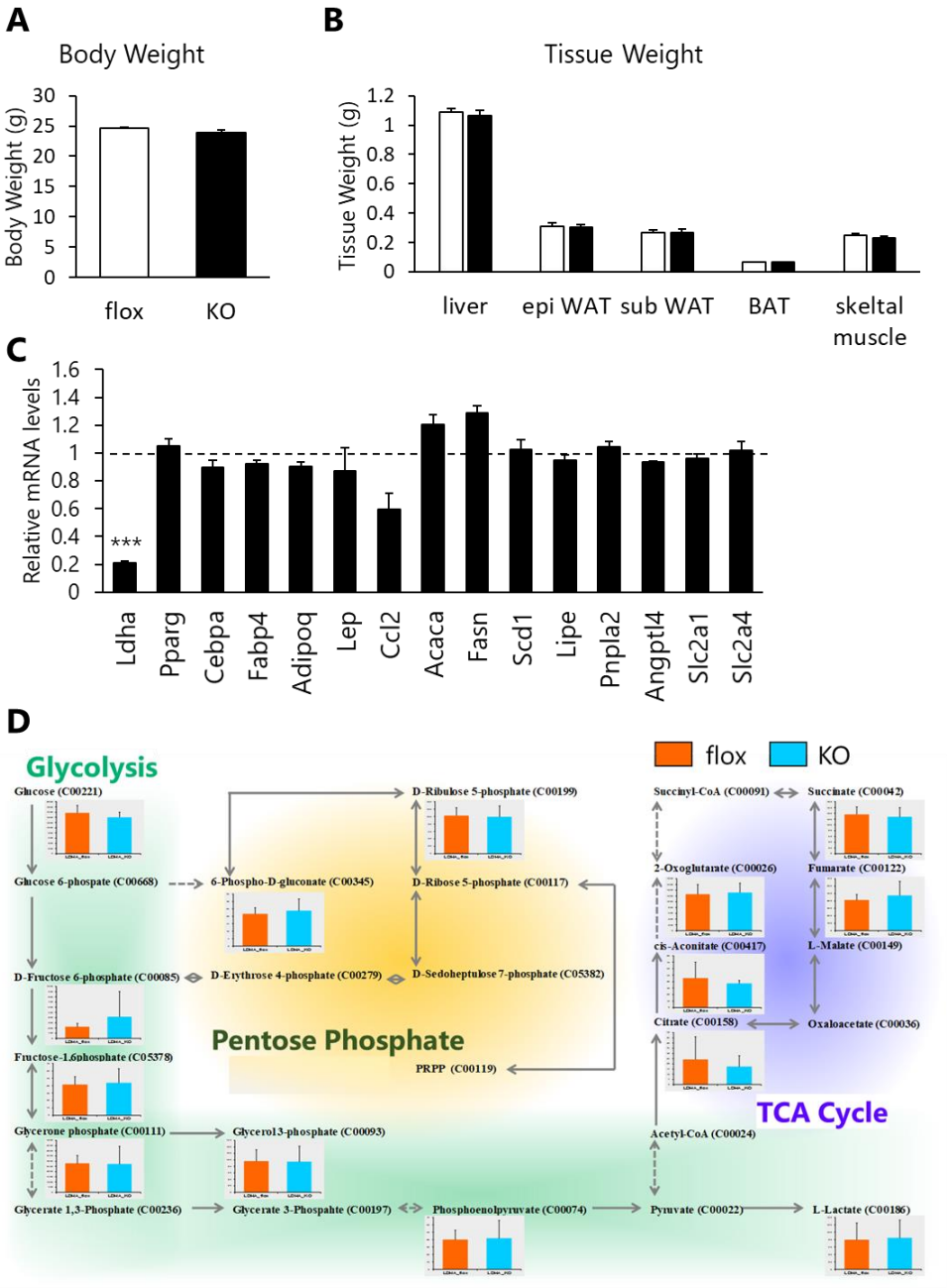
Supplemental Figure.2 LDHA expression levels in adipose tissue and adipocytes

(A) Gene expression levels of adiponectin and DPP4 in each adipose tissue fraction. n=6. (B) Gene expression level of LDHA in each fraction of adipose tissue (stromal vascular fraction, and mature adipocyte fraction). n=5. (C) Gene expression level of LDHA during adipocyte differentiation in 3T3-L1 adipocytes (left) and mouse primary adipocytes (right). n=3. Data are presented as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001.



Supplemental Figure.3 The regulation of LDHA expression in adipocytes

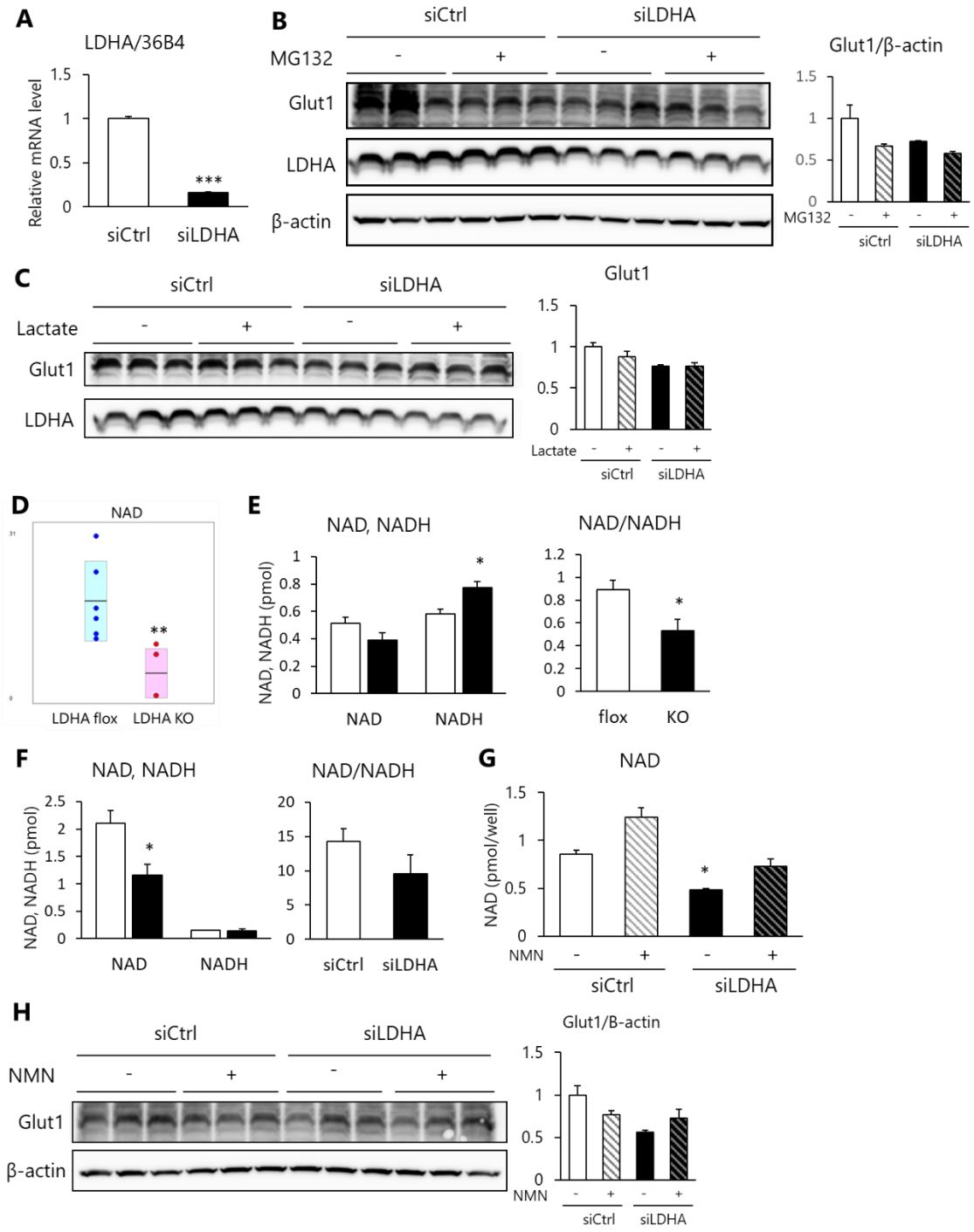
Gene expression level of LDHA in 3T3-L1 adipocytes treated with (A) insulin, (B) glucose concentration. n=3. Data are presented as mean ± SEM. ***p<0.001.



720

Supplemental Figure.4 The phenotypes of AdLDHAKO mice

(A and B) (A) Body weight and (B) tissue weight of LDHA floxed and AdLDHAKO mice on a normal diet. n=6. (C) the amount of the metabolites in the subcutaneous adipose tissue. Data from the global metabolomic analysis. n=6. (C) mRNA expression levels of genes related to adipocyte functions in subcutaneous adipose tissue. The bars indicate the relative number normalized by the floxed mice. n=3. Data are presented as mean ± SEM. *p<0.05, **p<0.01 ***p<0.001.



Supplemental Figure.5 Glut1 protein in 3T3-L1 adipocytes

(A) the LDHA gene expression in 3T3-L1 adipocytes treated with siRNA. n=4. (B) Protein expression levels of Glut1, LDHA and β-actin in 3T3-L1 adipocytes treated with MG132 for 3 hours after the LDHA gene was silenced by siRNA. (C) Protein expression levels of Glut1 and LDHA in 3T3-L1 adipocytes treated with sodium lactate for 20 hours after the LDHA

gene was silenced by siRNA. (D) NAD content of subcutaneous WAT in metabolomic analysis (n=6). (E) The amount of NAD and NADH (left) and the ratio of NAD/NADH (right) in subcutaneous WAT from LDHA flox and AdLDHAKO mice (F) The amount of intracellular NAD and NADH (left) and the ratio of NAD/NADH (right) in 3T3-L1 adipocytes treated with siRNA (n=4). (G and H) (G) The amount of intracellular NAD and NADH (H) protein expression of Glut1, LDHA and β-actin in 3T3-L1 adipocytes treated w/o NMN after LDHA gene were silenced by siRNA (n=3).Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

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11. Record of research achievements

11-1. Conference presentation

Date 2018/8	Conference Name (City, Country) 第 23 回アディポサイエンス・シンポジウム (大阪)
Conference type Domestic	Presentation form Poster
Presentation Title 脂肪細胞における乳酸産生の意義	

Date 2018/9	Conference Name (City, Country) 第 91 回日本生化学会大会（京都）
Conference type Domestic	Presentation form Oral
Presentation Title 乳酸産生臓器としての脂肪組織の生理学的・病態学的意義の解明	

Date 2018/10	Conference Name (City, Country) 第 39 回日本肥満学会 (神戸)
Conference type Domestic	Presentation form Oral
Presentation Title 乳酸産生臓器としての脂肪組織の生理学的・病態学的意義の解明	

Date 2019/5	Conference Name (City, Country) 第 62 回日本糖尿病学会年次学術集会 (仙台)
Conference type Domestic	Presentation form Oral
Presentation Title 乳酸の脂肪細胞機能に対する作用	

Date 2019/7	Conference Name (City, Country) 第 11 回大阪内分泌代謝研究を語る会 (大阪)
Conference type Domestic	Presentation form Oral
Presentation Title 乳酸産生臓器としての脂肪組織の生理学的・病態学的意義の解明	

Date 2019/8	Conference Name (City, Country) 第 24 回アディポサイエンス・シンポジウム (大阪)
Conference type Domestic	Presentation form Poster
Presentation Title 乳酸による脂肪細胞機能制御	

Date 2019/11	Conference Name (City, Country) 第 40 回日本肥満学会 (東京)
Conference type Domestic	Presentation form Oral
Presentation Title 乳酸の脂肪細胞機能に対する作用	

Date 2020/7	Conference Name (City, Country) 第 93 回内分泌学会(web 開催)
Conference type Domestic	Presentation form Oral
Presentation Title 脂肪細胞乳酸産生酵素 LDHA による脂質代謝制御	

Date 2021/3	Conference Name (City, Country) 第 41 回日本肥満学会 (web 開催)
Conference type Domestic	Presentation form Oral
Presentation Title 脂肪細胞乳酸産生は脂肪細胞への糖取り込みを制御する	

Date 2021/5	Conference Name (City, Country) 第 64 回日本糖尿病学会年次学術集会 (web 開催)
Conference type Domestic	Presentation form Oral
Presentation Title 脂肪細胞乳酸産生は脂肪細胞への糖取り込みを制御する	

880

11-2. Journal publication

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