— Rapid Communication —

# Differential Effects of Two Types of Obesity on Ketone Body Utilization in Skeletal Muscle

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**Previously, we showed that obesity affects the gene expression of acetoacetyl-CoA synthetase (AACS), the enzyme responsible for the utilization of ketone bodies for lipid synthesis, in adipocytes. Therefore, we examined whether obesity also changes the AACS level in skeletal muscle. AACS mRNA expression was decreased in the skeletal muscle of leptin-deficiency-induced obese rats. In undifferentiated C2C12 myoblast cells, a high concentration of glucose induced the expression of AACS mRNA but decreased the AACS level in differentiated C2C12 cells. Moreover, leptin directly increased the AACS mRNA level in C2C12 myocytes. The expression level of succinyl-CoA: 3-oxoacid CoA-transferase (SCOT), another enzyme that induces ketone body consumption, was not changed in C2C12 cells. Our results suggest that AACS gene expression is differently affected by leptin and glucose during muscle differentiation and leptin plays a regulatory role not only in lipid consumption but also in ketone body utilization via AACS in skeletal muscle.**

**Key words** —— acetoacetyl-CoA synthetase, obesity, leptin, ketone body utilization

## INTRODUCTION

Overnutrition induces obesity and causes various physiological disorders, such as unusual metabolism of lipids and ketone bodies.[1] For example, hepatic ketogenesis and $\beta$-oxidation are strongly impaired in the genetically obese Zucker rat.[2] On the other hand, in the case of dietary obesity, long-term diet-induced obesity increases ketogenesis but decreases lipogenesis in hepatocytes.[3] Therefore, regulation of lipid and ketone body metabolism seems to differ according to the type of obesity.

Ketone bodies, D(−)-$\beta$-hydroxybutyrate and acetoacetate, are important energy sources, and mitochondrial succinyl-CoA: 3-oxoacid CoA-transferase (SCOT; EC 2.8.3.5) has been regarded as the enzyme responsible for the activation of acetoacetate for energy generation.[4] However, in the cytosol, acetoacetate is known to be directly activated through the ligase reaction catalyzed by acetoacetyl-CoA synthetase (AACS; acetoacetate-CoA ligase, EC 6.2.1.16)[5,6] for the synthesis of biologically important lipidic substances, such as cholesterol and fatty acids.[7] Previously, we showed that AACS is highly expressed in lipogenic tissues, such as brain, liver, and adipose tissue.[6,8,9] In rodent adipose tissues, AACS mRNA expression is abundant in the male subcutaneous white adipose tissue (WAT), and is increased during adipogenesis.[9] These findings suggest that AACS plays important roles in lipogenesis from ketone bodies. However, the pathological importance as well as physiological role of AACS in lipolytic tissues, such as skeletal muscle, has yet to be clarified.

The different types of obesity induce different effects on energy metabolism. In fact, we have demonstrated that the gene expression patterns of AACS and other lipogenic enzymes in WATs are different between genetically obese and dietary obese rats.[10] Thus, the question arises of how AACS is involved in the obesity-induced metabolic disorders in lipid consumption in skeletal muscle. To clarify the effect of obesity on ketone body metabolism of muscle, we investigated whether the two different types of obesity affect the gene expression of these enzymes in soleus muscle.

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## MATERIALS AND METHODS

**Animals** —— The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Genetically Obese Rat** —— Three pairs of lean and obese male Zucker rats (8 weeks old, Sankyo Labo Service Co., Tokyo, Japan) were killed after acclimatization for at least 3 days. The soleus muscle was excised for Northern blot analysis.

**High Fat Diet-induced Obese Rat** —— Four-week-old male rats of the Sprague-Dawley (SD) strain (Tokyo Laboratory Animals Science Co., Tokyo, Japan) were used after acclimatization for at least 3 days. They were given food and tap water *ad libitum* and maintained in a light-dark cycle of 12 hr (light on at 8 a.m.). To induce obesity nutritionally, rats were given a high-fat chow (type F2HFD2, Oriental Yeast Co., Tokyo, Japan; 60.0% fat, 24.5% protein, and 7.5% carbohydrate) for 6 weeks. Control rats were fed a regular chow (type MF, Oriental Yeast Co.; 5% fat, 24% protein, and 54% carbohydrate) for the same period. Then, the animals were killed; the soleus muscle was excised for Northern blot analysis.

**Preparation of RNA** —— RNA was prepared from the soleus muscle and C2C12 cells using RNeasy Plant Mini Kit (Qiagen, Germantown, MD, U.S.A.). The integrity of RNA was confirmed by its electrophoresis on a denaturing agarose gel containing formaldehyde.

**Measurement of Plasma Glucose and Total Ketone Bodies** —— Plasma glucose concentration was determined with a glucose assay kit (Glucose CII-Test Wako, Wako Pure Chemical Industries, Tokyo, Japan), which was developed from the mutarotase-glucose oxidase method.[11] Determination of plasma ketone bodies[12] was carried out using ketone body assay kit (Ketone Test Sanwa, Sanwa Kagaku Co., Tokyo, Japan).

**Northern Blot Analysis** —— The cDNA fragments of AACS and SCOT were labeled with deoxycytidine 5′-[$\alpha$-$^{32}$P] triphosphate and aliquots of RNA (10 μg each) were subjected to Northern blot analysis as described previously.[13]

**Cell Culture** —— The mouse C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.) containing glucose at 4.5 mg/ml and supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), then maintained at 37°C in an atmosphere of 5% $CO_2$. When the cells were 70–80% confluent, they were induced to differentiate into myotubes by changing to a low-serum differentiation medium (DMEM supplemented with 2% horse serum) for 7 days, and the medium was replaced with serum-free DMEM containing glucose at 1 mg/ml with 1% bovine serum albumin (BSA). After 2 hr, cells were treated with glucose or human recombinant leptin protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) for 24 hr. After fixation of the cells in 4% formalin at room temperature for 10 min, glycogen was visualized by periodic acid-Schiff (PAS) staining.[14] Neutral lipids (triacylglycerols) were visualized with oil red-O as described elsewhere.[15] PAS- and oil red-O-stained cells were counter-stained with hematoxylin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

**Statistical Analysis** —— All data are presented as means ± S.E. and were analyzed using unpaired Student's *t*-tests. *P* values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

**Differential Effects of Genetic and Diet-induced Obesity on AACS Gene in Rat Skeletal Muscle**

First, we investigated the effects of obesity on the gene expression of ketone body-utilizing enzymes, using diet-induced obese rats and genetically obese Zucker (*fa/fa*) rats. Six-week feeding of rats with the high-fat diet (HFD) resulted in about a 6-fold increase in blood ketone body level and a 16.7% increase in blood glucose level compared with the feeding with a normal diet (Table 1). On the other hand, in the obese Zucker rats, blood ketone body level was markedly low (> 52.8%) compared with that of lean rats, but such a difference was not observed in blood glucose level. Figure 1 shows the gene expression levels of two ketone body-utilizing enzymes, AACS (Fig. 1A and 1C) and SCOT (Fig. 1B and 1D) in the soleus muscle. AACS mRNA expression was lower (> 42.6%) in obese Zucker rats than in lean littermates (Fig. 1C). However, such a difference was not observed in HFD-induced obese rats (Fig. 1A). In contrast, SCOT mRNA expression was lower (> 39.3%) in HFD-induced obese rats than in nor-

**Table 1.** Body Weight and Blood Parameters of Two Types of Obese Rats

| | Diet-induced obese rat | | Zucker rat | |
|---|---|---|---|---|
| | Normal diet | High-fat diet | Lean (+/+) | Fatty (*fa/fa*) |
| Body weight (g) | 597.6 ± 39 | 631.9 ± 27 | 211.8 ± 8.6 | 278.1 ± 13* |
| Plasma glucose (mg/dl) | 132.5 ± 3.7 | 154.7 ± 13* | 140.3 ± 19 | 152.5 ± 23 |
| Plasma total ketone body (μmol/l) | 217.8 ± 30 | 1308 ± 410*** | 365.1 ± 50 | 176.5 ± 67** |

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the normal diet or lean littermates.

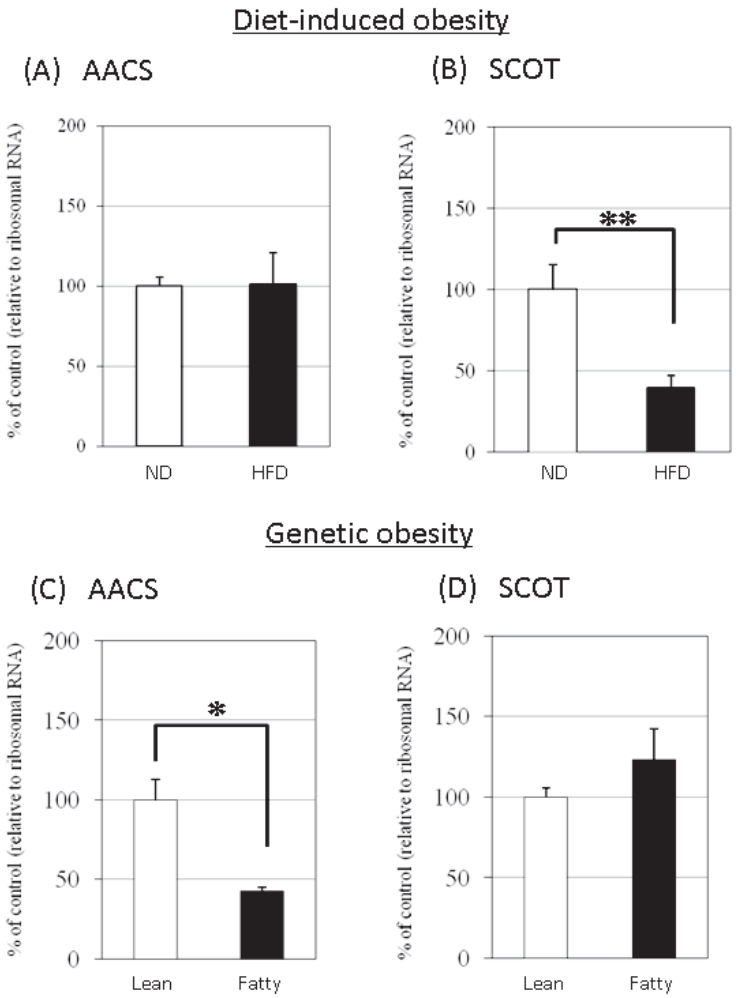


**Fig. 1.** Expression of AACS and SCOT mRNA in Soleus Muscles of HFD-fed Rats and Obese Zucker Rats

Total RNAs were prepared from soleus muscles of HFD-fed rats (A and B) and obese Zucker rats (C and D), and subjected to Northern blot analysis with AACS (A and C) and SCOT (B and D) cDNA probes. ND, HFD, fatty and lean indicate normal diet-fed rats, HFD-fed rats, obese Zucker rats and their lean littermates, respectively. Each bar represents the means ± S.E. ($n = 3$). *$p < 0.05$ and **$p < 0.01$ compared with the ND or lean littermates.

mal diet-fed rats (Fig. 1B), but such difference was not observed in obese Zucker rats (Fig. 1D). Previously, we reported that AACS mRNA expression in the subcutaneous WAT is closely related to the type of obesity.[10] These results suggest that the type of obesity also affects the regulation of AACS expression in skeletal muscle.

### Effects of High Glucose on AACS mRNA Expression were Different between Myoblasts and Differentiated Muscle Cells

Serum glucose level was significantly increased by feeding on an HFD (Table 1). In order to investigate the effects of overnutrition on ketone body utilization in skeletal muscle cells, we examined

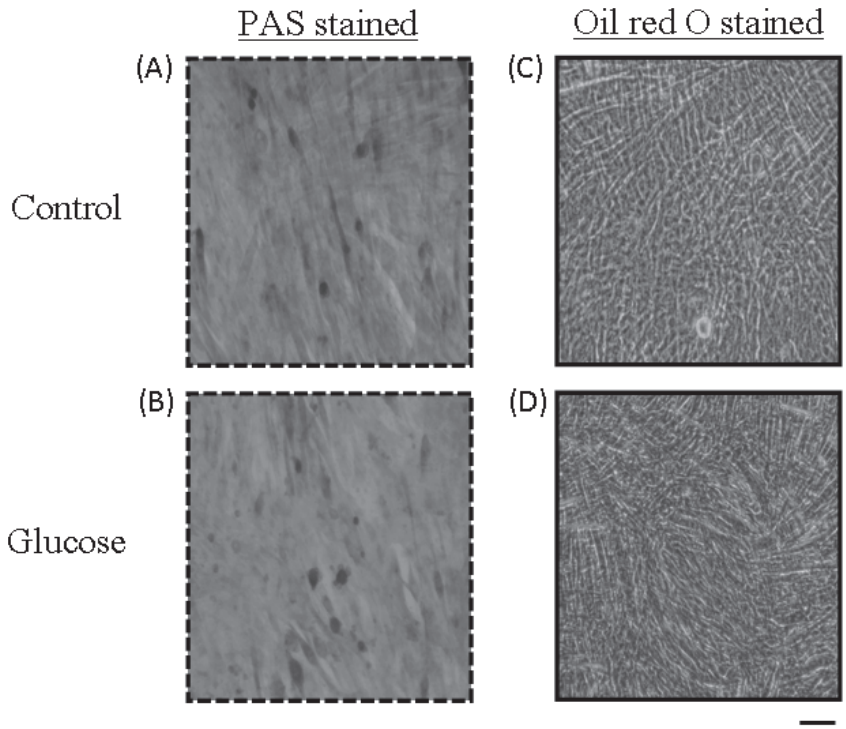


**Fig. 2.** Effect of Glucose Treatment on Glycogen and Lipid Accumulation in C2C12 Cells

C2C12 myoblast cells were maintained in differentiation medium for 2 days and treated with glucose (B, D) for 6 days. Glycogen was stained with periodic acid-Schiff reagent (A, B) and lipid droplets were stained with oil red O (C, D). Scale bar = 40 μm.

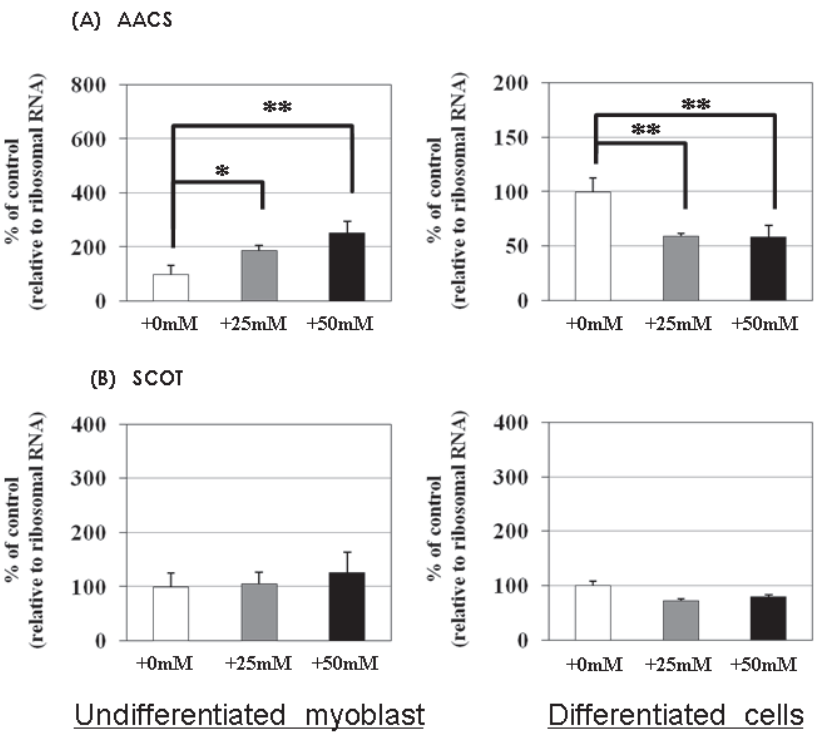


**Fig. 3.** Effect of Glucose on Gene Expression Levels of AACS and SCOT in C2C12 Cells

C2C12 cells were induced (left panels) or not induced (right panels), then treated with glucose. After 6 days, total RNAs were isolated from each cells, and subjected to Northern blot analysis for AACS (A) and SCOT (B). Means ± S.E. ($n$ = 3). $^{*}p < 0.05$; $^{**}p < 0.01$. All samples were compared with the + 0 mM glucose-treated cells.

the effects of a high glucose concentration on the gene expressions of AACS and SCOT in C2C12 myoblast cells. Glycogen and lipids were not accumulated in C2C12 cells in this overnutrition state (Fig. 2). AACS mRNA level was decreased by the treatment with a high level of glucose in differentiated C2C12 cells, although it was increased in undifferentiated cells (Fig. 3A). In contrast, SCOT mRNA was not affected by glucose treatment in C2C12 cells (Fig. 3B). These results suggest that high levels of glucose suppress the ketone body utilization by AACS in differentiated muscle cells but up-regulate this utilization in myoblasts. The AACS gene level in whole soleus muscle of diet-induced obese rats (Fig. 1A) may be due to these opposite effects of glucose on undifferentiated and differentiated muscle cells.

**Leptin Controls Ketone Body Utilization in Muscle Cells**

Blood glucose level was not significantly increased in obese Zucker rats (Table 1). However, AACS was significantly decreased in these rats (Fig. 1C). Since a lack of leptin response is well described in obese Zucker rats,[16] we next examined the effect of leptin signaling on ketone body utilization in skeletal muscle. Figure 4 shows the effects of leptin treatment on the gene expression of ketone body-utilizing enzymes in differentiated C2C12 cells. Leptin significantly increased the levels of AACS gene in a dose-dependent manner (Fig. 4A). This effect was not observed in undifferentiated C2C12 cells (data not shown). In contrast, mRNA levels of SCOT were not affected by any concentration of leptin (Fig. 4B). Since AACS gene expression was increased in C2C12 cells by glucose but significantly suppressed in the soleus muscle of obese Zucker rat (Fig. 1C), it is likely that leptin acts as a key regulator of ketone body-utilization via AACS in skeletal muscle. On the other hand, SCOT was not affected by either glucose or leptin in C2C12 cells, while SCOT mRNA was significantly decreased in diet-induced obesity (Fig. 1B). Thus, it is possibile that ketone body consumption via mitochondrial SCOT is regulated by other factors, such as neural signaling, circulating ketone bodies, and hormones.

Leptin is well known as the key regulator of lipid oxidation in skeletal muscle.[17] Since ketone bodies are mainly produced from the $\beta$-oxidation of fatty acids, our present data suggest that leptin regulates not only lipid consumption but also recycling

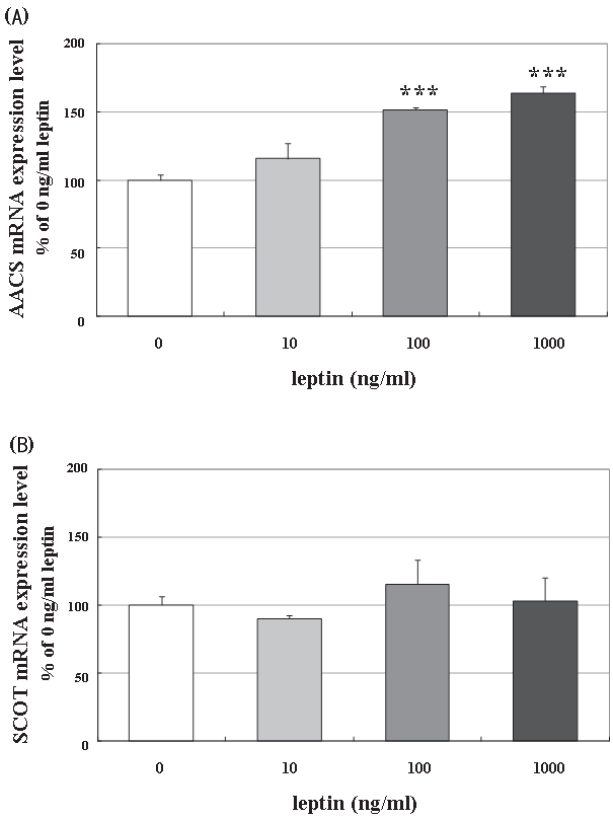


**Fig. 4.** Effect of Leptin Treatment on AACS and SCOT mRNA Expression Levels in Mouse Differentiated C2C12 Cell Line

C2C12 cells were maintained in differentiation medium for 10 days and treated with leptin protein (10, 100, 1000 ng/ml). After 24 hr total RNAs were isolated, and subjected to Northern blot analysis for AACS (A) and SCOT (B). Each bar represents the mean ± S.D. ($n$ = 3). $^{***}p < 0.001$ compared with 0 ng/ml leptin.

of ketone bodies into fatty acids via AACS in skeletal muscle and suppresses an abnormal increase of ketone bodies in circulating blood.

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