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Effect of fasting on PPAR γ and AMPK activity in adipocytes

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ABSTRACT

We investigated the effects of fasting on gene expression and intracellular signals regulating energy metabolism in adipose tissue. Following fasting for 15 h or 39 h, epididymal fat pads were isolated from Wistar rats. PPAR γ mRNA levels decreased in the adipose tissues isolated from rats fasted for 39 h, whereas adipocyte lipid-binding protein (aP2) and lipoprotein lipase (LPL) mRNA levels increased. Overnight fasting increased the AMP/ATP ratio and AMP-activated protein kinase (AMPK) in adipose tissue, but not in muscle or liver tissue. In addition, the effect of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) on PPAR γ expression in primary cultured adipocytes was investigated. AICAR reduced PPAR γ mRNA levels but increased aP2 and LPL mRNA levels. Thus, fasting-induced AMPK activation may affect on the regulation of gene expression in adipocytes.

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1. Introduction

Obesity is a major health problem commonly associated with insulin resistance, type 2 diabetes, hypertension, dyslipidemia and atherosclerosis. Numerous studies have demonstrated that elevating PPAR γ activity with thiazolidinediones improves insulin sensitivity [1]. On the other hand, little is known about the effects of nutritional state, such as a low calorie diet, on the expression and activity of PPAR γ in adipose tissue. Herein, we evaluated the expression of PPAR γ and related genes in adipose tissue from fasted animals, and assessed the signals regulating glucose and lipid homeostasis in adipose tissue. In particular, we focused on the relationship between fasting and AMP-activated protein kinase (AMPK) in adipocytes.

AMPK is a heterotrimeric enzyme consisting of a catalytic α subunit and regulatory β and γ subunits [2]. AMPK is activated following an increase in the AMP/ATP ratio rather than the intracellular depletion of ATP or accumulation of AMP [3]. Therefore, AMPK is regarded as a fuel gauge whose activation inhibits ATP-consuming pathways. AMPK has emerged as a key regulator of glucose metabolism. Exercise-induced recruitment of Glut4 to the plasma membrane followed by glucose uptake is mediated via AMPK [4]. Thus, considerable work has elucidated the significance of AMPK in glucose and fat homeostasis and its possible usefulness as a therapeutic target in type 2 diabetes. However, little information is available surrounding its roles in adipose tissue, compared with liver, skeletal muscle. Therefore, we propose a possible role for AMPK in adipocytes isolated from fasted animals.

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2. Materials and methods

2.1. Adipocyte preparation

Male Wistar rats weighing 180–200 g were fed ad libitum with a standard diet. To examine the effect of fasting, animals were sacrificed 15 h or 39 h after the removal of food at 18:00. Epididymal fat pads, livers and soleus muscles were collected and homogenized in lysis buffer or Isogen (Nippon Gene) as described previously [5]. For adipocyte primary culture, isolated adipocytes were obtained as described previously [5]. The adipocyte primary culture was maintained in DMEM containing 5% calf serum, with or without 1 mM 5-aminimidazole-4-carboxamide-ribonucleoside (AICAR) (Sigma), 3 mM metformin (Sigma), or 10 nM TNF α (Sigma) for 24 h at 37 °C. Cells were then collected in a lysis buffer or Isogen. To assess the role of PPAR γ in fasting or the AICAR-induced reaction, a specific PPAR γ inhibitor, HX531 was used [6]. Wistar rats were fed with food containing 0.1% HX531 for 1 week, after which time the effects of starvation were evaluated. Isolated adipocytes were incubated with or without 10 μ M HX531 and 1 mM AICAR to measure the expression of genes showing a PPAR response element. Furthermore, to evaluate the effect of AMPK inhibitor, on fasting-induced PPAR γ and adipocyte lipid-binding protein (aP2) expression, rats were treated intraper-

itoneally 20 mg/kg compound C, the specific inhibitor of AMPK, prior to fasting [7].

2.2. Immunoblot analysis

Immunoblot was carried out as described previously [5] using anti-AMPK phosphothreonine 172 antibody (Cell Signaling Technology), anti-AMPK α 1, α 2 antibody (Abcam), anti-phospho-acetyl-CoA carboxylase (ACC) antibody (Upstate Biotechnology), anti-ACC antibody (Upstate Biotechnology).

2.3. AMPK enzymatic activity

AMPK enzymatic activity was measured as described previously [8] using synthetic peptide with sequence HMRSAMSGHLVKRR as substrate.

2.4. Real-time PCR

The mRNA levels of PPAR γ , aP2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured with real-time PCRs as described previously [5]. The mRNA levels of lipoprotein lipase (LPL), leptin and RNA polymerase II (Pol2), an additional housekeeping gene, were measured using real-time PCR. The sense and antisense primers used (GenBankTM

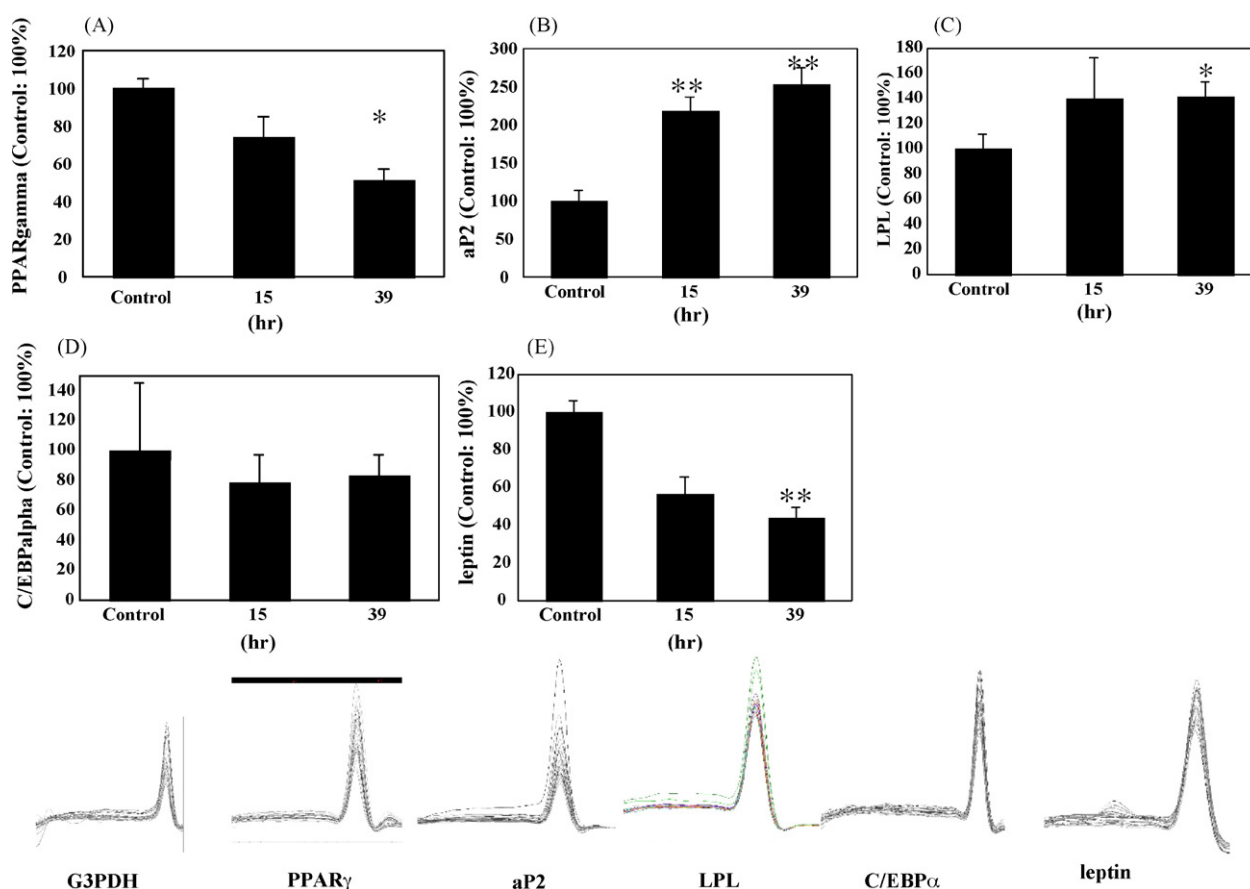


Fig. 1 – Expression of adipocyte specific genes in adipose tissue from starved rats. Wistar rats were fasted for indicated periods, and sacrificed to obtain epididymal fat pads. Total RNA was extracted from each tissue using Isogen, after which real-time PCR was performed. The levels of PPAR γ (A), aP2 (B), LPL (C), C/EBP α (D) and leptin (E) mRNA are shown. Values are expressed as means \pm S.E. of six determinations. * p < 0.05, ** p < 0.005 by ANOVA.

accession numbers are in parentheses) with the concentrations of Mg^{2+} /dimethylsulfoxide (DMSO) in buffer and annealing temperature were as follows: LPL (NM_012598), nt 956–974 and 1167–1186 with 3 mM Mg^{2+} /2% DMSO at 60 °C; leptin (NM_013076), nt 151–170 and 477–496 with 4 mM Mg^{2+} /2% DMSO at 60 °C; Pol2 (XM_343922), nt 1778–1800 and 1831–1855 with 3 mM Mg^{2+} at 58 °C. Corresponding fragments amplified in the 2 mM Mg^{2+} PCR buffer with Ex Taq DNA polymerase (TaKaRa Co., Osaka, Japan) were gel-purified and quantitated using an Agilent2100 Bioanalyzer™ (Agilent Technologies., Palo Alto, CA), yielding each standard to calculate the exact copy number of each mRNA in the samples. The mRNA values calculated as copy numbers in each sample were normalized for a housekeeping gene (GAPDH or Pol2).

2.5. Nucleotide measurement

Following treatment with or without overnight fasting, the Wistar rats were sacrificed, and epididymal fat pads, livers and soleus muscles were collected. These samples were washed

with PBS before the addition of 5% perchloric acid. Acid-insoluble materials were removed by centrifugation at $10,000 \times g$ for 2 min and the pH was adjusted to 7.0 using 3 M K_2CO_3 to 7.0. The amounts of ATP and AMP were measured as described previously [9].

3. Results

Although PPAR γ mRNA levels decreased significantly to 51% during the fasting for 39 h (Fig. 1A), mRNA levels of aP2 and LPL, markedly increased to 217% and 141%, respectively, in adipose tissues from rats starved for 15 h (Fig. 1B and C). The activity of AMPK, one of the key enzymes regulating glucose and lipid homeostasis in adipose tissue, was subsequently investigated. Fasting for 15 h resulted in elevated levels of AMPK phosphothreonine 172 and phospho-ACC in adipose tissue (Fig. 2A). In addition, an elevation in the enzymatic activity of AMPK to 210% was observed in fasted adipose tissue (Fig. 2B). In contrast, p-AMPK levels remained unaltered with

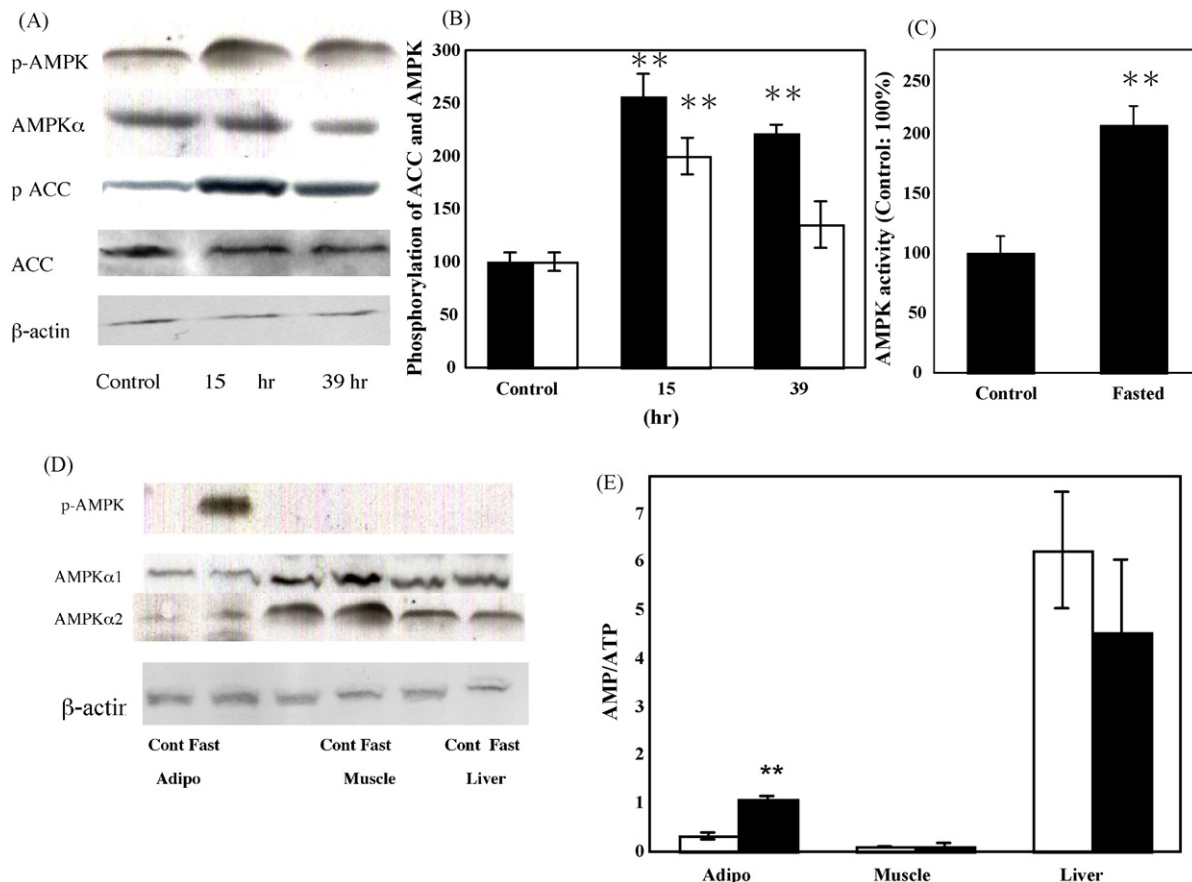


Fig. 2 – Effects of fasting on signals regulating glucose and lipid metabolism. Fasting for 15 h and 39 h increased the AMPK phosphothreonine 172 (p-AMPK) levels and phospho-ACC (p-ACC) levels in adipose tissue. Typical results of Western blotting (A) and quantitated one (filled bars: p-ACC, open bars: p-AMPK, (B) are shown. Values are expressed as means \pm S.E. of five determinations. ** $p < 0.01$ by ANOVA. Immunoprecipitable AMPK enzymatic activity was assayed by measuring the phosphorylation of synthetic peptide. Values are expressed as means \pm S.E. of six determinations. ** $p < 0.01$ by ANOVA (C). Although both AMPK α 1 and AMPK α 2 were expressed in muscle and liver as well as adipose tissue, a fasting-induced increase in p-AMPK levels was detected only in adipose tissue (D). Fasting for 15 h increased AMP/ATP ratio in adipose tissue, but not in soleus muscle or liver. Filled bars represent the results of fasted rats and Open bars represent those of control rats (E). Values are expressed as means \pm S.E. of seven determinations. ** $p < 0.01$ by ANOVA.

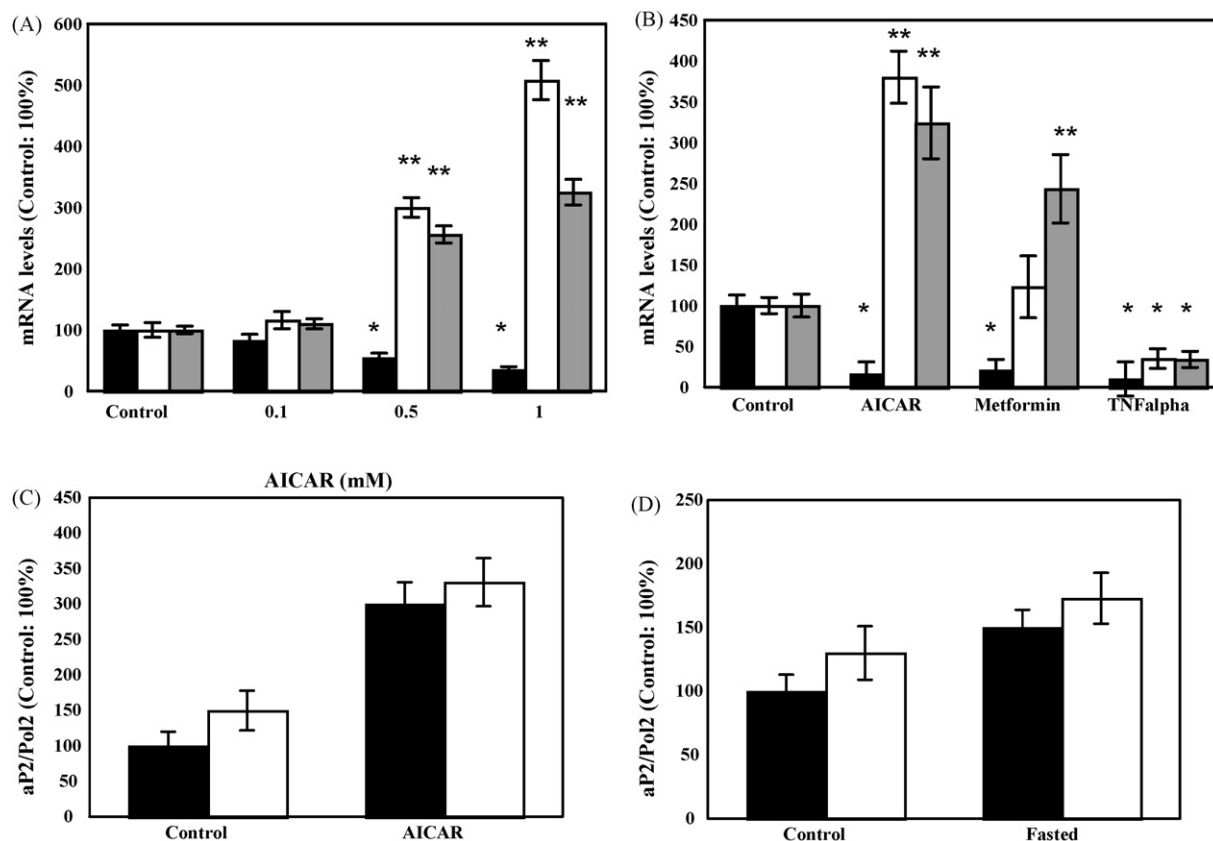


Fig. 3 – Effects of AICAR and other agents on the gene expression in adipocytes. Primary cultured adipocytes were incubated with various concentrations of AICAR (A) or other agents (1 mM AICAR, 3 mM metformin or 10 nM TNF α) (B) for 24 h. Incubation with AICAR reduced the PPAR γ (black bars) mRNA levels and elevated aP2 (white bars), LPL (gray bars) and leptin (vertical line bars) mRNA levels. In contrast, treatment with TNF α reduced both PPAR γ and aP2 mRNA levels. Values are expressed as means \pm S.E. of four determinations. * p < 0.05, ** p < 0.01 by ANOVA. Isolated adipocytes from epididymal fat were pre-incubated with (white bar) or without (black bar) 10 μ M HX531 in DMEM for 1 h, then incubated with or without 1 mM AICAR for 24 h (C, left). Wistar rats were bred with (white bar) or without (black bar) 0.1% HX531 containing food. They were fed ad libitum or fasted for 15 h, then epididymal fat tissues were collected to obtain mRNA (D, right). Levels of the aP2 and house keeping gene, Pol2 mRNA in adipocytes was measured using real-time PCR as described in Section 2. Values were expressed as means \pm S.E. of three determinations.

fasting in the soleus muscle or liver (Fig. 2C). An increased ratio of AMP/ATP is known to activate AMPK kinase, therefore, we measured nucleotide levels in these tissues. Fasting for 15 h led to a significant increase in the AMP/ATP ratio in adipose tissue, but not in muscle or liver tissue (Fig. 2D). These results strongly suggest that the elevation of the AMP/ATP ratio causes activation of AMPK in adipose tissue.

The possibility that AMPK activation might regulate expression of adipocyte specific genes was then investigated. Incubation with AICAR for 24 h resulted in a dose-dependent decrease in PPAR γ mRNA to 31.7% and an increase in aP2, and LPL mRNA to 481% and 302%, respectively, in primary cultured adipocytes (Fig. 3A). Incubation with metformin yielded similar results, whereas incubation with TNF α led to a corresponding reduction in PPAR γ and aP2 mRNA (Fig. 3B). Incubation with 1 mM AICAR and 3 mM metformin for 24 h elevated immunoreactive p-AMPK to 260% and 187%, respectively. These results suggested that the activation of AMPK results in elevation of the PPAR γ activity *in vivo* as in the case of fasting, however, the

expression of PPAR γ itself was decreased simultaneously. To assess the role of PPAR γ in these apparently conflicting results, the effects of the PPAR γ inhibitor, HX531 on fasting and incubation with AICAR were studied. The results showed that HX531 affected neither fasting nor AICAR-induced up-regulation of aP2 (Fig. 3C), but indicated that some other signal may be involved in this process. Similarly, HX531 did not suppress elevation of LPL (data not shown). Administration of metformin *in vivo* resulted in elevation of aP2 mRNA level, whereas pretreatment with compound C suppressed AMPK activity in fasted rats, which abolished fasting-induced reduction of PPAR γ and increase of aP2 mRNA levels (Fig. 4).

4. Discussion

A restricted calorie diet is a common treatment for type 2 diabetes and metabolic syndrome associated with obesity. *In vivo* studies have demonstrated that fasting or a low calorie

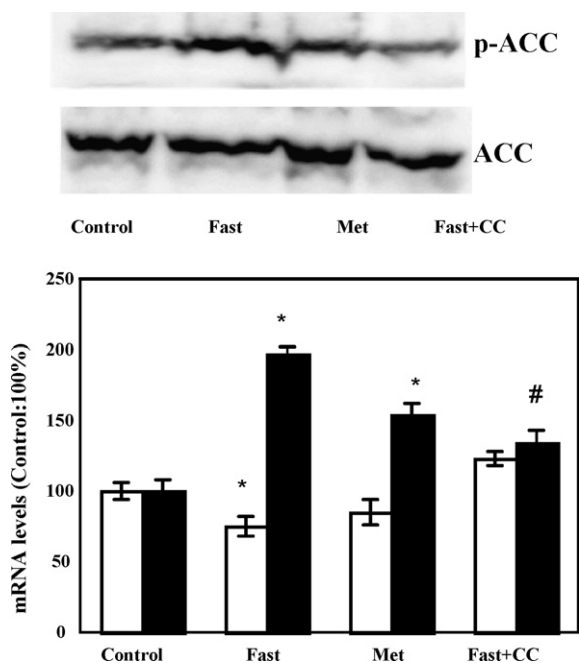


Fig. 4 – Effects of *in vivo* administration of metformin and compound C on the gene expression in adipocytes. To evaluate the pharmacological activation or inhibition of AMPK *in vivo*, rats were separated into 4 groups ($n = 4$); fed (Control), fasted (Fast), treated with metformin (Met), treated with compound C (Fast + CC). After oral administration of metformin (200 mg/(kg day)) for 1 week, epididymal fat was obtained in feeding condition. On the other hand, rats were treated intraperitoneally with compound C (20 mg/kg) before fasting for 15 h. PPAR γ (black bar) and aP2 (white bar) mRNA levels were measured with real-time PCR (lower) and phospho-ACC (p-ACC) levels with Western blotting (upper). Values were expressed as means \pm S.E. of four determinations. * $p < 0.05$ vs. Control, # $p < 0.05$ vs. Fast.

diet lead to improvement of insulin sensitivity [10]. Moreover, Vidal-Puig et al. demonstrated that a low calorie diet reduced the expression of PPAR γ in obese humans [11], although no confirmatory reports have as yet been published. However, little is known about the effects of fasting and feeding on signal transductions regulating energy homeostasis in adipocytes. Herin, we found that fasting for 15 h increased the expression of aP2 and LPL without up-regulation of PPAR γ expression. Since LPL is regulated largely post-transcriptionally and post-translationally [12], our results do not mean that fasting increase LPL activity. As expected, leptin mRNA levels were reduced during the fasting.

AMPK was activated during overnight fasting with a concomitant increase in the AMP/ATP ratio observed in adipose tissue only. Recently, Daval et al., reviewed the function of AMPK in adipose tissue [13]. They described that fasting and exercise activate AMPK activity in adipose tissue, however they did not state the tissue specificity and AMP/ATP ratio. We hypothesized that an elevation in AMPK activity might influence gene expression in adipocytes. AICAR, an analog of adenosine, is phosphorylated to form AICAR

monophosphate (ZMP). ZMP, like AMP, phosphorylates and activates AMPK [14]. Incubation with AICAR increased the expression of aP2 and LPL, but reduced the expression of PPAR γ mRNA significantly. Our results showed that AMPK played a paradoxical role in suppressing the concomitant expression of PPAR γ while increasing adipocyte specific genes, which are up-regulated by PPAR γ . Currently, we are unable to explain the mechanisms underlying these responses. On the other hand, our results shown in Fig. 3C and D indicated that fasting and AICAR-induced increase in aP2 and LPL mRNA are mediated via other factor than PPAR γ . Supraphysiological activation of PPAR γ ameliorates insulin sensitivity, whereas moderate reduction of PPAR γ activity also improves it [6]. Our results of PPAR γ consists with these facts. We simultaneously assessed the effects of other agonists on the expression and *in vivo* activity of PPAR γ . Treatment with TNF α reduced the expression of PPAR γ , in parallel with aP2 and LPL, probably through the activation of NF- κ B. Metformin, which is known to activate AMPK [15], exhibited similar results to those observed with AICAR. These results suggest that activation of AMPK partially mimic the change of gene expression provoked with fasting. Administration of metformin *in vivo* yielded the compatible result. Moreover, the result that pharmacological inhibition of AMPK, with compound C prevented fasting-induced effects supported our hypothesis.

Recently, it has been advocated that effects of calorie restriction, including decreased adiposity, repression of PPAR γ and increased free fatty acid release, is mediated via Sirt1, an NAD-dependent protein deacetylase [16]. Unfortunately, we have no idea of relation between AMPK and Sirt1 in the role of energy sensing in adipose tissue, however, it is possible that their interaction might regulate total glucose and lipid homeostasis. Calorie restriction, exercise and high plasma adiponectin level activate AMPK in peripheral tissue, which contribute to longevity. On the other hand, SIR2, yeast orthologue of mammalian Sirt1, promotes elongated life span associated with calorie restriction [17]. These facts suggest that both factors might act in common pathway or synergistically. Further study will provide new standpoints for understanding the role of adipocyte and AMPK.

In summary, the present study is the first to evaluate the effects of fasting on the expression and transcriptional activity of PPAR γ in adipose tissues. Our results showed that: (1) fasting decreased the expression of PPAR γ mRNA and increase the expression of aP2 and LPL mRNA, (2) fasting resulted in an elevated AMP/ATP ratio and activated AMPK activity in adipose tissue, and (3) AMPK may be involved in the fasting-induced gene regulation adipocytes.

Conflict of interest

There are no conflicts of interest.

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