Dissociation between PGC-1α and GLUT-4 Expression in Skeletal Muscle of Rats Fed a High-Fat Diet

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Summary It has recently been reported that a 4-wk high-fat diet gradually increases skeletal muscle peroxisome proliferator activated receptor (PPAR) γ coactivator-1α (PGC-1α) protein content, which has been suggested to regulate GLUT-4 gene transcription. However, it has not been reported that a high-fat diet enhances GLUT-4 mRNA expression and protein content in skeletal muscle, suggesting that an increase in PGC-1α protein content is not sufficient to induce muscle GLUT-4 biogenesis in a high-fat fed animal. Therefore, we first evaluated the relationship between PGC-1α and GLUT-4 expression in skeletal muscle of rats fed a high-fat diet for 4 wk. The PGC-1α protein content in rat epitrochlearis muscle significantly increased by twofold after the 4-wk high-fat diet feeding. However, the high-fat diet had no effect on GLUT-4 protein content and induced a 30% decrease in GLUT-4 mRNA expression in rat skeletal muscle (p<0.05). To clarify the mechanism by which a high-fat diet downregulates GLUT-4 mRNA expression, we next examined the effect of PPARδ activation, which is known to occur in response to a high-fat diet, on GLUT-4 mRNA expression in L6 myotubes. Incubation with 500 nM GW501516 (PPARδ activator) for 24 h significantly decreased GLUT-4 mRNA in L6 myotubes. Taken together, these findings suggest that a high-fat diet downregulates GLUT-4 mRNA, possibly through the activation of PPARδ, despite an increase in PGC-1α protein content in rat skeletal muscle, and that a posttranscriptional regulatory mechanism maintains GLUT-4 protein content in skeletal muscle of rats fed a high-fat diet.

Key Words PGC-1α, GLUT-4, PPARδ, skeletal muscle, high-fat diet

Skeletal muscle is the site of more than 80% of insulin-mediated glucose uptake in vivo (1). Furthermore, maximal insulin- and contraction-stimulated glucose transport activity is linearly related to the content of GLUT-4, which is a predominant isoform of the glucose transporters in skeletal muscle (2, 3). Therefore, the GLUT-4 levels in skeletal muscle may be an important determinant of whole-body glucose disposal. Despite the importance of GLUT-4 on whole-body glucose metabolism, the precise mechanism by which GLUT-4 expression is regulated in skeletal muscle is not fully understood. Peroxisome proliferator activated receptor (PPAR) γ coactivator-1α (PGC-1α), a transcriptional coactivator, was cloned from a differentiated brown fat cell line, and it is now widely accepted as a key regulator of adaptive mitochondrial biogenesis in skeletal muscle (4). In addition, it has been reported that enhanced expression of PGC-1α triggers a significant increase in GLUT-4 mRNA and protein content in myocytes and mice skeletal muscle (5, 6). These findings therefore provide evidence that PGC-1α plays an important role in the regulation of GLUT-4 as well as mitochondrial biogenesis in skeletal muscle.

Hancock et al. (7) have recently demonstrated that the PGC-1α protein content in rat skeletal muscle gradually increased after a 4-wk high-fat diet, concomitant with an increase in a wide range of mitochondrial proteins. It therefore seems possible that the GLUT-4 protein content also might be elevated in skeletal muscle of rats fed a high-fat diet. However, to our knowledge, no study has demonstrated that a high-fat diet causes a significant increase in muscle GLUT-4 mRNA or GLUT-4 protein content (8–11). These findings led us to consider the possibility that enhanced expression of PGC-1α protein content is not sufficient to induce GLUT-4 biogenesis in skeletal muscle of animals fed a high-fat diet, and that some factors induced by high-fat diet feeding might counteract the enhancing effect of PGC-1α on GLUT-4 biogenesis in skeletal muscle. In this context, the present investigation first evaluated the relationship between PGC-1α and GLUT-4 expression in skeletal muscle of rats fed a high-fat diet. We also attempted to elucidate the potential mechanism by
which a high-fat diet prevents the increase in GLUT-4 expression despite an elevated expression of PGC-1α protein content.

**EXPERIMENT**

**Materials.** Anti-PGC-1α and anti-UCP-3 antibodies were purchased from Calbiochem (San Diego, CA). Calpastatin antibody was from SANTA CRUZ (Santa Cruz, CA). A polyclonal antibody specific to GLUT-4 was the generous gift of Dr. Mike Mueckler (Washington University, St. Louis, MO). GW501516 and fenofibrate were obtained from Calbiochem and Cayman Chemical (Ann Arbor, MI), respectively. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). Reagents for SDS-PAGE were from Bio-Rad (Hercules, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Animal care.** Male Wistar rats weighing 100–120 g were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were housed in rooms lighted from 9:00 AM to 9:00 PM. The room temperature was maintained at 20–22°C.

After a period of acclimation to the new facilities, animals were given either a rodent chow diet (CLEA Japan) or a high-fat diet and water ad libitum for 4 wk. The high-fat diet contained, as a percent of calories, 50% lard, 27% sucrose, and 23% casein, supplemented with a vitamin mix (22 g/kg, AIN 93 vitamin mix, CLEA Japan), minerals (51 g/kg, AIN93G mineral mix, CLEA Japan), and methionine (4.4 g/kg, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The energy content of the high-fat diet was 5.1 kcal/g, whereas that of the rat chow was 3.4 kcal/g. The committee for Animal Experimentation in the School of Sport Sciences at Waseda University approved this research.

**Tissue collection.** After 4 wk on the diets, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and the epitrochlears and triceps muscles were dissected out and clamp-frozen in liquid nitrogen at −80°C until analysis. The intra-abdominal fat pads consisting of mesenteric, retroperitoneal, and epididymal fat pads were removed and weighed. Blood samples were drawn via cardiac puncture for measurement of serum free fatty acid (FFA) concentrations. Serum FFA concentrations were determined using a kit (NEFA-C Test) obtained from Wako Pure Chemical.

**Cell culture and treatment.** L6 myocytes (Dainippon Sumitomo Pharma Co., Ltd., Tokyo, Japan) were cultured in 100-mm polystyrene culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and passaged by trypsinization using 0.25% trypsin. Myoblasts were maintained in a humidified incubator under an atmosphere of 5% CO₂ at 37°C. Myoblast differentiation was induced by switching to medium containing 2% horse serum when myoblasts were confluent. Experimental treatments were begun 4 d later, when myotubes were evident. The myotubes were treated with either 500 nM GW501516 or 100 μM fenofibrate, which were dissolved in DMSO. The same amount of DMSO was added to the control group. L6 myotubes were treated with either 500 nM GW501516 or 100 μM fenofibrate for 1 or 5 d. The culture medium was changed daily.

**Western blot analysis.** Myotubes and frozen epitrochlearis muscles were homogenized in ice-cold RIPA buffer (Upstate, Lake Placid, NY) containing: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Protease inhibitor cocktail (SIGMA). Protein concentrations were measured with a BCA protein assay kit (PEIRCE, Rockford, IL) according to the manufacturer’s instructions. Samples were prepared in Laemmli sample buffer (Wako Pure Chemical). Equal amounts of sample protein were subjected to SDS-PAGE and then transferred to PVDF membranes at 220 mA for 2 h. After transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBST; 20 mM Tris base, 137 mM NaCl, pH 7.6) supplemented with 10% nonfat powdered milk at room temperature. Membranes were incubated overnight with antibodies specific for GLUT-4, PGC-1α, UCP-3, and calpastatin at concentrations of 1:200–10,000 at 4°C. The HRP-conjugated secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG) was used at a concentration of 1:5,000–10,000. Bands were visualized by ECL and quantified using densitometry.

**Semiquantitative RT-PCR.** Total RNA from triceps muscle and myotube were isolated using TRIzol reagent (Invitrogen). The DNase-treated total RNA (2 μg) was reverse transcribed (RT) into cDNA by using random primer with a Reverse Transcription System (Promega, WI). Aliquots of each RT reaction were added to a PCR master mix (Promega) mixture containing Taq DNA polymerase, dNTPs, MgCl₂, reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, and 10 pmol of both sense and antisense primers (GLUT-4, forward, 5′-CCATTTTTGGGCTCTACATT-3′; reverse, 5′-GTTGTTTCTACCTTCTT-3′). PCR was carried out using a thermal cycler (PCR Thermal Cycler Dice, TAKARA, Japan). The samples were amplified for 40 cycles after an initial denaturation at 95°C for 2 min, using the following PCR cycle conditions: 95, 57 and 72°C for 1 min each. In the present investigation, 18s rRNA expression was simultaneously measured as an internal standard by using a QuantumRNA 18s Internal Standard Kit (Ambion, Austin, TX). The PCR products were run on a 2% agarose gel and visualized with SYBR Safe (Molecular Probes, Eugene, OR). The transcript intensity is expressed relative to 18s rRNA.

**Mitochondrial enzyme activities.** For the enzyme activity measurements, 10% homogenates were made from the triceps muscles in 175 mM KCl, 10 mM GSH, and 2 mM EDTA, pH 7.4. This homogenate was frozen and thawed three times and mixed thoroughly before the enzyme activities were measured. As a marker of
oxidative enzyme, the citrate synthase (CS) activity was measured using Srere’s method (12). For the 3-β-hydroxyl-CoA dehydrogenase (HAD) assay, an aliquot of the homogenate was centrifuged at 700 × g for 10 min at 4°C. The HAD, a key enzyme of the fatty acid β-oxidation pathway in skeletal muscle, was measured using Bass’s method (13).

Muscle glycogen concentrations. Muscle glycogen concentrations were determined by enzymatic methods according to Lowry and Passonneau (14) after acid hydrolysis.

Statistical analysis. The data are presented as the means±SE. The significance of differences between the two groups was assessed using Student’s t-test. For multiple comparisons, significance was determined by one-way analysis of variance (ANOVA) followed by a post hoc comparison using the Tukey significant difference method. Statistical difference was defined as p<0.05.

RESULTS AND DISCUSSION

Body weight and intra-abdominal fat significantly increased in response to a 4-wk high-fat diet by 17 and 138%, respectively (Table 1). As shown in Fig. 1A, the high-fat diet feeding caused a twofold increase in muscle PGC-1α protein content, concomitant with 15–20% increase in CS and HAD enzyme activities in rat triceps muscle (Table 1). These results confirmed the recent findings by Hancock et al. (7), demonstrating that a high-fat diet gradually increased PGC-1α protein content in rat skeletal muscle, and provided further evidence that this adaptation appears to mediate high-fat diet-induced muscle mitochondrial biogenesis.

Recent evidence implies that PGC-1α is involved in GLUT-4 biogenesis in skeletal muscle. Michael et al. (5) have reported that forced expression of PGC-1α activates transcription of the GLUT-4 gene in myogenic cells in culture. Wende et al. (6) have also demonstrated that skeletal muscle overexpression of PGC-1α upregulates GLUT-4 mRNA and protein content in mouse skeletal muscle. Our results showing that a 4-wk high-fat diet upregulated muscle PGC-1α protein content (Fig. 1A) led us to hypothesize that GLUT-4 expression in skeletal muscle might increase in response to a high-fat diet. In the present investigation, we first measured GLUT-4 mRNA expression in rat skeletal muscle, since PGC-1α is a transcription coactivator and has been shown to activate GLUT-4 transcription in myocytes (5). In contrast to our hypothesis, GLUT-4 mRNA expression decreased in the triceps muscle of the rats fed a high-fat diet (Fig. 1B), despite a twofold increase in muscle PGC-1α protein content. This dissociation between the PGC-1α protein content and GLUT-4 mRNA expression in rats fed a high-fat diet suggests that a substantial increase in PGC-1α protein is not sufficient to activate GLUT-4 gene transcription in skeletal muscle of rats fed a high-fat diet. In this context, Miura et al. (15), reported that transgenic mice that overexpressed PGC-1α did not upregulate GLUT-4 mRNA expression in skeletal muscle. This result supports our data that upregulation of PGC-1α does not always result in an increase in GLUT-4 mRNA expression. In addition, our results also indicate that some factors induced by a high-fat diet may decrease GLUT-4 gene

**Table 1.** Effects of 4-wk high-fat diet on body weight, intra-abdominal fat, serum FFA concentration and muscle mitochondrial enzyme activities in rats.

|                           | Control          | High-fat diet    |
|---------------------------|------------------|------------------|
| Body weight (g)           | 340±10           | 399±13***        |
| Intra-abdominal fat (g/100 g body weight) | 4.1±0.3          | 6.6±0.4***      |
| Serum FFA concentration (mEq/L) | 1.1±0.1          | 4.1±0.8*        |
| Mitochondrial enzymes     |                  |                  |
| CS activity (μmol/g/min)   | 39±2             | 45±2*           |
| HAD activity (μmol/g/min)  | 16±1             | 20±1*           |

Means±SE (n=5 per group). FFA, free fatty acid; CS, citrate synthase; HAD, 3-β-hydroxyl CoA dehydrogenase. * and *** indicate significant difference from control at levels of p<0.05 and p<0.001, respectively.
transcription and/or cause a degradation of GLUT-4 mRNA, despite an increase in PGC-1α protein content.

As shown in Table 1, serum FF A concentrations were markedly elevated in the rats fed a high-fat diet, compared with the chow-fed group. Raising plasma FF A, which are endogenous ligands of PPARs, has been shown to activate PPARδ in rat skeletal muscle (16). In addition, since treatment with GW601742X, another PPARδ activator, has been reported to induce a decrease in GLUT-4 mRNA expression in rat skeletal muscle (17), we thought it plausible that activation of PPARδ by raising serum FF A affects the gene expression of GLUT-4 in skeletal muscle of rats fed a high-fat diet. To explore this possibility, we performed additional experiments to examine the effects of GW501516, a specific PPARδ activator, on GLUT-4 expression in L6 myotubes. First, we observed the effects of GW501516 on UCP-3 protein content to assess the bioactivity of GW501516, since PPARδ has been shown to regulate UCP-3 gene expression in skeletal muscle and myotubes (18). Consequently, a 5-d treatment of L6 myotubes with 500 nM GW501516 induced a 3-fold increase in UCP-3 protein content (Fig. 2A). As shown in Fig. 2B, incubation with the activator for 24 h decreased GLUT-4 mRNA expression by 30%. Our result is consistent with the findings of Jucker et al. (17), and suggests that PPARδ activation inhibits transcription of the GLUT-4 gene and/or causes degradation of GLUT-4 mRNA; this result also may partially explain the decrease in GLUT-4 mRNA in skeletal muscle induced by the high-fat diet.

As shown in Fig. 2C, long-term (5 d) treatment of GW501516 significantly decreased the GLUT-4 protein content in L6 myotubes, suggesting that activation of PPARδ downregulates GLUT-4 protein content at a transcriptional level. In contrast, no significant decrease in GLUT-4 protein content occurred in epitrochlearis muscle of rats fed a high-fat diet, compared with chow-fed rats (Fig. 1C), despite the ~30% decrease in muscle GLUT-4 mRNA expression (Fig. 1B). Based on these findings, it is likely that a posttranscriptional regulatory mechanism compensates for the decreased GLUT-4 mRNA induced by PPARδ activation, resulting in preservation of muscle GLUT-4 protein content in rats fed a high-fat diet.

It has been reported that diets with a relatively high fat content increase calpastatin activity, which is an endogenous inhibitor of proteolytic enzyme calpain, in the skeletal muscle of slaughtered pigs (19). In addition, Otani et al. (20) have clearly demonstrated that human GLUT-4 is cleaved by calpain-2 in vitro and that a transgenic mouse that overexpresses calpastatin has a three-fold higher expression of GLUT-4 protein. On the basis of these findings, we hypothesized that calpastatin might be involved in the maintenance of GLUT-4 protein content in skeletal muscle of rats fed a high-fat diet. However, there was no significant difference in calpastatin protein content between control and high-fat diet groups (Fig. 3), suggesting that calpastatin might not be involved in the high-fat diet-induced preservation of muscle GLUT-4 protein content. Garcia-Roves et al. (21) have shown that prevention of glycogen supercompensation prolongs the increase in GLUT-4 protein content in glycogen-depleted muscle, even after GLUT-4 mRNA expression has returned to control levels. It is therefore possible that glycogen-related proteolysis systems play a role in posttranscriptional regu-
lutation of muscle GLUT-4 protein content in rats fed a high-fat diet. However, in the present investigation, there was no significant difference in muscle glycogen concentrations between control and high-fat diet-fed groups (data not shown). Future extensive studies are expected to elucidate the mechanism by which a high-fat diet regulates GLUT-4 protein stability.

Finck et al. (22) have shown that muscle-specific overexpression of PPARα decreases the GLUT-4 protein content and mRNA expression in skeletal muscle. indicating that PPARα may also be involved in the regulation of GLUT-4 gene expression in skeletal muscle. However, PPARα is known to be a predominant form of PPARs in skeletal muscle and muscle cell lines (18). Furthermore, in the present investigation, incubation with a PPARα activator, fenofibrate, did not affect either GLUT-4 or UCP-3 expression in L6 myotubes (Fig. 2). It is therefore unlikely that activation of PPARα is involved in the regulation of muscle GLUT-4 expression in rats fed a high-fat diet.

In conclusion, the present investigation has demonstrated that a high-fat diet induces a decrease in muscle GLUT-4 mRNA expression, despite an increase in the PGC-1α protein content in rat skeletal muscle. The mechanism responsible for this phenomenon appears to be activation of PPARβ by elevated serum FFA concentrations. Our results also suggest that a high-fat diet maintains GLUT-4 protein content, possibly through posttranscriptional regulation.

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