Insulin-Nonspecific Reduction in Skeletal Muscle Glucose Transport in High-Fat–Fed Rats

K. Koshinaka, Y. Oshida, Y.-Q. Han, M. Kubota, A.Y.I. Viana, M. Nagasaki, and Y. Sato

High-fat feeding diminishes insulin-stimulated glucose transport in skeletal muscle. However, conflicting results are reported regarding whether phosphatidylinositol (PI)-3 kinase–independent glucose transport is also impaired in insulin-resistant high-fat–fed rodents. The aim of the present study was to study whether non–insulin-dependent mechanisms for stimulation of glucose transport are defective in skeletal muscle from high-fat–fed rats. Rats were fed normal chow diet or high-fat diet for 4 weeks and isolated epitrochlearis muscles were used for measuring glucose transport. Insulin-stimulated glucose transport was significantly lower in rats fed the high-fat diet compared with chow-fed rats ($P < .05$). Hypoxia-stimulated glucose transport was also reduced in high-fat–fed rats ($P < .05$). Nevertheless, hypoxia-stimulated adenosine monophosphate–activated protein kinase (AMPK) phosphorylation (Thr^{172}) level was not affected by high-fat feeding. Glucose transport by sodium nitroprusside stimulation was reduced in high-fat–fed rats ($P < .05$). Protein content of glucose transporter (GLUT)-4 and AMPK-α, and glycogen content were comparable between both groups. Our findings provide evidence that high-fat feeding can affect not only insulin but also non–insulin-stimulated glucose transport. A putative defect in common steps in glucose transport may play a role to account for impaired insulin-stimulated glucose transport in rats fed a high-fat diet.

IMPAIRED INSULIN action to stimulate glucose disposal in insulin-sensitive tissues is a hallmark of the insulin resistance associated with obesity.[1] In skeletal muscle, insulin induces translocation of the glucose transporter (GLUT)-4 from the intracellular pool to the plasma membrane in order to facilitate uptake glucose. Phosphatidylinositol (PI)-3 kinase is considered to be essential for insulin-stimulated GLUT-4 translocation.[2] Although the precise mechanisms underling obesity-related decrease in insulin-stimulated glucose transport are still not clear, it has been widely accepted that impaired early insulin signaling leading to insufficient activation of PI-3 kinase is a cause of reduced insulin-stimulated glucose transport in obese subjects[3] and high-fat–fed rodents.[4-6] Recently, however, it has been reported that PI-3 kinase activity in skeletal muscle is not always impaired in obese subjects,[7,8] obese first-degree relatives of type 2 diabetic (FDR) subjects,[9,10] or high-fat–fed rodents,[11] despite defective insulin-stimulated glucose transport. Additional impairments seem to be likely.

It is known that exercise,[12] muscle contraction,[2,13,14] or hypoxia[14-16] can induce GLUT-4 translocation. These stimuli enhance glucose transport via a PI-3 kinase–independent[2,17-19] and an adenosine monophosphate–activated protein kinase (AMPK)-dependent pathway.[14] Some reports that glucose transport by exercise,[12] muscle contraction,[13,20,21] or hypoxia[13,21] stimulation was inhibited in high-fat–fed rodents, suggest that a defect in common steps in glucose transport may also be important to account for impaired insulin-stimulated glucose transport in rodents fed a high-fat diet. However, conflicting results are reported regarding whether exercise[22] or hypoxia[4] stimulation of glucose transport is defective in skeletal muscle from high-fat–fed rodents. The aim of the present study was to examine whether non–insulin-dependent mechanisms for stimulation of glucose transport are defective in skeletal muscle from high-fat–fed rats. To characterize defective glucose transport, insulin (PI-3 kinase–dependent), hypoxia (PI-3 kinase–independent, AMPK-dependent), 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR; PI-3 kinase–independent, AMPK-dependent), and sodium nitroprusside (SNP; PI-3 kinase–independent, AMPK-independent) were used as stimulators of glucose transport. Furthermore, hypoxia-stimulated AMPK activation was also measured. AMPK is considered as an attractive molecular target for the treatment of insulin resistance[23]; however, only a few studies have examined the effect of insulin resistance on AMPK activation. To our knowledge, studies on whether high-fat feeding could cause AMPK activation have not been reported yet.

MATERIALS AND METHODS

Materials

2-Deoxy-D-[1,2-^{3}H]glucose (2DG) and L-[1-^{14}C]glucose (L-glucose) were from Perkin Elmer Life Sciences, Boston, MA. Antibody against GLUT-4 was from Biogenesis, South Coast, UK. Antibodies against AMPK-α and phospho-specific AMPK (Thr^{172}) were from Cell Signaling Technology, Beverly, MA. Antibody against Akt 1/2 was from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies against phospho-specific Akt 1 (Ser^{473} and Thr^{308}) were from Upstate Biotechnology, Lake Placid, NY. Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences, Buckinghamshire, UK.

Animal Care

All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of Nagoya University. Male Wistar rats (Chubu Kagakushizai, Nagoya, Japan) were obtained at 3 weeks of age and maintained in a 12:12-hour reversed light-dark environment at an ambient temperature of 23°C. Rats were randomly divided into 2 groups and fed commercially available normal chow diet or high-fat diet for 4 weeks. The chow diet (MF, Oriental Yeast, Chiba, Japan) contained, as percent of calories, 59% carbohydrate, 29% protein, and 12% fat. High-fat diet contained 27% carbohydrate, 23%

From the Department of Sports Medicine, Graduate School of Medicine; and Research Center of Health, Physical Fitness and Sports, Nagoya University, Nagoya, Japan.

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Address reprint requests to Y. Sato, MD, PhD, Department of Health Science, Faculty of Psychological and Physical Sciences, Aichigakuin University, 12, Araike, Iwasaki-cho, Nishin City, Aichi 970-0195, Japan.

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protein, and 50% fat.[21] The calorie contents of the chow and high-fat diets were 3.6 kcal/g and 5.1 kcal/g, respectively. After the feeding period, overnight-fasted (16 hours) animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). After blood samples were drawn from a tail vein, epitrochlearis muscles were dissected out. Some epitrochlearis muscles were used for measuring glucose transport and the remaining were stored at −80°C until biochemical assay. After muscle dissection was completed, retroperitoneal, mesenteric, and epididymal fat-pads were removed and weighted.

Muscle Incubations: Effects of Insulin, SNP, and AICAR

Muscles were incubated for 70 minutes in Krebs-Henseleit buffer (KHB) containing 8 mmol/L glucose, 36 mmol/L mannitol, and 0.1% bovine serum albumin (BSA). Muscles were then incubated for 30 minutes in KHB containing 2 mmol/L sodium pyruvate, 36 mmol/L mannitol, and 0.1% BSA with or without insulin (2 mU/mL), SNP (a nitric oxide [NO] donor; 10 mmol/L), or AICAR (a pharmacological activator of AMPK; 2 mmol/L). All incubations were performed at 30°C. The gas phase was maintained at 95% O_2/5% CO_2.

Muscle Incubations: Effects of Hypoxia

For study of the effect of hypoxia on glucose transport, muscles were incubated for 80 minutes in KHB containing 8 mmol/L glucose, 36 mmol/L mannitol, and 0.1% BSA under hypoxic condition (95% N_2/5% CO_2).[15] Thereafter, muscles were incubated for 20 min in oxygenated KHB supplemented with 2 mmol/L sodium pyruvate, 36 mmol/L mannitol, and 0.1% BSA. All incubations were performed at 30°C.

Glucose Transport

Glucose transport was measured as described previously.[24] Muscles were incubated for 20 min in KHB containing 4 mmol/L 2DG (0.3 μCi/mL), 36 mmol/L mannitol, 0.1 % BSA. L-Glucose (0.06 μCi/mL) was used to estimate extracellular space. Insulin, SNP, or AICAR was added to the buffer if needed. All incubations were performed at 30°C. The gas phase was maintained at 95% O_2/5% CO_2. Glucose transport rate is expressed as micromoles of 2DG accumulated per milliliter of intracellular water per 20 minutes.

Hypoxia Stimulation for AMPK

After 80 minutes of hypoxia stimulation, as described above, muscles were clamp frozen. The muscles were homogenized as described by Musi et al.[25] The muscles were homogenized in buffer [20 mmol/L tris(hydroxymethyl)aminomethane (Tris), 1% Triton-X 100, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 0.1 mmol/L benzamidine, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), pH7.4] on ice, and it was centrifuged at 14, 000 × *g* for 20 minutes at 4°C. The supernatants were used as samples.

GLUT-4 Protein Content

Muscles were homogenized in homogenization buffer (25 mmol/L HEPES, 2 mmol/L EDTA, 250 mmol/L sucrose, 3.3 mg/L leupeptin, 100 mg/L trypsin inhibitor, 1 mmol/L PMSF, pH 7.4) on ice. After centrifugation at 175,000 × *g* for 60 minutes, the pellets were resuspended in the homogenization buffer with 1% Triton-X 100. This solution was recentrifuged at 175,000 × *g* for 60 minutes, and the supernatants obtained were used as samples.

Table 1. Characteristics of the Rats

	Chow-Fed	High-Fat–Fed
Body weight (g)	212 ± 3 (24)	240 ± 2 (23)*
Visceral fat weight (g)	9.0 ± 0.4 (18)	20.1 ± 0.8 (19)*
Food intake (kcal/d)	74 ± 1 (7)	88 ± 1 (8)*
Plasma glucose (mg/dL)	111 ± 3 (8)	110 ± 3 (8)
Plasma insulin (ng/mL)	0.4 ± 0.1 (8)	1.0 ± 0.2 (8)*

NOTE. Values are means ± SE (n). Visceral fat weight is the sum of mesenteric, retroperitoneal, and epididymal depots weights.

*$P < .05$ *vs* chow-fed.

Insulin Stimulation for Akt

Overnight-fasted animals were anesthetized as mentioned above. A bolus of saline with or without (control) insulin (10 U/kg body weight) was injected via vena cava inferior. After 120 seconds, the left (control) and right plantaris/gastrocnemius muscles were excised and clamp frozen. Muscle extracts were prepared as described by Kawanaka et al.[26]

Western Blotting

Treated samples were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. After membrane blocking with 5% nonfat dry milk for 2 hours, the blots were incubated overnight with anti–GLUT-4, AMPK-α, Akt 1/2 antibodies, antiphospho-AMPK-α (Thr^{172}), or antiphospho-Akt 1 (Ser^{473} and Thr^{308} antibodies. The blots were then rinsed in Tris-buffered saline with 0.05% Tween 20 and incubated with horseradish peroxidase–conjugated donkey anti-rabbit IgG for 2 hours. Immunoreactive bands were detected by the ECL reagent and subsequently analyzed by densitometry.

Analytical Determination

Plasma glucose level was determined by YSI 2300 STAT glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH). Plasma insulin level was assayed using a radioimmunological assay kit (Phadeseph Insulin RIA, Pharmacia, Stockholm, Sweden). Glycogen content was measured as described previously.[27]

Statistical Analyses

Values are presented as means ± SE. When 2 mean values were compared, analysis was performed by unpaired *t* test. When multiple mean values were compared, analysis was performed by 2-way analysis of variance (ANOVA). If a significant F value was found, further analysis was performed by the Scheffé's method. Difference was considered statistically significant when $P < .05$.

RESULTS

Characterization of High-Fat—Fed Rats

Table 1 summarizes body weight, visceral fat weight, food intake, and plasma glucose and insulin levels. High-fat feeding for 4 weeks resulted in significant increase in body weight and visceral fat weight compared with chow feeding ($P < .05$). Food intake in high-fat–fed rats was significantly greater than in chow-fed rats ($P < .05$). Fasting plasma glucose levels were comparable between both groups, but fasting plasma insulin levels were significantly elevated in high-fat–fed rats ($P < .05$).

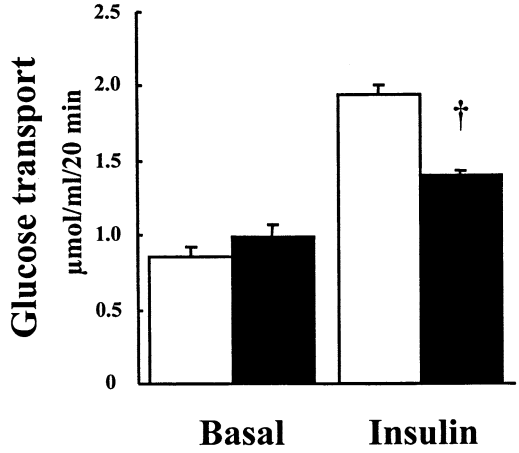

Fig 1. Glucose transport in rats fed either chow (□) or high-fat diet (■) for 4 weeks. Epitrochlearis muscles were incubated in the absence (basal) or presence of insulin (2 mU/ml). Values are mean ± SE (n = 6-8). †$P < .05$ *v* chow-fed.

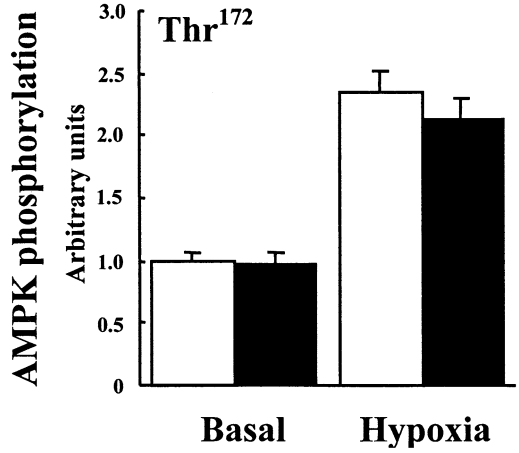

Fig 3. Effect of high-fat feeding on hypoxia-stimulated AMP-activated protein kinase (AMPK) phosphorylation level. Epitrochlearis muscles were incubated for 80 minutes under hypoxia and frozen. Quantification of AMPK phosphorylation level at the Thr^{172} site was expressed relative to chow-fed basal values. Values are mean ± SE (n = 5-6). (□) Chow-fed; (■) high-fat–fed.

Insulin-Stimulated Glucose Transport in High-Fat–Fed Rats

The effect of high-fat feeding on basal and insulin-stimulated glucose transport is shown in Fig 1. Glucose transport by insulin stimulation was significantly lower in muscles from high-fat–fed rats than from chow-fed rats. Basal glucose transport was not affected by diet.

Hypoxia, SNP, and AICAR-Stimulated Glucose Transport in High-Fat–Fed Rats

Figure 2 shows the non–insulin-stimulated glucose transport. Hypoxia-stimulated glucose transport was significantly lower in muscles from high-fat–fed rats compared with from chow-fed rats ($P < .05$). We also found significant reduction in SNP-stimulated glucose transport in high-fat–fed rats. Although a detrimental tendency was observed in AICAR-stimulated glucose transport in high-fat–fed rats, the difference between both groups was not statistically significant.

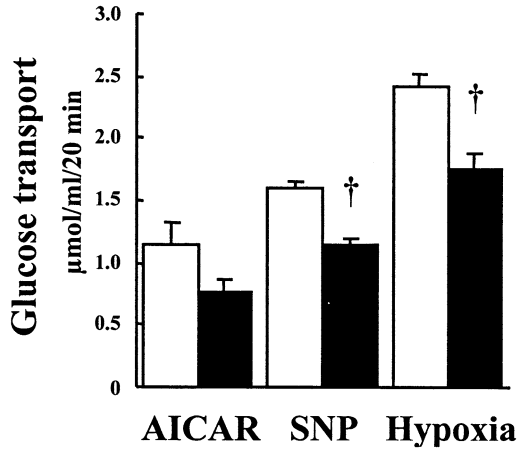

Fig 2. Glucose transport in rats fed either chow (□) or high-fat diet (■) for 4 weeks. Epitrochlearis muscles were incubated in the absence (basal), or in the presence of sodium nitroprusside (SNP; 10 mmol/L), or 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR; 2 mmol/L). For hypoxia stimulation, epitrochlearis muscles were incubated for 80 minutes under hypoxia. Values are mean ± SE (n = 6-8). †$P < .05$ *v* chow-fed.

Effect of High-Fat Feeding on AMPK

To study whether reduced glucose transport by hypoxia stimulation is accompanied with blunted activation of AMPK, we also measured hypoxia-stimulated AMPK phosphorylation level at the Thr^{172} site in high-fat–fed rats (Fig 3). A 2- to 3-fold higher phosphorylation level was given by hypoxia stimulation compared with the basal condition. High-fat feeding did not affect basal or hypoxia-stimulated these phosphorylations. No significant difference was observed in AMPK-α protein content between both groups (Table 2).

GLUT-4 Protein and Glycogen Content

High-fat feeding did not affect GLUT-4 protein content or glycogen content (Table 2).

Effect of High-Fat Feeding on Akt

To determine whether high-fat feeding impairs the downstream of PI-3 kinase, we measured insulin-stimulated Akt phosphorylation level at the Ser^{473} and Thr^{308} sites (Fig 4). In chow-fed rats, insulin caused 5-fold (Ser^{473}, Fig 4A) and 4-fold (Thr^{308}, Fig 4B) increases in phosphorylation level compared

Table 2. Protein and Glycogen Content

	Chow-Fed	High-Fat–Fed
AMPK-α	1.00 ± 0.04	1.00 ± 0.04
GLUT-4	1.00 ± 0.08	1.06 ± 0.1
Glycogen content (mg/g wet weight)	5.5 ± 0.3	5.3 ± 0.09

NOTE. Values are means ± SE (n = 5-7). AMPK-α and GLUT-4 are expressed in arbitrary units.

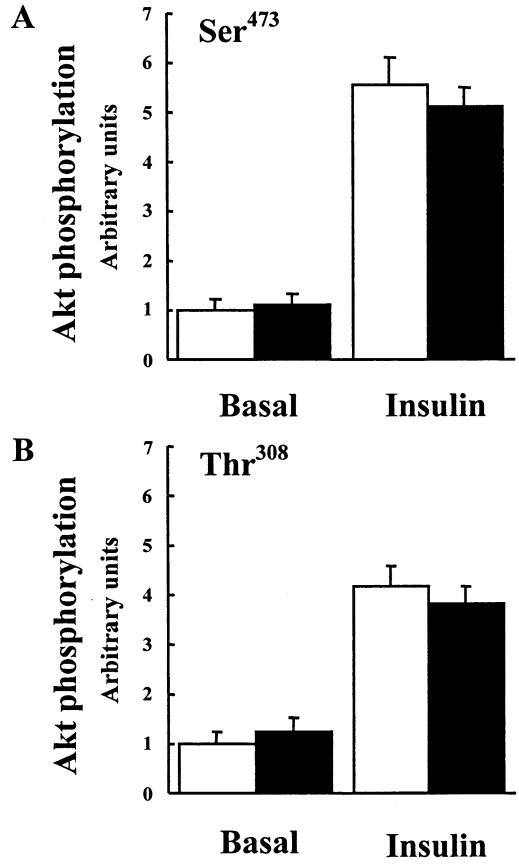

Fig 4. Effect of high-fat feeding on insulin-stimulated Akt phosphorylation level. Animals were anesthetized and then given an intravenous bolus of saline (basal) or insulin (10 U/kg body weight). Plantaris/gastrocnemius muscles were quickly dissected and frozen 120 seconds after administration of saline or insulin. Quantification of Akt phosphorylation level at (A) Ser^{473} and (B) Thr^{308} sites was expressed relative to chow-fed basal values. Values are mean ± SE (n = 5-6). (□) Chow-fed; (■) high-fat–fed.

with the basal condition (Fig 4B). High-fat feeding did not affect basal or insulin-stimulated phosphorylations. No significant difference was observed in Akt 1/2 protein content between both groups (chow-fed *v* high-fat–fed, 1.00 ± 0.15 *v* 1.07 ± 0.19 arbitrary units).

DISCUSSION

In skeletal muscle, there are at least 2 distinct signaling cascades that stimulate GLUT-4 translocation and glucose transport. Insulin induces glucose transport via a PI-3 kinase–dependent pathway,[2,17-19,28,29] whereas muscle contraction,[2,17-19] hypoxia,[17] or SNP[30] activates glucose transport via a PI-3 kinase–independent pathway. In the present study, we confirmed that high-fat feeding decreased insulin-stimulated glucose transport. A major finding of the present study was that both hypoxia and SNP-stimulated glucose transport were also impaired in insulin-resistant high-fat–fed rats. This result clearly demonstrates that resistance of glucose transport is not limited only against insulin but also to non-insulin stimuli.

AMPK is thought to be an important mediator of muscle contraction or hypoxia-stimulated glucose transport in skeletal muscle. It has been reported that exercise,[25] muscle contraction,[14,18,19,31] or hypoxia[14,31] stimulates AMPK activation. AICAR acutely induces GLUT-4 translocation[29,32] and glucose transport.[14,18,28,29,32-35] To determine whether decreased glucose transport by hypoxia stimulation could be accounted for reduced AMPK activation, hypoxia-stimulated AMPK phosphorylation level at the Thr^{172} site was measured in high-fat–fed rats. Our data demonstrated that the defect in hypoxia-stimulated glucose transport in high-fat–fed rats occurred with normal AMPK activation. It has been reported that exercise,[12] muscle contraction,[13,20,21] or hypoxia stimulation of glucose transport[13,21] is impaired, whereas glucose transport by exercise[22] or hypoxia[4] stimulation is normal in high-fat–fed rodents. It is not clear what causes the difference. Also we cannot apply this result to the case of muscle contraction because muscle contraction and hypoxia are similar[17] but not the same.[14] However, normal AMPK activation has been also reported in non–insulin-dependent diabetes mellitus (NIDDM)[25] and obese Zucker rats,[31,33] and in the fatty acid–induced insulin-resistant condition.[34] These results indicate that AMPK activation is highly preserved in the insulin-resistant condition, and that the decrease in hypoxia-stimulated glucose transport in high-fat–fed rats can not be explained by AMPK arrangement.

SNP can induce GLUT-4 translocation.[30] It has been reported that PI-3 kinase inhibition leads to little[19] or no reduction[28,30] in SNP-stimulated glucose transport. NO synthase (NOS) inhibitor has no effect on the glucose transport by exercise,[19] muscle contraction,[19,30] or insulin[28,30] stimulation. Although SNP could slightly stimulate AMPK-α[1][19] hypoxia-stimulated glucose transport is mainly AMPK-α2–dependent.[14] A study demonstrated that AICAR-stimulated glucose transport was completely abolished by a NOS inhibitor.[28] However, we found no effect of the NOS inhibitor on AICAR-stimulated glucose transport (K. Koshinaka, unpublished observations). The physiological role of the NO-induced glucose transport and its signaling is still obscure. Taken together these findings suggest essentially independence of the NO signaling from PI-3 kinase and AMPK pathway. We observed that SNP-stimulated glucose transport was reduced in high-fat–fed rats. Bergeron et al[33] reported that AICAR-stimulated glucose transport was impaired in obese Zucker rats in a skeletal muscle fiber type-dependent manner. These investigators suggested a possibility of impaired downstream pathway of AMPK on GLUT-4 translocation in the rats. Although the defective tendency in AICAR-stimulated glucose transport was not statistically significant in the present study, insulin-nonspecific reduction in glucose transport could lead to conclusion of the possible defect in common steps in glucose transport.

GLUT-4 is considered as one of the common components of the glucose transport system in skeletal muscle. GLUT-4 protein content is known to be closely related to muscle contraction and insulin-stimulated glucose transport.[36] However, our results demonstrated that GLUT-4 content may not be a main cause of the decreased glucose transport by insulin or non-insulin stimuli. Hansen et al[13] demonstrated that the impaired both insulin and muscle contraction stimulation of glucose transport were accompanied with a decrease in GLUT-4 trans-

location. Furthermore, Rosholt et al[12] provided evidence that high-fat feeding blunted GLUT-4 intrinsic activity. Although we did not measure GLUT-4 translocation or intrinsic activity, impaired functional aspects of glucose transport system might play a role in the reduced glucose transport in high-fat–fed rats.

It has been accepted that impaired early insulin signaling leading to insufficient activation of PI-3 kinase is a cause of reduced insulin-stimulated glucose transport. However, PI-3 kinase activity in skeletal muscle is not always impaired in obese subjects,[7,8] FDR subjects,[9,10] or high-fat–fed rodents.[11] Also, Akt, a downstream molecule of PI-3 kinase, phosphorylation/activity by insulin stimulation is normal in obese,[7,8] FDR subjects,[9,10] and high-fat–fed rodents.[11] We found that the insulin-stimulated Akt phosphorylation level at the Ser^{473} and Thr^{308} sites was normal in plantaris/gastrocnemius muscles from high-fat–fed rats. Our result partially supports the speculation of the cause of blunted insulin-stimulated glucose transport in epitrochlearis muscle from high-fat–fed rats. These results might indicate that a defect in insulin signaling is not enough to account for obesity-related impaired insulin-stimulated glucose transport.

Interestingly, the insulin-nonspecific reduction in glucose transport observed in the present study was also observed in NIDDM,[16] high-sucrose feeding,[37] high-starch feeding,[37] and dexamethasone treatment,[38] whereas it was not observed in high-fat feeding,[4,22] genetic obese rodents,[31,35] or NIDDM.[39] It is likely that insulin-nonspecific reduction in glucose transport is not an obesity-specific and also not a common phenomenon in the insulin-resistant state.

In the present study, it remains unclear why glucose transport is impaired. Recently some effectors including atypical protein kinase C (PKC),[29] p38 mitogen-activated protein kinase,[40] and adenosine[41] have been considered as modulators of both insulin and non–insulin-stimulated glucose transport. In addition to a possible defect in glucose transport system, these inactivation or inadequate production might have some role in reduced glucose transport. We cannot exclude the possibility that insulin-nonspecific reduction in glucose transport was owing to defective individual signaling, not common steps because we did not measure the downstream cascade of Akt, AMPK, or NO signaling and these signaling effects are still obscure. Nevertheless, it could be concluded that the mechanisms that can induce non–insulin-stimulated glucose transport would, at least partly, contribute to blunted insulin-stimulated glucose transport in high-fat fed rats.

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