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Title:

Fucoxanthin promotes translocation and induction of glucose transporter 4  
in skeletal muscles of diabetic/obese KK-*A<sup>y</sup>* mice

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Running title: Fucoxanthin regulates GLUT 4 translocation and expression  
in skeletal muscle

Keywords: fucoxanthin, glucose transporter 4, skeletal muscle,  
PGC-1 $\alpha$ , insulin receptor, Akt

## ABSTRACT

Fucoxanthin (Fx) isolated from *Undaria pinnatifida* suppresses the development of hyperglycemia and hyperinsulinemia of diabetic/obese KK-*A<sup>y</sup>* mice after two weeks of feeding 0.2% Fx-containing diet. In the soleus muscle of KK-*A<sup>y</sup>* mice that were fed Fx, glucose transporter 4 (GLUT4) translocation to plasma membranes from cytosol was promoted. On the other hand, Fx increased GLUT4 expression levels in the extensor digitorum longus (EDL) muscle, although GLUT4 translocation tended to increase. The expression levels of insulin receptor (IR) mRNA and phosphorylation of Akt, which are in upstream of the insulin signaling pathway regulating GLUT4 translocation, were also enhanced in the soleus and EDL muscles of the mice fed Fx. Furthermore, Fx induced peroxisome proliferator activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), which has been reported to increase GLUT4 expression, in both soleus and EDL muscles. These results suggest that in diabetic/obese KK-*A<sup>y</sup>* mice, Fx improves hyperglycemia by activating the insulin signaling pathway, including GLUT4 translocation, and inducing GLUT4 expression in the soleus and EDL muscles, respectively, of diabetic/obese KK-*A<sup>y</sup>* mice.

## Introduction

Glucose transporter 4 (GLUT4), which is a 12-transmembrane protein, is the predominant isoform of the glucose transporters expressed abundantly in skeletal muscle and adipose tissue (Joost et al. 2002). Insulin stimulates GLUT4 translocation to plasma membranes from cytosol and promotes glucose uptake (Huang and Czech 2007). Furthermore, glucose uptake stimulated by insulin is linearly related to the content of GLUT4 in plasma membranes (Goodyear et al. 1990; Henriksen et al. 1990). Skeletal muscle expressing GLUT4 accounts for up to 40% to 60% of body mass and thereby regulates glucose homeostasis. However, several reports have shown that GLUT4 translocation was decreased in type 2 diabetes because of insulin resistance (Gaster et al. 2001; Schimmack et al. 2005). Therefore, the regulation of GLUT4 translocation and expression is a potential target for the treatment of diabetes mellitus.

Our previous report demonstrated that fucoxanthin (Fx, Fig. 1), which is a carotenoid found in edible brown seaweeds, such as *Undaria pinnatifida* and *Hijikia fusiformis*, improved hyperglycemia in diabetic/obese KK-*A<sup>y</sup>* mice, whereas it did not affect blood glucose level of normal C57BL/6J mice (Hosokawa et al. 2010). Further, Fx down-regulated the mRNA expression of pro-inflammatory adipocytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), in white adipose tissue (WAT). However, the mechanism of the anti-diabetic effect by Fx is not clear yet. An Fx diet reduced the serum insulin levels of KK-*A<sup>y</sup>* mice showing hyperinsulinemia.

These results indicate that Fx may improve insulin resistance in *KK-A<sup>y</sup>* mice.

In the insulin signaling pathway, insulin receptor (IR), insulin receptor substrate (IRS), and Akt/protein kinase B, as well as GLUT4, are important molecules. In addition, peroxisome proliferator activated receptor  $\gamma$  coactivator- $\alpha$  (PGC-1 $\alpha$ ) has been reported to regulate GLUT4 expression in skeletal muscle and myocytes (Michael et al. 2001). PGC-1 $\alpha$  is preferentially expressed in muscle enriched with type I fibers (Lin et al. 2002), and it is involved in oxidative energy production, while PGC-1 $\alpha$  expression level is low in type IIb fibers. The soleus and extensor digitorum longus (EDL), which are typical muscle types, are mainly composed of type I fibers and type IIb fibers, respectively (Henriksen et al. 1990). In obesity and type 2 diabetes, PGC-1 $\alpha$  expression has been reported to decrease in muscles (Mootha et al. 2003). Therefore, PGC-1 $\alpha$  is an important molecule to regulate GLUT4 expression in skeletal muscles, such as the soleus and EDL.

In this study, to address the mechanism of the anti-diabetic effect by Fx, we examined the effects of Fx on GLUT4 expression and translocation in typical skeletal muscles, the soleus and EDL. Furthermore, the expression levels of IR, Akt and PGC-1 $\alpha$  were also measured in skeletal muscles.

## **Materials and methods**

### *Materials*

Commercial seaweed powder from *Undaria pinnatifida* was purchased

from the market in Hakodate, Japan. Anti-GLUT4, anti-PGC-1 $\alpha$  and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-Akt and anti-phospho-Akt (p-Akt) (Ser473) were obtained from Cell Signaling Technology (Beverly, MA, USA).

#### *Fucoxanthin preparation*

Crude seaweed lipids containing Fx were extracted from the commercial seaweed powder by acetone. Fx was purified from the crude seaweed lipids by silica gel column chromatography with *n*-hexane/acetone (8:2, v/v), as in our previous report (Tsukui et al. 2007). Its purity was more than 98% by HPLC. Fx was stored at -30°C and mixed in AIN93G diet before feeding every two days.

#### *Animal experiments*

Diabetic/obese KK-*A<sup>y</sup>* mice (female, 4 weeks old) were obtained from CREA Japan (Tokyo, Japan) and were housed at 23 $\pm$ 1°C and 50% humidity with a 12 h light/12 h dark cycle. The mice were allowed free access to water and food. The control diet was prepared according to AIN-93G (Reeves et al. 1993). After acclimation feeding of the control diet for 1 week, KK-*A<sup>y</sup>* mice were assigned to 2 groups, and provided with the control diet (AIN-93G) or the experimental diet containing 0.2% Fx added to the control diet, for 2

weeks. Then, the mice were anatomized under anesthesia by diethyl ether. The soleus and EDL muscles were removed and immediately stored in RNA later™ (Sigma Chemical Co., St. Louis, MO, USA) for quantitative real-time PCR analysis, or were frozen in liquid nitrogen for Western blot analysis. All procedures for the use and care of animals for this research were approved by the Ethical Committee for Experimental Animal Care at Hokkaido University.

#### *Blood glucose and serum insulin levels*

Blood glucose was determined using a G. Checker (Gunze, Kyoto, Japan) blood glucose meter without fasting. Serum insulin was determined using a mouse insulin ELISA kit (Shibayagi, Gunma, Japan).

#### *Western blot analysis*

The total fraction (Total), plasma membrane fraction (PM) and post-plasma membrane fraction (Post-PM) containing mainly cytosol of skeletal muscle were prepared according to a previous report (Nishiumi and Ashida 2007). The total, PM and Post-PM fractions were loaded into an SDS-PAGE system, and separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies against GLUT4 (1 : 5000 dilutions), GAPDH (1 : 200,000), Akt (1 : 2000), p-Akt (1 : 2000), and PGC-1 $\alpha$  (1 : 3000) for 1 h and then were

incubated with a secondary antibody rabbit or mouse IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. Proteins were detected with a chemiluminescence detection kit (ECL system, Amersham, USA).

### *Quantitative real-time PCR*

Total RNA was extracted from skeletal muscles using RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan). Then, cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd, Tokyo, Japan). The quantitative real-time PCR analysis of individual cDNA was performed using an ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd, Tokyo, Japan); IR: Mm01211875\_m1, 18S RNA: Mm02601778\_g1. The PCR cycling conditions were 50°C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

### *Statistical analysis*

Results were expressed as mean±SEM. Statistical analysis was performed using an unpaired Student's *t*-test.  $P<0.05$  and  $P<0.01$  were considered to have significant differences.

## Results

### *Blood glucose level and serum insulin level in KK- $A^y$ mice*

Blood glucose levels without fasting were significantly lower in the diabetic/obese KK- $A^y$  mice fed Fx diet than those of control mice after 2 weeks of feeding (Fig. 2A). Fx markedly decreased blood glucose levels to  $176.4 \pm 15.8$  mg/dl, compared to  $389.2 \pm 23.3$  mg/dl in the control mice. In addition, Fx significantly reduced the serum insulin levels of KK- $A^y$  mice showing hyperinsulinemia after 2 weeks of feeding (Fig. 2B). We also examined the gene expression of the gluconeogenesis enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver (data not shown). However, Fx did not alter PEPCK mRNA expression and slightly increased G6Pase mRNA expression in the liver. These data show that the improvement of hyperglycemia and hyperinsulinemia by Fx is not responsible for the down-regulation of gluconeogenesis in KK- $A^y$  mice.

### *Translocation and expression of GLUT4 in the soleus and EDL muscles*

Insulin stimulates GLUT4 translocation to plasma membranes and activates glucose uptake in muscle (Huang and Czech 2007). However, GLUT4 translocation is attenuated in diabetic/obese KK- $A^y$  mice with insulin resistance (Liu et al. 2010). Therefore, we examined the effect of Fx

on GLUT4 translocation in the skeletal muscle of KK-*A<sup>y</sup>* mice. As shown in Fig. 3, Fx significantly increased GLUT4 translocation to plasma membranes in the soleus muscle (Fig. 3A) and tended to increase translocation in EDL muscles (Fig. 3B). Moreover, Fx significantly increased GLUT4 expression in EDL muscles (Fig. 3D), but not in the soleus muscles (Fig. 3C). These data suggest that the responses associated with translocation and expression of GLUT4 by Fx are different in muscle types.

*IR mRNA expression and Akt phosphorylation by Fx in soleus and EDL muscles*

The insulin signaling pathway is triggered by the activation of IR tyrosine kinase. Akt/protein kinase B is then phosphorylated through phosphorylation of the tyrosine site of IRS-1. In obese, insulin-resistant subjects, GLUT4 translocation to the plasma membrane is attenuated by the inhibition of insulin signaling pathways such as IR, IRS-1 and Akt activation (Zierath et al. 1996). Therefore, to clarify the activation of GLUT4 translocation associated with improvement of insulin resistance by Fx, we investigated IR mRNA expression and Akt phosphorylation in the soleus and EDL muscles. Fx significantly increased IR mRNA expression in both the soleus (Fig. 4A) and EDL (Fig. 4B) muscles. Furthermore, Fx also activated Akt phosphorylation in the soleus (Fig. 4C) and EDL muscles (Fig. 4D). The ratio of p-Akt to Akt expressions increased by more than 1.7 fold and 1.8 fold in the soleus muscle and EDL muscle, respectively, of Fx-fed

mice compared to control.

#### *Expression level of PGC-1 $\alpha$ by Fx in the soleus and EDL muscles*

PGC-1 $\alpha$  is an important co-activator that has been implicated in the regulation of mitochondrial biogenesis and the oxidative metabolism of muscle (Bonen 2009). PGC-1 $\alpha$  has been reported to activate GLUT4 expression in myocytes (Michael et al. 2001). In KK-*A<sup>y</sup>* mice fed Fx, GLUT4 expression levels were enhanced in EDL muscles as shown in Fig. 3D. We therefore examined PGC-1 $\alpha$  expression levels in muscles. Fx up-regulated PGC-1 $\alpha$  expression levels in EDL muscles (Fig. 5B) and its level was 190% higher compared to the control group. PGC-1 $\alpha$  levels were also significantly increased by Fx in the soleus muscle (Fig. 5A).

#### **Discussion**

GLUT4, which is highly expressed in skeletal muscle and adipose tissue, plays a pivotal role in whole body glucose homeostasis. By insulin and other stimuli, GLUT4 is acutely distributed to plasma membranes from intracellular disposition and promotes glucose uptake (Huang and Czech 2007). In type 2 diabetes mellitus, insulin signaling is impaired, and GLUT4 translocation to the plasma membrane is attenuated (Shepherd and Kahn 1999). In addition, it has been reported that overexpression of GLUT4 in the muscles of diabetic mice alleviates insulin resistance and elevates both basal

and insulin-stimulated glucose transport (Gibbs et al. 1995). Therefore, natural compounds and chemicals that facilitate GLUT4 translocation and induction exhibit beneficial effects on the prevention and improvement of type 2 diabetes and on insulin resistance.

Recently, epigallocatechin, which is a major polyphenol in green tea, has been reported to decrease blood glucose levels and to increase glucose uptake through GLUT4 translocation in rat skeletal muscles (Ueda et al. 2008). Dietary anthocyanin-rich bilberry extract has also been shown to ameliorate hyperglycemia by inducing GLUT4 expression in the skeletal muscles of diabetic/obese *KK-A<sup>y</sup>* (Takikawa et al. 2010). We previously reported that 0.2% Fx-containing diet fed for 4 weeks markedly improved hyperglycemia of *KK-A<sup>y</sup>* (Hosokawa et al. 2010; Maeda et al. 2007). In this study, we observed that the blood glucose level of *KK-A<sup>y</sup>* significantly decreased compared to the control mice even after 2 weeks of feeding 0.2% Fx-containing diet. In addition, Fx also improved serum insulin levels of *KK-A<sup>y</sup>* mice with hyperinsulinemia during 2 weeks of feeding with Fx diet. Insulin-mediated glucose uptake by GLUT4 is the rate-limiting step in carbohydrate metabolism. In especially, GLUT4 expression in *KK-A<sup>y</sup>* mice is known to be low (Hofmann et al. 1991). Therefore, to address the mechanism for the improvement of hyperglycemia in *KK-A<sup>y</sup>* mice by Fx, GLUT4 translocation to plasma membranes and induction were investigated in typical skeletal muscles, i.e., the soleus and EDL muscles.

Muscles can be classified by fiber types, and they have different metabolic activity. Type I fibers are slow-twitch oxidative fibers. Type IIa and IIb fibers

are fast-twitch oxidative and fast-twitch glycolytic fibers, respectively. Type I and type IIa fibers have high insulin sensitivity and express high levels of GLUT4, whereas type IIb fibers have low insulin sensitivity and GLUT4 expression (Henriksen et al. 1990; James et al. 1989). In previous reports, the soleus muscle was composed mainly of type I fibers, and the EDL muscle was composed of type IIa and IIb fibers (Burkholder et al. 1994; Hirai et al. 2011). Thus, the soleus muscle is rich in oxidative fiber, and the EDL muscle is high in glycolytic fiber.

As shown Fig. 3A, Fx activated GLUT4 translocation in the soleus muscle of KK-*A<sup>y</sup>* mice. In addition, GLUT4 translocation tended to increase in EDL muscle by Fx. Insulin signaling is known as a major upstream pathway of GLUT4 translocation. To elucidate the mechanism of GLUT4 translocation in the muscles of KK-*A<sup>y</sup>* mice fed Fx, we examined IR mRNA expression levels and Akt phosphorylation. Fx significantly enhanced IR mRNA expression levels in the soleus and EDL muscles. Furthermore, Fx also increased the phosphorylation of Akt in the soleus and EDL muscles. Therefore, Fx seems to improve insulin resistance in the muscles of KK-*A<sup>y</sup>* mice through promotion of GLUT4 translocation, depending on the activation of insulin signaling via up-regulation of IR mRNA expression and activation of Akt.

On the other hand, GLUT4 expression was induced in EDL muscle with highly glycolytic activity, with the remaining ratio of GLUT4 distribution in cytosol and plasma membranes by Fx. These results show an abundance of GLUT4 in plasma membranes increased in the EDL muscle, and this process

results in an increase in glucose uptake by Fx. It has been reported that GLUT4 expression and translocation are different in the soleus and EDL muscles. Henriksen et al. (1990) reported that GLUT4 content was higher in the soleus muscle than that in the EDL muscle. On the other hand, Oh et al. (2007) showed that GLUT4 expression was increased by exercise in the EDL muscle, but not in the soleus muscle. Therefore, to inducing GLUT4 expression in the EDL muscle using Fx is an effective way to activate glucose metabolism and to improve hyperglycemia in *KK-A<sup>y</sup>* mice.

Notably, Fx significantly increased PGC-1 $\alpha$  expression levels in the soleus and EDL muscles. PGC-1 $\alpha$  is a critical activator of mitochondrial biogenesis. In several previous studies, reduction of PGC-1 $\alpha$  expression was observed in obesity and in type 2 diabetes (Mootha et al. 2003; Patti et al. 2003). Further, overexpression of PGC-1 $\alpha$  in muscle led to increase GLUT4 expression and insulin-stimulated glucose uptake (Benton et al. 2008). PGC-1 $\alpha$  has been also reported to induce proteins that are characteristic of type I fibers in addition to the activation of genes involved in oxidative energy production in muscles enriched with type IIb fibers (Lin et al. 2002). In the present study, GLUT4 expression was increased in the EDL muscle through PGC-1 $\alpha$  induction by Fx. These data suggest that induction of PGC-1 $\alpha$  in muscle is an important mechanism to improve hyperglycemia by regulating of GLUT4 in *KK-A<sup>y</sup>* mice fed Fx. However, GLUT4 expression did not increase in the soleus muscle with highly GLUT4 expression level, although PGC-1 $\alpha$  was induced. It is required to further examine the molecular mechanism for GLUT4 induction by Fx in skeletal muscles.

In summary, this is the first study to show that Fx promotes translocation and expression of GLUT4 in the soleus and EDL muscles, respectively, in diabetes/obesity *KK-A<sup>y</sup>* mice. Moreover, Fx also increases IR mRNA expression, Akt phosphorylation and PGC-1 $\alpha$  expression in the soleus and EDL muscles. These results suggest that Fx improves hyperglycemia in diabetic/obese *KK-A<sup>y</sup>* mice through the activation of the insulin signaling pathway including GLUT4 translocation and expression.

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## Figure legend

**Fig. 1.** Fucoxanthin structure.

**Fig. 2.** Effects of fucoxanthin on blood glucose and serum insulin levels in KK-*A<sup>y</sup>* mice. The mice were fed 0.2% fucoxanthin (Fx)-containing diet or control diet for 2 weeks. The blood glucose and serum insulin levels without fasting in KK-*A<sup>y</sup>* mice were measured using G. Checker and ELISA, respectively. (A) Blood glucose concentration; (B) Serum insulin concentration. Values are presented as  $\pm$  SEM (n = 6-7); \**P* < 0.05, \*\**P* < 0.01 vs. control.

**Fig. 3.** Effects of fucoxanthin on GLUT4 translocation and expression in the skeletal muscles of KK-*A<sup>y</sup>* mice. The mice were fed 0.2% fucoxanthin (Fx)-containing diet or control diet for 2 weeks. After separation of plasma membrane (PM) and post-plasma membrane (Post-PM) containing cytosol in soleus (A) and EDL (B) muscles, GLUT4 expression was detected by Western blotting. GLUT4 translocation was expressed as the ratio of GLUT4 expression of PM to that of Post-PM. GLUT4 expression levels in the soleus (C) and EDL (D) muscles were measured by Western blotting using tissue lysates. GLUT4 expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level and expressed relative to the control. Values are presented means  $\pm$  SEM (n = 6-7); \**P* < 0.05, \*\**P* < 0.01 vs. control.

**Fig. 4.** Effects of fucoxanthin on IR mRNA expression and Akt phosphorylation in skeletal muscles. The mice were fed 0.2% fucoxanthin (Fx)-containing diet or control diet for 2 weeks. IR mRNA expression levels in the soleus (A) and EDL (B) muscles were measured by real-time PCR. IR mRNA expression was normalized to 18S RNA expression level and expressed relative to control. Akt and p-Akt expression in the soleus (C) and EDL (D) muscles were measured by Western blotting. The ratio of p-Akt to Akt is expressed relatively to the control. Values are presented as means $\pm$ SEM (n = 6-7); \* $P$ <0.05, \*\* $P$ <0.01 vs. control.

**Fig. 5.** PGC-1 $\alpha$  expression in the skeletal muscles of KK- $A^y$  mice fed fucoxanthin. The mice were fed 0.2% fucoxanthin (Fx)-containing diet or control diet for 2 weeks. PGC-1 $\alpha$  expression levels in the soleus (A) and EDL (B) muscles were measured by Western blotting. PGC-1 $\alpha$  expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level and expressed relative to the control. GAPDH expression levels are the same as Fig. 3. Values are presented means $\pm$ SEM (n = 6-7); \* $P$ <0.05, \*\* $P$ <0.01 vs control.



Fig.2

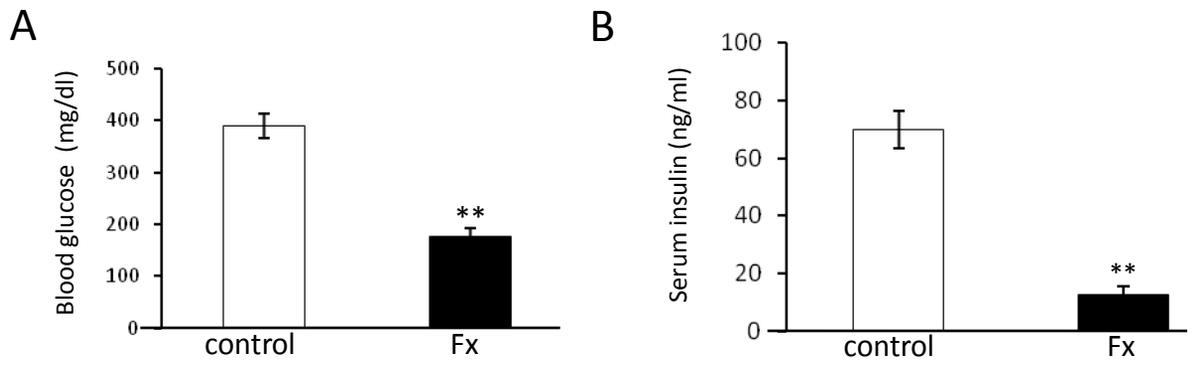
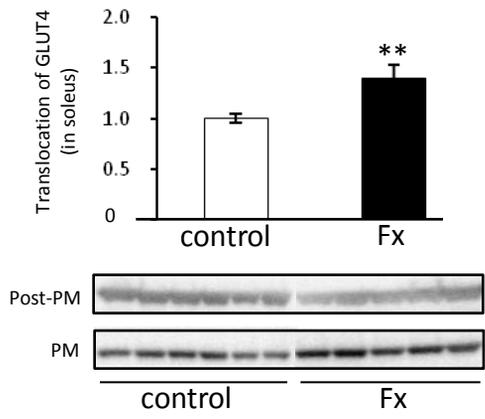
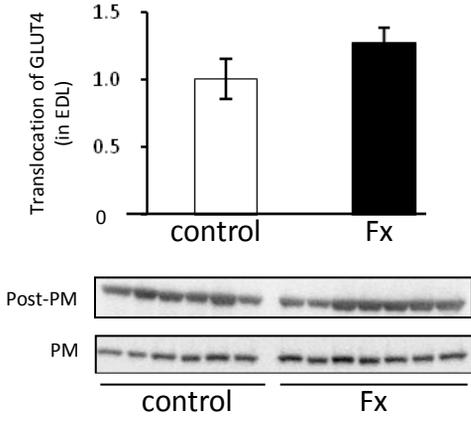


Fig.3

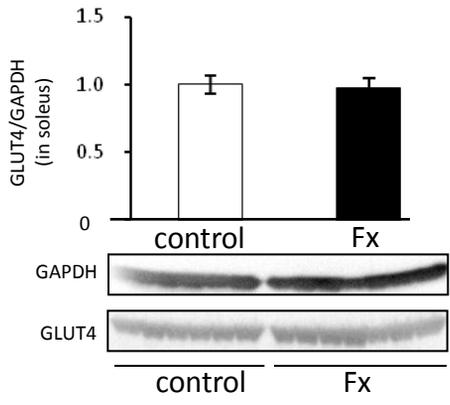
A



B



C



D

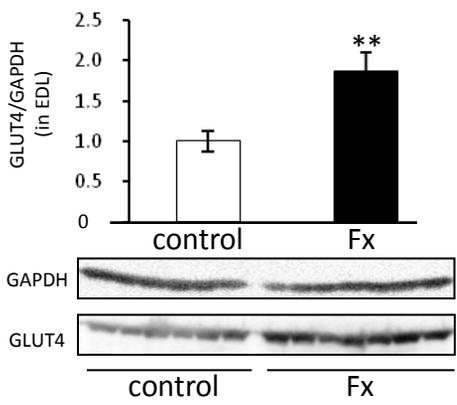
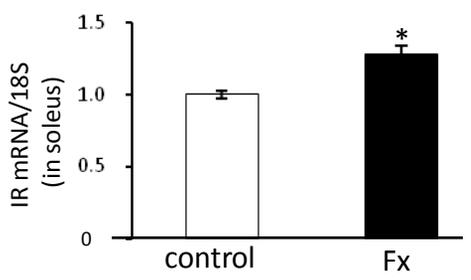
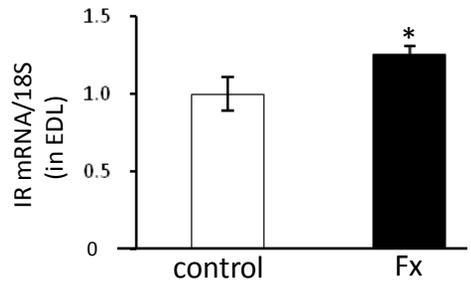


Fig.4

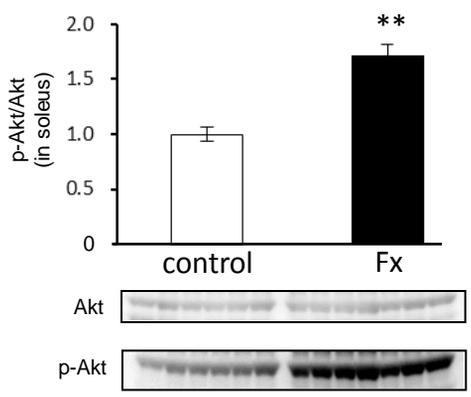
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B



C



D

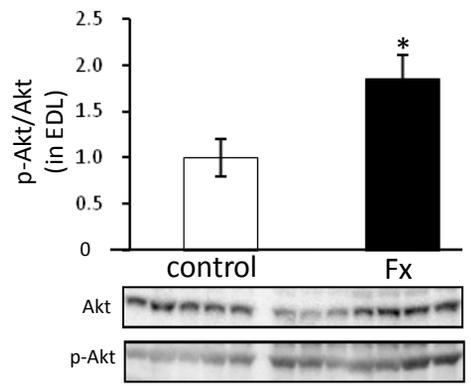
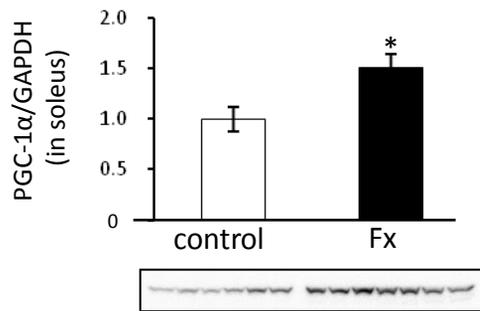


Fig.5

A



B

