AKT2 regulates development and metabolic homeostasis via AMPK-dependent pathway in skeletal muscle

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Abstract

Skeletal muscle is responsible for the majority of glucose disposal in the body. Insulin resistance in the skeletal muscle accounts for 85-90% of the impairment of total glucose disposal in patients with type 2 diabetes. However, the mechanism remains controversial. This study aims to investigate whether AKT2 deficiency causes deficits in skeletal muscle development and metabolism, we analyzed the expression of molecules related to skeletal muscle development, glucose uptake and metabolism in mice of 3-month and 8-months old. We found that AMPK phosphorylation and MEF2A expression were downregulated in AKT2 KO mice, which can be inverted by AMPK activation. We also observed reduced mtDNA abundance and reduced expression of genes involved in mitochondrial biogenesis in the skeletal muscle of AKT2 KO mice, which was prevented by AMPK activation. Moreover, AKT2 KO mice exhibited impaired AMPK-signaling in response to insulin stimulation compared to WT mice. Our study establishes a new and important function of AKT2 in regulating skeletal muscle development and glucose metabolism via AMPK-dependent signaling.

Key words:

AKT2, skeletal muscle, glucose metabolism, AMPK, mitochondria
Abbreviations

ACC, Acetyl-coA carboxylase; AMPK, AMP-activated protein kinase; MEF2, Myocyte enhancer factor 2; GSK3β, glycogen synthase kinase 3 beta; HFD, High fat diet; HOMA-IR, Homeostasis model assessment- insulin resistance; MTFA, Mitochondrial transcription factor A; NRF1, nuclear respiratory factor 1; PGC-1α, Peroxisome proliferator activated receptor gamma coactivator 1 alpha; PPARα, peroxisome proliferators-activated receptor alpha; type 2 diabetes, T2D.
Introduction

AKT, also known as protein kinase B (PKB) [1], is a serine/threonine kinase that plays a key role in insulin-stimulated glucose uptake [2]. There are three isoforms of AKT family, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ. The three AKTs are highly related to each other in both structure and amino acid sequence (~85%) and are activated by phosphatidylinositol-3 kinase [3]. Previous studies comparing the three proteins support the notion that these isoforms have similar biochemical characteristics [4]. However, the three isoforms of AKT exhibit tissue-specific expression and distinct functions. In mice, AKT1 loss of function causes diminished fetal growth but maintain normal glucose regulation[5]. AKT2 deficiency results in insulin resistance and a type 2 diabetes mellitus-like syndrome [6], and global knockout of AKT3 leads to reduced brain size but normal glucose homeostasis [7]. It is well documented that loss of AKT2 is associated with metabolic disorder with glucose imbalance, growth deficiency[8], loss of adipose tissue [9], but the mechanisms behind are not fully understood.

After extensive proliferation, myoblasts undergo differentiation and fusion with each other or existing myofibers to build functional muscle tissue [10]. Many transcription factors are involved in the control of myogenesis. The MyoD family play important roles in the control of muscle differentiation [10]. The myogenic activity of these transcriptional factors is enhanced through their interaction with the myocyte enhancer factor 2 (MEF2) family [10]. In vertebrates, the MEF2 isoforms active in skeletal muscle are A, C and D [11]. Combined deletion of the MEF2A, C and D results in a blockade to generation, suggesting essential roles of MEF2A, C and D in satellite cell differentiation [10]. In addition, MEF2A is involved in mitochondrial development in cardiomyocytes[12]. It is reported that loss of AKT2 results in growth deficiency by exhibiting decreased body weight [13-15], and impaired myotube maturation in the skeletal muscle [16]. However, the impact of AKT2 deficiency in muscle development and the possible implication of MEF2 transcription factors-dependent signaling has not been previously investigated.
Skeletal muscle is responsible for the majority of glucose disposal in the body. Impaired glucose handling capacity leads to a state of systemic insulin resistance [17]. AKT2-null mice exhibited insulin resistance with elevated plasma triglycerides [13]. Interestingly, reduced AKT2 expression and impaired insulin-stimulated AKT2 activation are reported to occur in diabetic insulin resistant human skeletal muscle [18, 19]. Thus, these researches suggest that AKT2 plays an important role in systemic glucose homeostasis.

AMP-activated protein kinase (AMPK) is a major regulator of energy substrate use in several organs[20]. In addition, AMPK activation has been shown to promote the translocation of GLUT4 to the plasma membrane, thus stimulating glucose uptake in skeletal muscle [21, 22]. Furthermore, chronic activation of AMPK reduces markers of skeletal muscle fragility [23] and enhances muscle fiber oxidation capacity by stimulating mitochondrial biogenesis [24]. It is reported that MEF2A is activated in response to AMPK activation in skeletal muscle [25]. However, whether AKT2 functions as regulator of AMPK and the regulatory signaling pathway in skeletal muscle remains to be investigated.

In this study, we hypothesized that AKT2 deficiency impairs the development of skeletal muscle and causes glucose metabolic disturbance. By using AKT2 KO mice and age-matched WT littermates, as well as C2C12 myoblasts, we aims to detect the signaling pathway that AKT2 regulates the development and glucose uptake of skeletal muscle, and its downstream molecules. We observed that the lean mass of AKT2 KO mice decreased significantly, indicating an important role for AKT2 in skeletal muscle development. Then we found that AMPK activity, MEF2A expression and mitochondrial biogenesis were impaired in the skeletal muscle of AKT2 deficient mice, which were inverted by AMPK activation. In addition, AKT2 KO mice exhibited impaired AMPK-dependent signaling in response to insulin stimulation and more deteriorated insulin resistance after high-fat-diet (HFD) feeding. Our results indicate that AKT2 is an important factor for AMPK-associated mitochondrial biogenesis and glucose metabolism in skeletal muscle, and thus establish a new important function
of AKT2 in the development and metabolism of skeletal muscle.
Materials and Methods

Animals

The AKT2 knockout (AKT2 KO) mouse were obtained from the investigation with experimental animals conforms to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All the manipulation of animals were performed in the animal center of China Pharmaceutical University. The age-matched male AKT2 KO mice (Akt2tm1.1Mbb/J, genetic background C57BL/6J, stock No:D000054) were purchased from purchased from Model Animal Resource Information Platform of Nanjing University[26]. Age and body weight-matched wild-type C57BL/6J mice (WT) were used as controls. For all experiments, mice between 3 and 8 months old were used. The mice were maintained on a 12:12 h light/dark cycle, with controlled temperature (22 +/- 2 °C) and humidity (55 +/- 5%) conditions. The mice received ad libitum access to water and standard lab chow, unless otherwise specified.

Injection of insulin

Mice were fasted overnight and anesthetized with phenobarbitol sodium (200mg/kg body weight, diluted to 10% solution with 0.4% sodium chloride) by intraperitoneal injection. For insulin treatment, 150 mU/g body wt of insulin or the same volume of PBS were intraperitoneally injected[27]. 15 min after insulin injection, mice were killed by cervical dislocation, and indicated hindlimb muscles were removed rapidly and immediately frozen in liquid nitrogen and stored at -80°C.

Glucose tolerance test and insulin tolerance test

Glucose tolerance and insulin tolerance were investigated in AKT2 KO and WT mice at different ages as previously described[28]. HOMA of insulin resistance (HOMA-IR) is calculated as follows: Fasting insulin (microU/L) x Fasting glucose (nmol/L)/22.5.
Peritoneal injection of AICAR

6-month-old AKT2 KO mice accepted peritoneal injection of AMPK activator AICAR (Toronto Research Chemicals Inc., North York, ON, Canada, 0.5 mg/g of body weight) or saline three times a week for continuous 4 weeks[29]. Then the skeletal muscles were dissected for further investigation.

Immunohistological staining

Skeletal muscle was routinely processed into paraffin sections. Sections of all cases were double-stained with haematoxylin and eosin stain (H&E) and periodic acid-Schiff stain (PAS). These sections were scanned by the digital slide scanner NanoZoomer 2.0RS (Hamamatsu), and the area of interstitial fibrosis was calculated by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, Maryland, USA).

Electron microscope

The embedded muscle tissue was sectioned and stained with uranium lead double staining (2% uranyl acetate saturated alcohol solution, lead citrate, each stained for 15 min), and the sections were dried overnight at room temperature. Images were observed under a transmission electron microscope (Hitachi).

Glycogen content in muscle tissue

Glycogen in skeletal muscle was measured by a glycogen content analysis kit (QIYI Biological Technology, Shanghai, China, QYS-234078). Glycogen concentration was determined by referring a glycogen standard solution in the kit. We used 8 WT mice of both 3-months and 8-months age, 6 AKT2 KO mice of 3-month-old, and 8 AKT2 KO mice of 8-month-old.

C2C12 myoblasts culture, differentiation and Transfection

C2C12 cells (gift from Dr Caiping Chen) were cultured in growth medium (GM) consisting of Dulbecco’s modified Eagle’s medium (DMEM, sigma, USA) with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin under a
humidified atmosphere of 5% CO₂ at 37°C. To induce differentiation, the medium was replaced by differentiation medium (DM) consisting of DMEM containing 2% horse serum (Gibco, USA). After 3 days, they were transfected with mouse AKT2-targeting siRNA (siAKT2) or siRNA for negative control (NC). On the fourth day after transfection, cells were collected for protein extraction.

Mitochondria extraction and ATP quantification

Cells were washed four times with PBS and then digested with trypsin and collected and transferred to mitochondrial extraction reagent (2mM HEPES, 0.22M Mannitol, 70mM D(+)-Sucrose, 0.1mM EDTA, 1%BSA). Cells were homogenized using tissue homogenizer and then centrifuged at 800 g for 10 min, the precipitates are cytoplasmic protein. The supernatants were transferred to new centrifuge tubes and then centrifuged at 12,000 g for 15 min, the precipitates are mitochondrial. ATP content of C2C12 cells was detected via ATP Assay Kit (Beyotime, S0026). Briefly, mitochondria was extracted from C2C12 cells treated with NC or siAKT2 for 24h, then total ATP content of the mitochondria with either treatment was quantified by ATP Assay Kit, and protein concentration was quantified by BCA Kit (Vazyme, E112). Final ATP content was calculated by: mt ATP content/ protein concentration. Triplicate for both treatment was prepared each time.

Western Blot

Protein samples from the cultured cells and mouse tissue samples were performed as previously described [28]. The blots were incubated with antibodies (Table 1) overnight at 4°C The protein bands were detected with enhanced chemiluminescence kit (Thermo Scientific, USA). The images were quantitatively analyzed by using the Image J.

Total RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was extraction and real-time PCR procedure were performed as previously described [28]. Primers used for amplifying sequence were shown in Table
2. All samples were amplified in triplicate, and the results were normalized to the expression of 18s rRNA.

**Quantification of mitochondrial DNA (mtDNA) copy number**

Total DNA extraction from isolated skeletal muscle tissue was carried out using the genomic DNA extraction Kit (Beyotime, D0063) according to the manufacturer’s recommendations. Mitochondrial gene *nd-5* was analyzed to assess mtDNA copy number (Table 2). All samples were amplified in triplicate. Results were expressed as ratio of *nd-5* to *β-actin*.

**Immunofluorescence**

C2C12 cells replaced with differentiation medium (DM) for 3 days, and then were transfected with mouse siAKT2 or NC. On the fourth day after transfection, cells were used for immunofluorescence detection as previously described [30].

**Statistics**

Data are shown as mean ± SEM (standard error of mean). All statistical analyses were performed using Prism (Graphpad, San Diego, CA, USA) software. Differences between two groups were compared by unpaired 2-tailed Student’s t test. Multiple groups were tested via two-way ANOVA followed by Bonferroni post-test. A *p*-value < 0.05 was considered statistically significant.
Results

1. Disturbance of glucose metabolism due to AKT2 deficiency

AKT2 KO mice were born healthy, and were able to grow to adulthood without abnormalities of lifespan, as previously described by other groups [31]. However, AKT2 KO mice showed a reduction in body weight beginning around postnatal day 40 (Fig. 1A), and from P90, the AKT2 KO mice were visibly distinguishable from their WT littermates (Fig. 1B), although there were no differences of tibial length between WT and AKT2 KO mice (suppl. Figure 1), nor in the daily food intake (Fig. 1C and D).

We investigated whether glucose metabolism was also affected due to AKT2 deficiency by evaluating global glucose metabolic status. AKT2 KO mice exhibited impaired glucose tolerance at 3 and 8 months of age (Fig. 1E and F; suppl. Figure 2). Interestingly, glucose intolerance was less pronounced in female AKT2 KO mice compared to age-matched male mice (suppl. Figure 2). Insulin sensitivity was also impaired in AKT2 KO at 3 and 8 months of age (Fig. 1G and H). Plasma insulin levels and HOMA-IR were elevated in AKT2 KO mice at 3 and 8 months of age (Fig. 1I and J). These results suggest that AKT2 deficiency in skeletal muscle impairs both the response of glucose tolerance and insulin sensitivity.

2. Tissue and age-related changes in AKT2 expression

Next we explored AKT1 and AKT2 expression in different tissues. AKT1 is more abundant in the heart, liver, kidney and WAT (Fig. 2A). AKT2 mRNA and protein were enriched in tissues highly associated with glucose metabolism including pancreas, liver, brain and soleus muscle (Fig. 2A and B). In addition, we observed a positive correlation between p-AKT2 expression and age in skeletal muscle (Fig. 2C). To determine if there is compensatory effect of AKT1 due to AKT2 absence, we assessed the abundance of AKT1 and AKT2 in soleus muscle of both WT and AKT2 KO mice. The result showed that AKT1 expression was similar in skeletal muscle of both...
genotypes (Fig. 2D), suggesting that there was no compensatory expression of AKT1 in the skeletal muscle deficient for AKT2.

3. Loss of skeletal muscle mass of mice deficient for AKT2

The analysis of tissue weight of WT and AKT2 KO mice at 3 and 8 months of age confirmed the reduction of WAT mass, as previously described [2,12,31], yet demonstrated also a reduction of lean mass of AKT2 KO mice, compared to WT (Fig. 3A; suppl. Figure 3A and B), without obvious morphological changes of pancreas at 8 months of age (suppl. Figure 4). To assess the role of AKT2 in skeletal muscle growth, we weighed different muscle groups in 3-month-old and 8-month-old mice. The hind limb soleus, quadriceps, gluteus and gastrocnemius, all weighed significantly less in AKT2 KO mice compared to age-matched WT mice (Fig. 3B and C). Further histological analysis revealed normal nuclear position and similar H&E staining of either longitudinal or transverse sections (Fig. 3D). However, the cross-sectional area of soleus muscle from AKT2 KO mice was significantly reduced compared to WT (Fig. 3E). Electron microscopy analysis also revealed smaller sarcomeres in the muscle of AKT2 KO mice (Fig. 3F), suggesting impaired myotube development. However, there were no abnormalities in Z-discs and myofibrils (Fig. 3F). These results suggested that, in AKT2-deficient mice, the reduction of body weight, which coincides with a decrease in the lean mass at the age of 3 months and 8 months, was partly caused by loss of muscle weight, suggesting an important role for AKT2 in regulating skeletal muscle development.

4. Disorder of Skeletal muscle development due to AKT2 loss-of-function

The phosphorylation of AMPKα (p-AMPKα) and the expression of MEF2A and PPARα were significantly decreased in AKT2 KO muscle (Fig. 4A), which is in agreement in transcriptional level (Fig. 4B). To investigate the existence of a direct
link between AKT2 deficiency and AMPKα hypophosphorylation, C2C12 myoblasts were transfected with either lentivirus carrying empty vector (scr) or lentivirus carrying small interference RNA knockdown of AKT2 (siAKT2). The results showed that p-AMPKα decreased in cells transfected with siAKT2 compared to controls at day 4 and 6 post differentiation (Fig. 4C). In agreement with the in vivo observations, MEF2A and PPARα were less abundant in siAKT2 treated cells (Fig. 4C). Moreover, morphological analysis of C2C12 cells also suggested that AKT2 knockdown resulted in retardation of cell differentiation (Fig. 4D). These results indicate that the AMPK-MEF2A axis is inhibited in skeletal muscle due to AKT2 loss-of-function.

5. Alterations of mitochondrial biogenesis in skeletal muscle of AKT2 KO mice

Further investigation by electron microscopy revealed no gross morphological changes of mitochondria (Fig. 5A). We also quantified mitochondrial DNA (mtDNA)/genomic DNA, and the result showed diminished mtDNA copies in the 8-month-old AKT2 KO mouse muscle (Fig. 5B and C). Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), mitochondrial transcription factor A (MTFA) and nuclear respiratory factor 1 (NRF1) are recognized as regulators of mitochondrial function and biogenesis [32-36]. Our results showed that AKT2 deficiency results in decreased mRNA expression of pgc-1α, mtfa and nrf-1 in the skeletal muscle deficient for AKT2 at the age of 8-month-old (Fig. 5D). To evaluate the function of mitochondria, we extracted mitochondria from C2C12 cells transfected with siAKT2 or control siRNA (NC), and the result showed that there was obvious decrease of ATP content in cells treated with siAKT2 (Fig. 5E). These results suggesting that AKT2 deficiency induces impaired mitochondrial biogenesis as well as mitochondrial function.

6. Disturbance of glucose metabolism in skeletal muscle of AKT2 KO mice
Next we try to explore whether there were defects on metabolic disorder in the skeletal muscle. Our result showed that GLUT4 protein abundance was significantly decreased in soleus muscle of AKT2 KO mice at the age of 3 and 8 months (Fig. 6A, C, D and F). We next evaluated the activity of glycogen synthase kinase 3 beta (GSK3β), a serine/threonine kinase essential for the regulation of glycogen synthesis [37]. Our result showed that, despite markedly reduced GLUT4 expression, GSK3β phosphorylation (p-GSK3β) did not show obvious changes in 3-month-old AKT2 KO skeletal muscle compared to WT (Fig. 6A, B, D and E). Interestingly, at the age of 8 months, p-GSK3β in skeletal muscle of AKT2 KO mice increased as much as 20% compared to WT (Fig. 6B and E). To verify the regulation of GLUT4 by AKT2, GLUT4 was checked in C2C12 cells transfected with NC or siAKT2. The result showed that GLUT4 expression decreased at both protein and mRNA level after AKT2 knockdown (Fig. 6 G-I), suggesting that AKT2 positively regulates GLUT4 expression in skeletal muscle.

To check the amount of glycogen stored in skeletal muscle of AKT2 KO mice, glycogen quantification and staining were performed in both genotypes of 3- and 8-month old mice. The results showed that the glycogen quantity was identical in soleus muscle of WT and AKT2 KO younger mice, but there was an increase of glycogen content in the skeletal muscle of AKT2-deficient 8-month-old mice (Fig. 6J-L). These data suggest that, on one hand AKT2 deficiency accounts for decreased glucose uptake in skeletal muscle. On the other hand, increased expression of p-GSK3β and glycogen content in adult AKT2 KO mice suggest that disturbance of glucose metabolism is a secondary effect due to AKT2 loss-of-function.

To further investigate the role of AKT2 in glucose metabolism in skeletal muscle, mice accepted 1u insulin injection for 15 min[27]. Upon insulin stimulation, AKT2 phosphorylation increased obviously in soleus muscle (Fig. 6M), while there was no obvious changes of p-AKT1 in soleus muscle of both genotypes treated with insulin or vehicle (Fig. 6M). AMPKα exhibited decreased activity after insulin stimulation in WT mice, while there was only slight decrease of AMPKα activity in the soleus muscle.
of AKT2 KO mice treated with insulin (Fig. 6M), and the alteration of ACC activity, which is a direct target of AMPK[38], is in agreement with that of AMPKα (Fig. 6M). Our results suggest that AKT2 plays a key role in insulin-stimulated AMPK activation in skeletal muscle, which cannot be compensated by AKT1.

7. Improvement of global and skeletal muscle metabolism by AMPK activator in AKT2-null mice

To check the role of AMPKα in AKT2 deficiency-induced skeletal muscle development and metabolism, an AMPK activator, AICAR, was continuously injected in 6-month-old AKT2 KO or WT mice for 4 weeks. Our results showed that AKT2 KO mice treated with AICAR did not show obvious changes of glucose tolerance (Fig. 7A), but had significantly improved insulin tolerance compared to untreated mice (Fig. 7B), and the weight of quacedrips, gastrocnemius and soleus muscle increased obviously in the group treated with AICAR compared to mice treated with saline (Fig. 7C). Furthermore, p-AMPKα as well as MEF2A, PPARα and NRF1 were significantly induced by AICAR treatment compared to saline-treated controls (Fig. 7D). Moreover, phosphorylation of GSK3β, which was highly stimulated in AKT2-deficient samples, reduced to the basal level after AICAR infusion (Fig. 7D). Therefore, our data suggest that the decrease of MEF2A, as well as molecules involved in glucose metabolism and mitochondrial biogenesis in skeletal muscle deficient for AKT2 is due to AMPKα inactivation, which may in turn contribute to aggravate insulin resistance and to impair glucose tolerance in serum (Fig. 8).
Discussion

In this study, we provide evidence that AKT2 positively regulates AMPK activation, which play an important role in the control of growth, mitochondrial biogenesis and function of skeletal muscle. We found that p-AMPKα, MEF2A, PPARα and molecules associated with mitochondrial biogenesis were downregulated in skeletal muscle of AKT2 KO mice, which can be reversed by AMPK activator. We also observed that AKT2 regulates GLUT4 expression and insulin-stimulated AMPK-activation in skeletal muscle. Therefore, our study establishes a new and important role for AKT2 in the developmental and metabolic regulation of glucose in skeletal muscle.

AKT1 and AKT2 KO mice exhibit remarkably different phenotypes [5, 39, 40], suggesting that the two isoforms regulate a subset of unique downstream genes and biological functions respectively. Our results showed that AKT2 is most abundant in organs that are active for glucose metabolism. We further found elevated p-AKT2 protein expression in soleus muscle at different ages. This result was also verified in vitro by checking different days of differentiation in C2C12 cells, indicating important role of AKT2 during skeletal muscle development. In AKT2 KO mice, there was no difference of AKT1 protein abundance compared to WT, suggesting the unique function of AKT2 in skeletal muscle, which is not compensated by other AKT isoforms.

Most research reported that the loss of body weight in AKT2 KO mice is largely due to decreased white adipose tissue (WAT), few focused on the alteration of skeletal muscle. We first observed a significant decrease in AKT2 KO mice. Further assessment showed decreased weight of hindlimb muscles, while the tibial length was equivalent in both genotypes, suggesting the decreased body mass is partly due to the loss of skeletal muscle despite WAT. Our further investigation of soleus muscle showed diminished cross-sectional area and smaller sarcomeres of AKT2 KO mice. Thus, our data suggests impaired skeletal muscle development due to AKT2...
deficiency.

It is reported that MEF2A can be activated by AMPK in skeletal muscle [41, 42]. As an important energy sensor, AMPK plays a key role in maintaining metabolic homeostasis [38, 43]. We first observed that in the skeletal muscle of AKT2 KO mice, AMPK activity decreased significantly than WT mice at the age of 3- or 8-months old, so in the following research work, we tried to prove AKT2-AMPK signaling-related development and metabolism. We further showed that p-AMPK protein abundance decreased significantly when AKT2 was knocked down in C2C12 cells, while AKT1 expression remained unchanged during the treatment. Therefore, our results suggest that AKT2 positively regulates AMPK activity at normal conditions in skeletal muscle. To further investigate the mechanisms underlying the role of AKT2 in skeletal muscle development, AKT2 KO mice were treated with AICAR injection for 4 weeks. AKT2 KO mice developed greatly alleviated insulin resistance after AICAR treatment, MEF2A was also significantly induced in soleus muscle of AKT2 KO mice. Thus, these results further confirmed that AKT2 regulates skeletal muscle development via AMPK activation.

Lack of AKT2 in skeletal muscle decreased both the insulin sensitivity and responsiveness of glucose transport [13]. It is proved that the activation of adipose precursors is dependent on PI3K/AKT2 signaling [44]; The process of lipogenesis requires AKT2, and AKT2 knockout enhances WAT browning [45]. So AKT2 deficiency is highly possible to cause adipocyte dysfunction that induces inflammation and contributes to global insulin resistance. Moreover, recent research also proved that AKT2 deficiency impairs the function of immune cells [46, 47]. However, recently it has been reported that AKT2 deficiency in skeletal muscle is not sufficient to affect development and glucose metabolism of skeletal muscle [48]. One possible explanation for the discrepancy could be that, during the research, the authors used young mice at ages between 8-12 weeks. As we show in the present work, AKT2 KO mice did not show significant decrease of body weight and glucose tolerance until 3-month of age. Therefore, we speculate that the lack of direct effects of AKT
deletion in skeletal muscle reported in by Jaiswal N et al., may be related to the age of the mice. Our study also proved that there was no obvious changes of the islets in AKT2 KO mice, while AKT2 deficiency impaired insulin-stimulated AMPK activity compared to WT. The protein expression of GLUT4 decreased in adipose tissue of obese and insulin resistant subjects [49]. However, the regulation of GLUT4 expression in skeletal muscle has not been well documented. Our data showed decreased GLUT4 expression in soleus muscle deficient for AKT2. We also used C2C12 myotubes, which express GLUT4 [50-52], to study the regulation of GLUT4 by AKT2. The result is in accordance with that in vivo., indicating decreased glucose uptake into skeletal muscle cell. Interestingly, we also observed that female exhibited alleviated glucose resistance compared to age-matched male with AKT2 deficiency. Previous research has similar results, that in males, insulin resistance progresses to a severe form of diabetes accompanied by pancreatic β cell failure. In contrast, female AKT2-deficient mice remain mildly hyperglycemic and hyperinsulinemic until at least one year of age [53]. It could be the reason of sexual hormone. The prevalence of diabetes was a slightly higher among men (9.1%) than women (8.4%) [54]. Glucose and lipid metabolism are directly modulated by estrogen and testosterone, and lack of estrogen or a relative increase in testosterone induces insulin resistance [55]. So the reason for female AKT2 KO mice exhibited alleviated glucose tolerance compared to age-matched male mice, could because that estrogen mitigates the development of insulin resistance. Therefore, our results suggest that AKT2 plays a key role in glucose homeostasis in skeletal muscle.

Our data confirmed that AKT2 deficiency impairs mitochondrial biogenesis, which in turn causes glycogen accumulation due to decreased glucose oxidation. Notably, there is research proved that AKT2 deficiency did not cause alteration of p-AMPK or GLUT4 expression in muscle [56]. However, the background of mice strain, also the gender and age of mice may cause the differences, as we showed that female mice exhibited delayed and alleviated glucose intolerance. Finally, after applying AICAR to AKT2 KO mice, these mice exhibited ameliorated glucose tolerance.
and insulin resistance, and expression of proteins related to muscle development and mitochondrial biogenesis increased to normal level, suggesting AMPK is a key mediator during AKT2-associated regulation of skeletal muscle metabolism. These data strengthened our conclusion that AKT2 and its activity plays a key role in glucose metabolism of skeletal muscle. However, The present studies do not allow one to distinguish between primary and secondary effects, so it is possible that the changes in insulin sensitivity in muscle are a result of the compensatory hyperinsulinemia. However, equivalent insensitivity of skeletal muscle to insulin is not observed in another model of hepatic insulin resistance[57], suggesting that the muscle phenotype in AKT2 KO mice is a cell-autonomous effect of the kinase deficiency in the skeletal muscle. Also, to distinguish whether the change of downstream protein abundance is caused by AKT2 deficiency or it is a compensatory effect, we used C2C12 cell to perform AKT2 knockdown, and our results suggest a direct regulation of these factors by AKT2. Future investigation of skeletal muscle-specific deletion of AKT2 will be more potent to support its role in the glucose metabolism in muscle cells.

In conclusion, skeletal muscle accounts for a great portion of the impairment of total glucose disposal in patients with type 2 diabetes, while the mechanism remains unclear. In the present study, we establishes an important function of AKT2 in regulating skeletal muscle development and glucose metabolism via AMPK-dependent signaling. Our work identified an important role of AKT2/AMPK signaling in skeletal muscle with insulin resistance. Therefore, our study gives new sights into the mechanism of AKT2 in regulating skeletal muscle development and insulin-stimulated glucose metabolism, suggesting that AKT2 may be a key target for the therapy of T2D-related metabolic disorder.
Clinical Perspectives:

- Our work identified an important role of AKT2/AMPK signaling in skeletal muscle with insulin resistance, suggesting that AKT2 may be a key target for the therapy of T2D-related metabolic disorder.

- In our study, we establishes a new and important function of AKT2 in regulating the development of skeletal muscle via AMPK-dependent signaling.

- We established that in normal state, AKT2 promotes mitochondrial biogenesis that regulates glucose metabolism in skeletal muscle.
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**Figure legends**

**Figure 1. Impaired glucose metabolism of AKT2 KO mice.** (A) Body weight curve of WT and AKT2 KO mice at different ages that range from 1 to 8 months old (WT, n=10; AKT2 KO, n=15). (B) Representative images of AKT2 KO mice with WT littermates at the age of 8-month-old. (C and D) Food intake of mice at the age of 3- and 8-month-old (n=10 for both genotypes). (E and F) Glucose tolerance with AUC of both genotypes at the age of 3-month-old (WT, n=6; AKT2 KO, n=11) and 8-month-old (n=10 for both genotypes). (G and H) Insulin tolerance with AUC of both genotypes at the age of 3-month-old (n=5 for both genotypes) and 8-month-old (n=4 for both genotypes). (I and J) Serum insulin and HOMA of insulin resistance of both genotypes at the age of 3-month-old (WT, n=8; AKT2 KO, n=11) and 8-month-old (WT, n=7; AKT2 KO, n=9). Statistical significance was determined using unpaired 2-tailed Student’s t test.

**Figure 2. AKT2 expression in different tissues and ages.** (A and B) Western blot and real-time PCR analysis of AKT1 and AKT2 protein abundance in multitissue panel using a C57BL/6 mouse (Representative western blot were from n=4). (C) p-AKT2 and AKT2 protein expression in skeletal muscle from embryo throughout development and into adulthood, mice were fast for 2h before sacrifice (n=3). (D) AKT1 and AKT2 expression in skeletal muscle of WT and AKT2 KO mice at the age of 3-month-old (n=4 for both genotypes). Statistical significance was determined using unpaired 2-tailed Student’s t test.

**Figure 3. Sarcopenia of AKT2 KO mice.** (A) Lean mass of mice at the age of 3- (n=15 for both genotypes) and 8-month-old (n=12 for both genotypes). (B and C) Muscle weight of different parts from mice hindlimb at the age of 3- (WT, n=10; AKT2 KO, n=12) and 8-month-old (n=10 for both genotypes). (D) H&E staining of Longitudinal (upper panel) and transverse sections of soleus muscle (middle panel), and WGA staining of soleus muscle (lower panel) from both genotypes (WT, n=4; AKT2 KO, n=8). scale bar: 100 μm for H&E; 50 μm for WGA. (E) Muscle area calculation based on WGA staining of soleus muscle (WT, n=4; AKT2 KO, n=6). (F) Representative transmission electron microscopic images showing sarcomeric morphology in WT and AKT2 KO soleus muscle (n=3 per genotype). scale bar: 4 μm. Statistical significance was determined using unpaired 2-tailed Student’s t test.

**Figure 4. Suppression of AMPK-MEF2A signaling due to AKT2 deficiency.** (A) Representative immunoblots of AKT1, AKT2, p-AMPKa, AMPKa, MEF2A in soleus muscle of mice from both genotypes at the age of 3- and 8-month-old (n=8 for both genotypes). (B) Relative mRNA expression of mef2a and ppara in skeletal muscle (n=5 for both genotypes). (C) Representative immunoblots of AKT1, AKT2 and MEF2A in C2C12 cells at different differentiating days. (D) Representative immunofluorescence staining of C2C12 cells with α-actinin and DAPI at day 4 and day 6 after transfection with either NC or siAKT2 (n=4) (Blue: nucleus; Red: α-actinin). scale bar: 20 μm. Statistical significance was determined using unpaired 2-tailed Student’s t test.
Figure 5. Impaired mitochondrial biogenesis of skeletal muscle with AKT2 deficiency. (A) Representative transmission electron microscopic images showing mitochondria morphology in WT and AKT2 KO soleus muscle (n=3 per genotype). (B and C) Quantification of mitochondrial copies in the skeletal muscle of WT and AKT2 KO mice at the age of 3- and 8-month-old (WT, n=10; AKT2 KO, n=6). The abundance of mitochondrial DNA was measured by real-time PCR to determine the copy number shown as the ratio of nd-5 vs β-actin. (D) Expression of mRNA for pgc-1α, mtfa and nrf1 in the skeletal muscle tissue of mice at the age of 3- and 8-month-old (n=5 for both genotypes). (E) ATP content in C2C12 cells transfected with NC or siAKT2 (Quantification was from 3 experiments). Statistical significance was determined using unpaired 2-tailed Student’s t test.

Figure 6. Glucose metabolic disorder in skeletal muscle with AKT2 deficiency. (A-F) Representative immunoblots of GLUT4, p-GSK3β and GSK3β in soleus muscle from WT and AKT2 KO mice, calculation of p-GSK3β/GSK3β ratio, and glut4 mRNA level in soleus muscle at the age of 3-month-old and 8-month-old (n=6 for both genotypes). (G) Representative immunoblots of AKT2, GLUT4 in C2C12 cells at different days after siAKT2 transfection (Representative western blot were from n=3). (H and I) Real-time PCR of akt2 and glut4 in C2C12 cells at 6 days after siAKT2 transfection (n=3). (J and K) Quantification of glycogen content in soleus muscle of mice from both genotypes at the age of 3- and 8-month-old (n=8 for both genotypes). Statistical significance was determined using unpaired 2-tailed Student’s t test. (L) Glycogen distribution shown by PAS staining in soleus muscle of mice from WT and AKT2 KO mice at the age of 3- and 8-month-old (n=8 for both genotypes). Scale bar: 100 μm. (M) Representative immunoblots of p-AKT2, AKT2, p-AKT1, AKT1, p-AMPKα, AMPKα, p-ACC and ACC in soleus muscle from WT and AKT2 KO mice with and without 1u insulin injection for 15 min (n=6 per genotype). Statistical significance was determined using two-way ANOVA followed by Bonferroni post-test.

Figure 7. Reverse of impaired skeletal muscle develop and glucose metabolism by AICAR in AKT2 KO mice. (A) Glucose tolerance were evaluated before and after 4 weeks’ AICAR application to AKT2 KO mice and calculation of AUC (n=4 for each group). (B) Insulin tolerance were evaluated before and after 4 weeks’ AICAR application to AKT2 KO mice and calculation of AUC (n=4 for each group). (C) Weight of quacedrips, gastrocnemius and soleus muscle of AKT2 KO mice treated with AICAR or saline for 4 weeks (n=4 for each group). (D) Representative immunoblots of soleus muscle from different groups (n=4 for both treatment). Statistical significance was determined using unpaired 2-tailed Student’s t test.

Figure 8. Schematic of AKT2 at a skeletal muscle in the network of gene regulation that underpins the program of muscle development and metabolism. During muscle development, AKT2 promotes GLUT4 expression, which transports glucose from blood into muscle cells. AKT2 also increases AMPK phosphorylation, which promotes the expression of MEF2A that positively regulate skeletal muscle development. However, when AKT2 is phosphorylated by stimulations
such as high-fat diet (HFD), it has the opposite function. On the other hand, activated AMPK promotes expression of genes related to mitochondrial biogenesis, including PPARα, PGC-1α, NRF-1 and EndoG. p-AMPK also inhibits phosphorylation of GSK3β upon AKT2 regulation, therefore increases glycogen synthesis in skeletal muscle cells.
| Antigen     | Provider             | Dilution | Cat. Number | Application |
|-------------|----------------------|----------|-------------|-------------|
| AKT2        | Cell Signaling Technology | 1:1000   | 3063        | WB          |
| p-AKT2\(^{\text{Ser}474}\) | Cell Signaling Technology | 1:1000   | 8599        | WB          |
| AKT1        | Cell Signaling Technology | 1:1000   | 2938        | WB          |
| p-AKT1\(^{\text{Ser}473}\) | Cell Signaling Technology | 1:1000   | 9018        | WB          |
| AKT         | Cell Signaling Technology | 1:1000   | 4691        | WB          |
| p-AKT\(^{\text{Thr}473}\) | Cell Signaling Technology | 1:1000   | 4060        | WB          |
| GSK-3β      | Proteintech          | 1:1000   | 22104       | WB          |
| AMPKα       | Cell Signaling Technology | 1:1000   | 2603        | WB          |
| p-AMPKα\(^{\text{Thr}172}\) | Cell Signaling Technology | 1:1000   | 2535        | WB          |
| ACC         | Cell Signaling Technology | 1:1000   | 3676        | WB          |
| p-ACC\(^{\text{Ser}79}\) | Cell Signaling Technology | 1:1000   | 11818       | WB          |
| MEF2A       | Cell Signaling Technology | 1:1000   | 9736        | WB          |
| PPARα       | WanLei               | 1:2000   | WL00978     | WB          |
| GLUT4       | Abcam                | 1:1000   | Ab188317    | WB (tissue) |
| GLUT4       | Proteintech          | 1:500    | 66846       | WB (C2C12 cell) |
| NRF1        | Proteintech          | 1:1000   | 12482       | WB          |
| GAPDH       | Proteintech          | 1:1000   | 60004       | WB          |
| α-actinin   | Sigma                | 1:500    | A7811       | IF          |

WB: western blot; IF: immunofluorescence
| Gene    | Oligonucleotides Sequences (5’→ 3’) |
|---------|-----------------------------------|
| pgc-1α  | aaggaagactaaaacggccca             |
|         | gttttctgggtgtgcaagga              |
| nrf-1   | tctctgagacgctgtctttca             |
|         | gcagttacccatcgctgc               |
| ppara   | accttgtgtatggccgagaa             |
|         | aagaggacagcatcgtagaa             |
| mtfa    | gtccataggcaccgtattgc             |
|         | cccatgtgaggaaaaacactt             |
| mef2a   | atccgtttactggtggttgtg           |
|         | tgtcaggacagcagatgagg             |
| 18s rRNA| gtaaccctgtgaaccccatt            |
|         | ccattcaatcgtgtagtgagc             |
| nd-5    | tggatgttgtacggacgaa             |
|         | tgcgttatagaggattgtttgt             |
| β-actin | tgttccctcccacaggtgt             |
|         | tccccagtggtaacatgcca             |
Figure 1

A. Body Weight (g) vs Age (months)

B. WT vs AKT2 KO

C. Food Intake (g/day) vs Age of 3 months

D. Food Intake (g/day) vs Age of 8 months

E. AUC of IP-GTT 3m

F. Serum glucose (mmol/L) vs Time (min)

G. AUC of IP-ITT 3m

H. Serum glucose (mmol/L) vs Time (min)

I. AUC of IP-ITT 8m

J. HOMA-IR vs Age of 3 months

K. HOMA-IR vs Age of 8 months
Figure 2

A

Heart Liver Kidney Pancreas Brain Soleus muscle WAT

AKT1 AKT2 Naphtol Blue

60kDa

B

Relative expression of AKT2

Heart Liver Brain Pancreas Kidney Soleus muscle WAT

P0 P2 P5 P15 P60 P90

p-AKT2Ser474 AKT2 Naphtol Blue

60kDa

D

WT AKT2 KO

AKT2 AKT1 GAPDH

60kDa 60kDa 36kDa
Figure 3

A

Lean mass (g)

WT
AKT2 KO
3 months 8 months

WT
AKT2 KO
p<0.0001
p<0.0001

B

Muscle Weight (g)

Gluteus
Quadriceps
Gastrocnemius
Soleus
3 months of age

WT AKT2 KO
p=0.0256
p=0.0001
p=0.0011
p=0.0017

C

Muscle Weight (g)

Gluteus
Quadriceps
Gastrocnemius
Soleus
8 months of age

WT AKT2 KO
p<0.0001
p=0.0011
p=0.0759

D

WT
AKT2 KO

HE

WT
AKT2 KO

WGA

E

Cell Area (A.U.)

WT AKT2 KO
p<0.0009

F

WT
AKT2 KO

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Figure 4

A

|            | 3 months         | 8 months         |
|------------|------------------|------------------|
|            | WT               | AKT2 KO          |
| AKT1       |                  |                  |
| AKT2       |                  |                  |
| p-AMPKα<sup>Thr172</sup> |       |                  |
| AMPKα      |                  |                  |
| MEF2A      |                  |                  |
| PPARα      |                  |                  |
| GAPDH      |                  |                  |

B

![Bar graphs showing fold change of mRNA](data:image/png;base64,...)

C

| Time (d) | scr | siAKT2 |
|----------|-----|--------|
| 0        |     |        |
| 4        |     |        |
| 6        |     |        |

D

![Immunofluorescence images](data:image/png;base64,...)
**Figure 5**

A.

B.

C.

D.

E.

Fold change of mRNA

ATP content

WT AKT2 KO

p=0.0110

p=0.0003

p=0.6225

p=0.3888

p=0.9341

p=0.0003

p=0.0084

p=0.0095

p=0.8634

p=0.0003

p=0.0084

p=0.0095

p=0.6225

p=0.3888

p=0.9341

p=0.0084

p=0.0095

ATP content (nmol/mg protein)

NC siAKT2

**"**

3 months

8 months

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

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nrf-1

pgc-1α,

mtfa,

nrf-1

pgc-1α,

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pgc-1α,

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pgc-1α,

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pgc-1α,

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nrf-1

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**Figure 6**

**A** 3 months

|       | WT | AKT2 KO |
|-------|----|---------|
| GLUT4 | 50kDa | 46kDa |
| p-GSK-3βSer9 | 46kDa | 36kDa |
| GSK-3β | 46kDa | 36kDa |
| GAPDH | 36kDa | 36kDa |

**B**

![Graph showing p-GSK-3β/GSK-3β levels](image1)

**C**

![Graph showing relative mRNA level of GSK-3β](image2)

**D** 8 months

|       | WT | AKT2 KO |
|-------|----|---------|
| GLUT4 | 50kDa | 46kDa |
| p-GSK-3βSer9 | 46kDa | 36kDa |
| GSK-3β | 46kDa | 36kDa |
| GAPDH | 36kDa | 36kDa |

**E**

![Graph showing p-GSK-3β/GSK-3β levels](image3)

**F**

![Graph showing relative mRNA level of GSK-3β](image4)

**G**

|       | NC | siAKT2 |
|-------|----|--------|
| AKT2 | 60kDa | 60kDa |
| p-AKTSer473 | 60kDa | 60kDa |
| AKT | 60kDa | 60kDa |
| GLUT4 | 50kDa | 36kDa |
| GAPDH | 36kDa | 36kDa |

**H**

![Graph showing relative mRNA level of AKT](image5)

**I**

![Graph showing relative mRNA level of GLUT4](image6)

**J**

**K**

![Graph showing glycogen levels](image7)

**L**

![Images of WT and AKT2 KO](image8)

**M**

|       | WT | AKT2 KO |
|-------|----|---------|
| p-AKT2Ser474 | 60kDa | 60kDa |
| AKT2 | 60kDa | 60kDa |
| p-AKT1Ser473 | < 60kDa | < 60kDa |
| AKT1 | < 60kDa | < 60kDa |
| p-AMPKαThr172 | 62kDa | 62kDa |
| AMPKα | 62kDa | 62kDa |
| p-ACCSer79 | 280kDa | 280kDa |
| ACC | 280kDa | 280kDa |
| GAPDH | 36kDa | 36kDa |

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Figure 7

A

**IP-GTT**

![Graph showing glucose level over time for different conditions](image)

B

**ITT**

![Graph showing glucose level over time for different conditions](image)

C

![Graph showing muscle weight for different groups](image)

D

|                | WT          | AKT2 KO     | AKT2 KO-AICAR |
|----------------|-------------|-------------|---------------|
| **AICAR**      | -           | -           | +             |
| **AKT2**       | -           | -           | +             |
| **p-AMPKα<sub>Thr172</sub>** | -           | -           | +             |
| **AMPKα**      | -           | -           | +             |
| **p-GSK-3β<sub>Ser9</sub>** | -           | -           | +             |
| **GSK-3β**     | -           | -           | +             |
| **MEF2A**      | -           | -           | +             |
| **PPARα**      | -           | -           | +             |
| **NRF-1**      | -           | -           | +             |
| **GAPDH**      | -           | -           | +             |

**AUC of OGTT**: p=0.1498, p=0.6690

**AUC of ITT 8m**: p<0.0001, p<0.0001

**Muscle Weight (g)**:

- Quadriceps: p=0.0286, p=0.0043, p<0.001
- Gastrocnemius
- Soleus
