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Photoactivation of GLUT4 translocation promotes glucose uptake via PI3-K/Akt2 signaling in 3T3-L1 adipocytes

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Insulin resistance is a hallmark of the metabolic syndrome and type 2 diabetes. Dysfunction of PI-3K/Akt signaling was involved in insulin resistance. Glucose transporter 4 (GLUT4) is a key factor for glucose uptake in muscle and adipose tissues, which is closely regulated by PI-3K/Akt signaling in response to insulin treatment. Low-power laser irradiation (LPLI) has been shown to regulate various physiological processes and induce the synthesis or release of multiple molecules such as growth factors, which (especially red and near infrared light) is mainly through the activation of mitochondrial respiratory chain and the initiation of intracellular signaling pathways. Nevertheless, it is unclear whether LPLI could promote glucose uptake through activation of PI-3K/Akt/GLUT4 signaling in 3T3L-1 adipocytes. In this study, we investigated how LPLI promoted glucose uptake through activation of PI-3K/Akt/GLUT4 signaling pathway. Here, we showed that GLUT4 was localized to the Golgi apparatus and translocated from cytoplasm to cytomembrane upon LPLI treatment in 3T3L-1 adipocytes, which enhanced glucose uptake. Moreover, we found that glucose uptake was mediated by the PI-3K/Akt2 signaling, but not Akt1 upon LPLI treatment with Akt isoforms gene silence and PI-3K/Akt inhibitors. Collectively, our results indicate that PI-3K/Akt2/GLUT4 signaling act as the key regulators for improvement of glucose uptake under LPLI treatment in 3T3L-1 adipocytes. More importantly, our findings suggest that activation of PI-3K/Akt2/GLUT4 signaling by LPLI may provide guidance in practical applications for promotion of glucose uptake in insulin-resistant adipose tissue.

Keywords: Glucose transporter 4; PI-3K/Akt; low-power laser irradiation; insulin resistance; 3T3-L1 adipocytes; type 2 diabetes.

1. Introduction

Insulin resistance is a hallmark in which peripheral tissues fail to properly respond to insulin stimulation, leading to abnormal glucose and lipid metabolism, which contributes to the risk of developing cardiovascular disease and type 2 diabetes. The effects of insulin are largely mediated by the activation of phosphatidylinositol3-kinase (PI3)-kinase/protein kinase B (PI3-K/Akt) signaling pathway. PI3-K/Akt signaling pathway regulates many physiological processes, including cell proliferation, survival, anti-apoptosis, development and metabolism.[1] In mammals, three highly conserved isoforms of Akt (PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3) encoded by three separate genes have been identified. All three genes products share a high degree of amino acid identity and seem to be regulated by similar mechanisms.[2] Recent studies have reported that PI3-kinase/Akt signaling pathway regulated glucose metabolism upon insulin exposure. Glucose transporter 4 (GLUT4) is a predominant downstream factor of PI3-k/Akt signaling pathway and responsible for glucose uptake in muscle and adipose tissues.[3] Once GLUT4 was activated by Akt, GLUT4 translocated from cytosol to cytomembrane and promoted glucose absorption. Nevertheless, if this process is disordered, glucose metabolism will be dysregulated and lead to hyperglycemia and insulin resistance.

Low-power laser irradiation (LPLI) has been found to regulate various biological processes in cell and animal models. It has been widely applied in neck pain care,[4] treatment of skeletal muscle regeneration,[5] wound healing[6] and diverse neurological diseases.[7,8] A large body of evidence has shown that LPLI can promote gene transcription,[9] movement[10] and differentiation[11] in different cell types, which are determined by activation of diverse signaling pathways, such as mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK),[5] Src,[12] protein kinase C (PKC)[13] and PI3-K/Akt.[14] Moreover, it has been reported that LPLI can significantly prevent cell apoptosis induced by Aβ in PC12 cells through activation of Akt/GSK3β signaling.[15] LPLI can efficiently penetrate into biological tissues including the central nervous system, producing noninvasive beneficial photobiomodulation effects such as promoting nerve regeneration and increasing ATP synthesis.[16] All the evidence suggests that LPLI regulates different biological processes through diverse signaling pathways in many cell types and ultimately affects cell physiological processes.

Although the effects of LPLI have been illustrated *in vitro* and *in vivo*, the roles of LPLI on GLUT4 activation and glucose uptake in 3T3-L1 adipocytes are poorly understood and the underlying molecular mechanisms are currently unknown. In the present study, we attempted to clarify how LPLI induced GLUT4 translocation and promoted glucose uptake. Based on various techniques and approaches, we found that GLUT4 was localized to the Golgi apparatus and translocated from cytoplasm to cytomembrane upon LPLI treatment in 3T3L-1 adipocytes. We also showed that glucose uptake induced by LPLI was almost completely prevented by PI3-K/Akt inhibitors. In addition, the results of shRNA-medicated knock down of Akt isoforms revealed that glucose uptake was mediated by the PI3-K/Akt2 signaling, but not PI3-K/Akt1 upon LPLI treatment. Collectively, we demonstrate that LPLI promotes glucose uptake through PI3-K/Akt2/GLUT4 signaling pathway. Our findings suggest that LPLI may have the potential therapeutic value in insulin-resistant adipose tissue.

2. Materials and Methods

2.1. *Cell culture and differentiation*

3T3-L1 fibroblast were grown in DMEM (GBICO, Co. Ltd., Grand Island, NY) containing 10% FCS, 50 units/mL penicillin and 50 μg/mL streptomycin. The cells were maintained in a humidified, 37°C incubator with 5% CO_2 and 95% air. For differentiation experiments, 3T3-L1 cells were induced 2 days post confluence and then were incubated in DMEM supplemented with 10% FCS, 500 μM 3-isobutyl 1-methylxanthine (IBMX), 0.25 μM dexamethasone and 10 μg/mL insulin (sigma) for 60 h. Finally, the cells were maintained in the same medium without IBMX and dexamethasone. All experiments were performed on day 8 after differentiation.

2.2. *Plasmids and reagents*

pEGFP-GLUT4 was kindly provided by Dr. Jeffrey E. Pessin.[17] pRFP-F-actin and pYFP-TGN38 were kindly provided by Dr. Cécile GauthierRouvière,[18] and Dr. Kai Simons,[19] respectively. Wortmannin

(50 nM) was procured from BIOMOL Research Laboratories, Inc. (Plymouth, PA). LY294002 (10 μM), insulin (1nM) and Akt inhibitor IV (10 nM) were procured from Sigma (St, Louis, MO). Akt inhibitor V (1 μM) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). The following antibodies were used: anti-phospho-Thr308-Akt, anti-phospho-Ser474-Akt, anti-Akt1, anti-Akt2, anti-total-Akt, GLUT4, pan-cadherin and GAPDH, β-actin (Cell Signaling Technology, Beverly, MA).

2.3. *Cell transfection and LPLI treatment*

Transient transfection was carried out using X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. 3T3-L1 adipocytes were seeded on 22-mm culture glasses or 60-mm plates one day prior to transfection. Cells were maintained in culture medium (Invitrogen) during transfection, and replaced with fresh culture medium 6 h later. About 48 h after transfection, cells were subjected to different treatments. 3T3-L1 adipocytes were serum-starved for at least 6 h in serum-free medium. For irradiation of these cells, the cells were irradiated with a He–Ne laser (632.8 nm, HN-1000; Guangzhou, China) in the dark with the fluence of 5 J/cm^2. The internal wells were filled with ink in order to minimize the scattered or reflected light. The power intensity was maintained at 10 mW/cm^2.

2.4. *GLUT4 translocation assay*

3T3-L1 adipocytes were co-transfected with GFP-GLUT4, YFP-TGN38 and RFP-F-actin expression plasmids. About 48 h after transfection, the cells were serum-starved for 6 h and then treated with LPLI (5 J/cm^2). The dynamic changes of GLUT4 in single living cell were observed by confocal microscopy (LSM510 META; Carl Zeiss Co., Ltd. Jena, Germany). Cells were observed with a 40 x oil objective lens (NA = 1.3). The stage of LSM was equipped with a temperature-controlled and CO$_2$-controlled small incubator (CTI-controller 3700 digital and Tempcontrol 37-2 digital; Zeiss, Jena, Germany), which maintained the cells at 37°C, 5% CO$_2$ during the whole experiment. GFP or YFP

fluorescence was excited with a 458- or 514-nm Ar–Ion laser, and the fluorescence emission was detected through a 465–510 nm or a 520–555 nm band pass filter, respectively. RFP fluorescence was excited with a 543-nm laser and the fluorescence emission was detected through a 650-nm long pass filter. During the experiments, the exciting power of 458-, 514-, or 543-nm laser was reduced to the minimal level (1–3%) to reduce the possible effects of exciting light. Data were analyzed with Zeiss Rel 3.2 image processing software (Zeiss, Germany).

2.5. *RNA interference*

Knockdown of Akt isoforms were performed using Akt-shRNAs. Akt1-shRNA, Akt2-shRNA and Akt1/2-shRNA were kindly provided by Dr. Nicolas Tricaud[20] and Dr. Juan P. Bolaños,[21] respectively. Briefly, 3T3-L1 adipocytes were infected with lentiviral vectors expressing Akt1-shRNA or Akt2 shRNA. After 72 h, these cells were selected with 2 μg/mL puromycin (Sigma) 2 days before to be used for different treatments. Akt1/2-shRNA was transfected into 3T3-L1 adipocyte using X-tremeGENE HP DNA transfection reagent according to the manufacturer's instructions. After 72 h, these cells were subjected to the next experiments.

2.6. *Glucose uptake assay*

The glucose consumption *in vitro* was measured by glucose oxidase method (GOD) using glucose assay kit (Applygen Company, China) according to manufacturer's instructions. 3T3-L1 adipocytes were serum-starved for 6 h and then treated with LPLI (5 J/cm^2) in the presence or absence of PI3-K/Akt inhibitors. After 2 h, glucose uptake in cells was detected with an Infinite 200 plate reader (TECAN, Mönnedorf, Switzerland).

2.7. *Subcellular fractionation*

Efficient extraction of transmembrane proteins was extracted with ProteoExtract$^®$ Transmembrane Protein Extraction Kit (Novagen) according to manufacturer's protocols. The extraction of transmembrane proteins was used in western blot analysis.

2.8. *Cell lysates collection*

For Western blot analysis, 3T3-L1 adipocytes were washed three times with PBS and then lysed with 200 mL of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1% TritonX-100, 0.1% SDS and 100 mM phenylmethylsulfonyl fluoride). The lysates were collected in microcentrifuge tubes and centrifuged. Protein concentrations were determined using the Bradford method. The lysates were stored at -80°C for Western blot analysis.

2.9. *Western blot analysis*

Western blot analysis was performed as described previously.[22] Briefly, the extracted proteins were separated in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were washed three times for 10 min each time with tris buffered saline with tween 20 (TBST) and incubated with indicated primary antibodies at 4°C overnight. After incubation, the membranes were labeled with goat anti-mouse conjugated to IRDyeTM 700 or goat anti-rabbit conjugated to IRDyeTM 800 secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA). Detection was performed using LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Inc., Lincoln, NE). Data were analyzed using LI-COR Image Studio Software (LI-COR, Biosciences, Lincoln, NE).

2.10. *Statistical analysis*

We performed each study at least three times under identical conditions. Data were represented as means $\pm$ SEM. Statistical analysis was applied using Student's paired t-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Subcellular localization of GLUT4*

In order to explore the subcellular localization of GLUT4, GFP-GLUT4, YFP-TGN38 and RFP-F-actin, expression plasmids were co-transfected into 3T3-L1 adipocytes. The subcellular localization of GLUT4 in single living cell was observed with confocal microscopy. As shown in Fig. 1(a), GLUT4 (green emission) was co-localized with trans-Golgi network (TGN38, yellow emission). This result indicates that GLUT4 is localized in the Golgi apparatus in 3T3-L1 adipocytes. Subsequently, the dynamic changes of GLUT4 in single living cell upon insulin (1 nM) exposure were observed, because insulin is a well-known factor which induces GLUT4 translocation. As shown in Fig. 1(b), GLUT4 was predominant in the cytosol under the normal condition, whereas it was translocated to the cytomembrane in response to insulin stimulation.

3.2. *LPLI induces GLUT4 translocation*

Next we investigate whether GLUT4 could be translocated from cytosol to cytomembrane upon LPLI treatment. To this end, GFP-GLUT4, YFP-TGN38 and RFP-F-actin expression plasmids were co-transfected into 3T3-L1 adipocytes and then treated with LPLI. The dynamic changes of GFP-GLUT4 in single living cell were monitored in real time by confocal microscopy. As shown in Fig. 2(a),
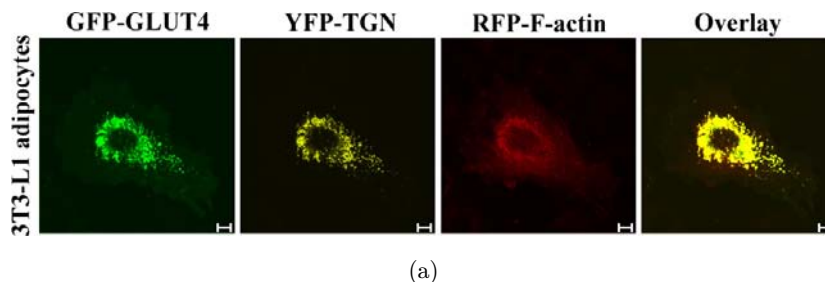

(a)

Fig. 1.   Subcellular localization of GLUT4. 3T3-L1 adipocytes were co-transfected with GFP-GLUT4, YFP-TGN38 and RFP-F-actin expression plasmids. About 48 h later, the transfected cells were observed with confocal microscopy. (a) The localization of GLUT4 in 3T3-L1 adipocytes. GLUT4 (green emission) was co-localized with Golgi apparatus (yellow emission). Bar = 10 μm. (b) 3T3-L1 adipocytes were treated with or without insulin (1 nM) and the localization of GLUT4 was observed. GLUT4 was localized to the cytomembrane. Bar = 10 μm.
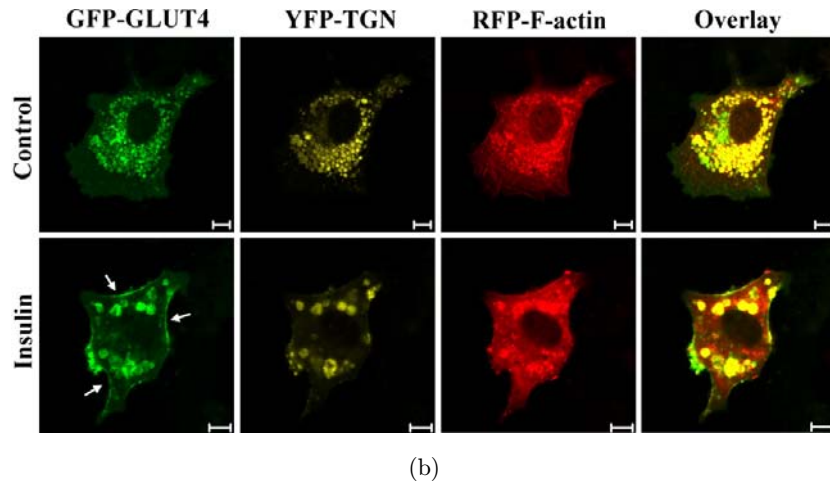
(b)

Fig. 1. (*Continued*)

GFP-GLUT4 was predominantly localized in the cytosol under normal conditions. When cells were stimulated with LPLI, GFP-GLUT4 was translocated from cytosol to cytomembrane within 90 min [see Fig. 2(b)]. This result indicates that LPLI can induce GLUT4 translocation in 3T3-L1 adipocytes. To further confirm the result that GLUT4 translocation was induced by LPLI, we isolated the cell membrane and cytoplasm of 3T3-L1 adipocytes and measured the subcellular localization of GLUT4
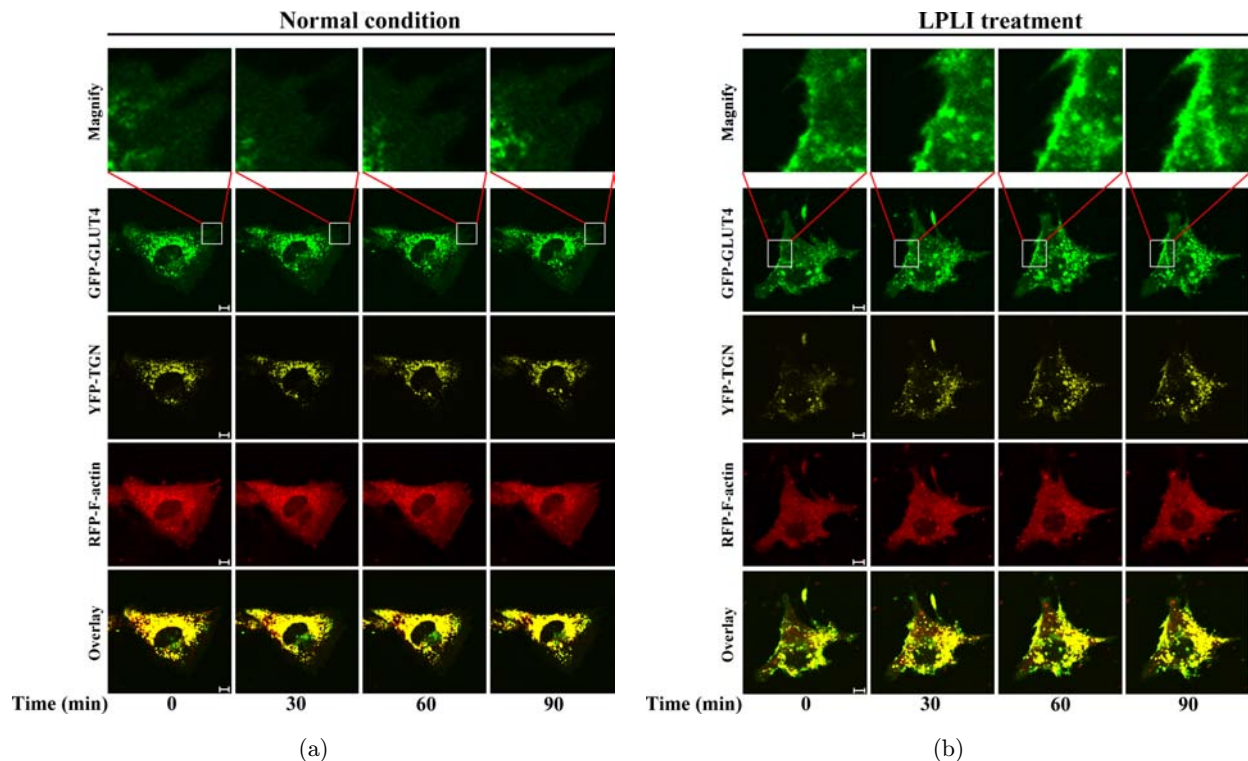

(a)

(b)

Fig. 2.   Real-time monitorings of the dynamic changes of GLUT4 in single living cell. 3T3-L1 adipocytes were co-transfected with GFP-GLUT4, YFP-TGN38 and RFP-F-actin expression plasmids. About 48 h later, the cells were serum-starved for 6 h and then treated with or without LPLI ($5\,\mathrm{J/cm^2}$) and the dynamic changes of GLUT4 was observed with confocal microscopy. (a) The time-elapse of the dynamic changes of GLUT4 in 3T3-L1 adipocytes without any treatment ($n=3$). Bar $=10\,\mu$m. (b) The time-elapse of the dynamic changes of GLUT4 in 3T3-L1 adipocytes treated with LPLI ($5\,\mathrm{J/cm^2}$) ($n=3$). Bar $=10\,\mu$m. (c) Western blot analysis of GLUT4 expression in cytosol and cytomembrane under LPLI ($5\,\mathrm{J/cm^2}$) treatment ($n=3$). (d) Quantitative analysis of the changes of GLUT4 expression. Data represent mean $\pm$ SEM ($n=3$, *$p < 0.05$ versus control group).
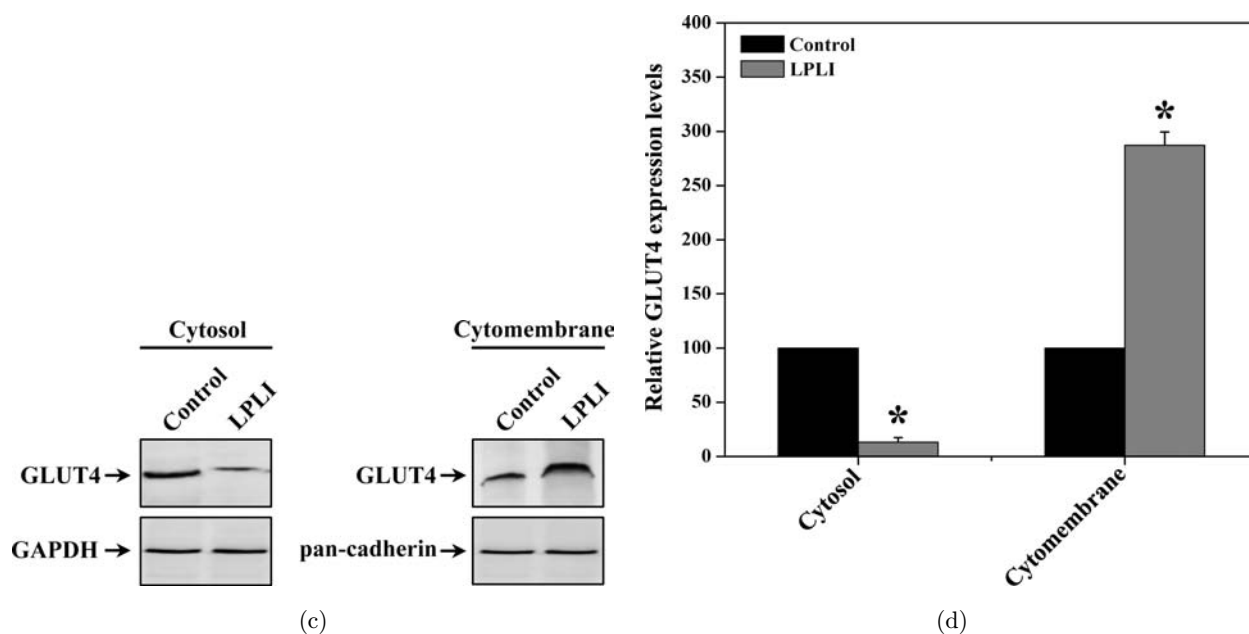
(c)

(d)

Fig. 2.   (*Continued*)

by western blot analysis. As show in Figs. 2(c) and 2(d), we found that GLUT4 mainly existed in the cytoplasm under normal condition, whereas a small number of GLUT4 existed in the cytomembrane.

When cells were treated with LPLI, GLUT4 mainly existed in the cytomembrane. Taken together, these results demonstrate that GLUT4 can be activated by LPLI.
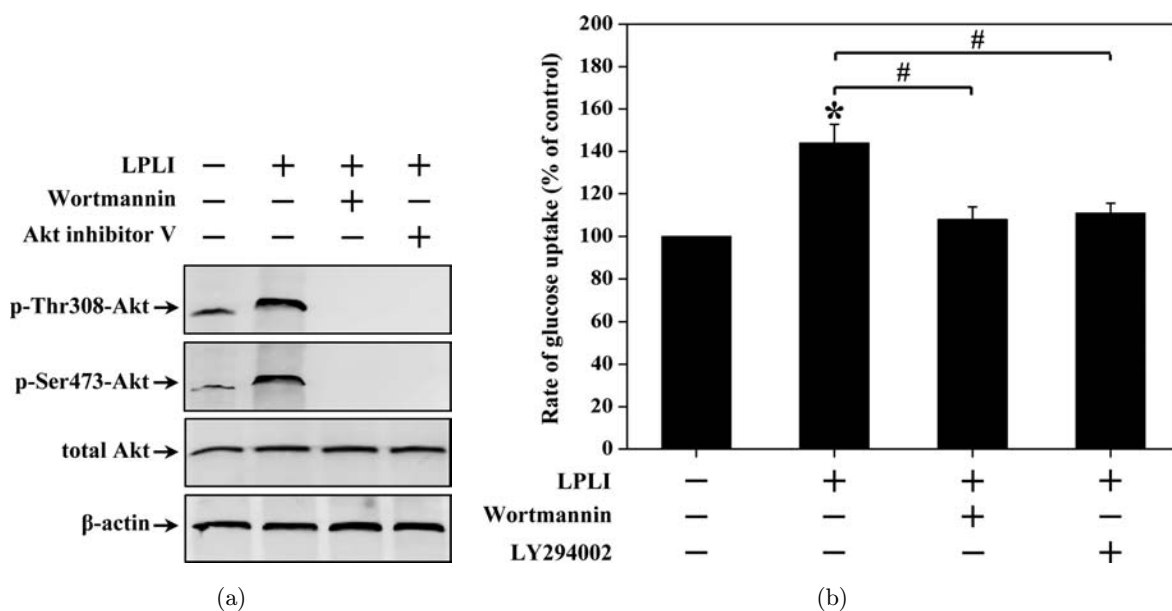

(a)

(b)

Fig. 3.   PI3-K/Akt regulates glucose uptake induced by LPLI. (a) 3T3-L1 adipocytes were serum-starved for 6 h and then treated with wortmannin or Akt inhibitor V for 2 h before LPLI (5 J/cm^2) treatment. The phosphorylation levels of Akt were detected by western blot analysis. (b) 3T3-L1 adipocytes were serum-starved for 6 h and then treated with wortmannin or LY294002 for 2 h before LPLI (5 J/cm^2) treatment. About 2 h later, the glucose uptake was measured. ($n = 3$, *$p < 0.05$ versus untreated group; #$p < 0.05$ versus indicated group), (c) was similar to (b). Akt specific inhibitors (Akt inhibitor IV or Akt inhibitor V) were used and the glucose uptake was measured. ($n = 3$, *$p < 0.05$ versus untreated group; #$p < 0.05$ versus indicated group).
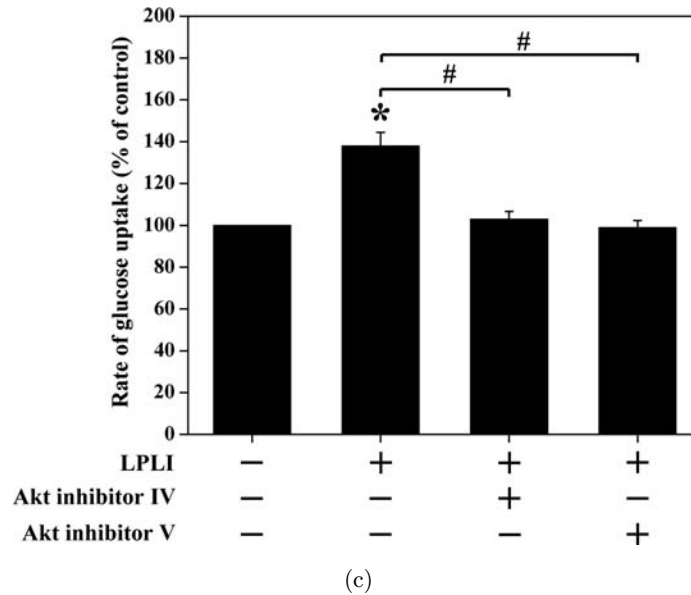
(c)

Fig. 3.   (*Continued*)

3.3. *PI3-K/Akt signaling mediates glucose uptake upon LPLI treatment*

We have demonstrated that LPLI can induce GLUT4 translocation in 3T3-L1 adipocytes. However, it is unclear whether LPLI could promote glucose uptake, which is mediated by the PI3-K/Akt/GLUT4 signaling axis. To further investigate whether LPLI could promote glucose uptake through activation of PI3-K/Akt signaling, 3T3-L1 adipocytes were treated with LPLI in the presence or absence of PI3-K or
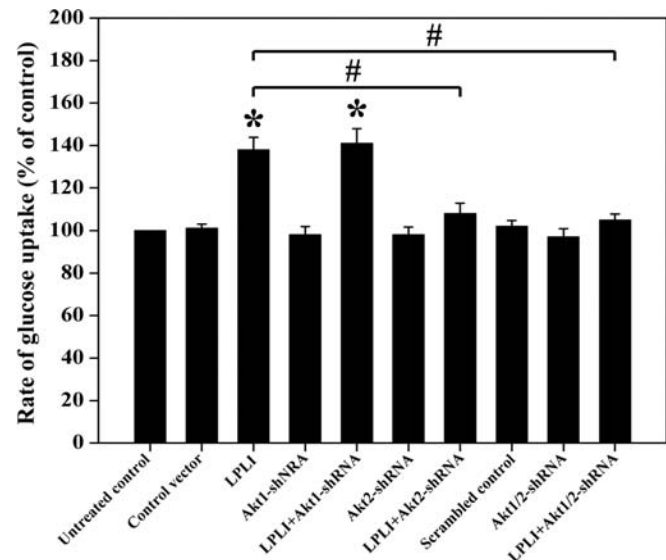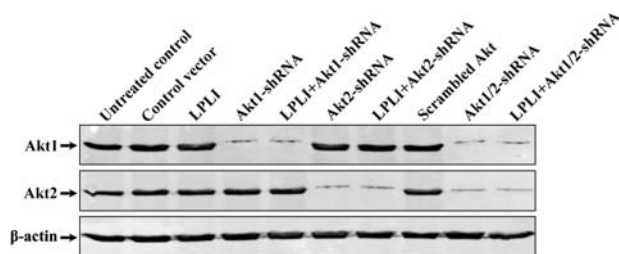

(a)

(b)

Fig. 4.   LPLI induces glucose uptake through Akt2, but not Akt1. 3T3-L1 adipocytes were infected with lentiviral vectors expressing Akt1-shRNA or Akt2 shRNA. Akt1/2-shRNA was transfected into 3T3-L1 adipocyte using X-tremeGENE HP DNA transfection reagent. (a) The expression levels of Akt isoforms (Akt1 and Akt2) were detected with western blot analysis and the effects of knockdown of Akt isoforms were shown ($n = 3$). (b) After downregulation of Akt isoforms, the 3T3-L1 adipocytes were serum-starved for 6 h and then treated with LPLI at the fluence of $5 \, \text{J/cm}^2$. About 2 h later, the glucose uptake was measured. ($n = 3$, *$p < 0.05$ versus untreated group; #$p < 0.05$ versus indicated group).

Akt specific inhibitors, respectively. As shown in Fig. 3(a), the phosphorylation levels of Akt were significantly increased by LPLI, whereas PI3-K/Akt inhibitors completely prevented the activation of Akt induced by LPLI. Subsequently, we measured the glucose uptake under LPLI treatment in the presence or absence of PI3-K or Akt specific inhibitors, respectively. As shown in Fig. 3(b), we found that glucose uptake was dramatically increased by LPLI. In contrast, PI3-K inhibitors (wortmannin and LY294002) almost completely prevented LPLI-induced glucose uptake compared to LPLI treatment only, revealing that PI3-K was indeed involved in LPLI-induced glucose uptake. Consistent with the above results, two Akt specific inhibitors (Akt inhibitor IV and Akt inhibitor V) significantly reduced LPLI-induced glucose uptake compared to LPLI treatment only [see Fig. 3(c)]. Taken together, these results demonstrate that LPLI induces glucose uptake through activation of PI3-K/Akt signaling axis.

3.4. *Akt2, but not Akt1 regulates glucose uptake upon LPLI treatment*

We next asked if glucose uptake could be regulated by Akt isoform-specific signaling upon LPLI treatment. RNA interference technology was used to knock down the expression levels of Akt1, Akt2 and Akt1/2 in 3T3-L1 adipocytes. As shown in Fig. 4(a), down-regulation of Akt2 specifically reduced LPLI-stimulated glucose uptake, whereas Akt1 knockdown had no effect on glucose uptake induced by LPLI. When Akt1 and Akt2 were simultaneously knocked down, glucose uptake induced by LPLI was dramatically reduced, but simultaneous knockdown of both Akt1 and Akt2 did not have a greater inhibitory effect than Akt2 knockdown. The expression levels of Akt1 and Akt2 in 3T3-L1 adipocytes were detected by western blot analysis [see Fig. 4(b)]. These results demonstrate that Akt2 regulates glucose uptake in response to LPLI stimulation, but not Akt1.

4. Conclusion

In this study, we showed a key regulatory mechanism of LPLI for GLUT4 translocation and glucose uptake in 3T3-L1adipocytes. Our major findings found that LPLI induced GLUT4 translocation from cytosol to cytomembrane and resulted in glucose uptake by activating the PI3-K/Akt signaling axis (Figs. 1 and 2). Further study revealed that promotion of glucose uptake caused by LPLI was controlled by Akt2 isoform-specific signaling axis, but not Akt1 (see Fig. 4). Understanding the molecular mechanisms and functional significance of LPLI-induced glucose uptake may lead to development of a new approach for diabetes therapy.

Akt is considered to be an important downstream target of insulin receptor substrate (IRS)/PI3-K signaling in regulation of glucose metabolism.[23] However, it remains completely unknown whether LPLI could promote glucose uptake through activation of PI-3K/Akt/GLUT4 signaling axis in 3T3L-1 adipocytes. Our study is designed to evaluate the relevance of Akt isoforms in control of GLUT4 translocation and glucose uptake upon LPLI treatment. In the present study, we found that LPLI not only induced activation of Akt, but also promoted GLUT4 translocation in 3T3L-1 adipocytes, which increased glucose uptake. Knockdown of Akt isoforms by shRNAs revealed that Akt2 mediated LPLI-induced glucose uptake, but not Akt1, which suggested that the functions of Akt isoforms in 3T3L-1 adipocytes were different, though their structures were very similar in mammalian cells. Recent studies suggested that Akt2 was required for glucose metabolism[24] and deficiency of Akt2 led to insulin resistance.[25] All the evidence suggests that Akt isoforms (Akt1 and Akt2) exert different functions in mammalian cells, although their regulation mechanisms are similar.

In summary, our data demonstrate that LPLI induced GLUT4 translocation and glucose uptake through PI3-K/Akt2 isoform-specific signaling axis, but not the PI3-K/Akt1. Activated Akt2 promoted downstream signaling pathways activaton that were closely related to regulation of glucose transport. These observations may provide a potentially therapeutic strategy for type 2 diabetes through increasing glucose absorption in the peripheral tissues.

Acknowledgments

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