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## Original Article

# Rubiscolin-6 activates opioid receptors to enhance glucose uptake in skeletal muscle

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## ABSTRACT

Rubiscolin-6 is an opioid peptide derived from plant ribulose biphosphate carboxylase/oxygenase (Rubisco). It has been demonstrated that opioid receptors could control glucose homeostasis in skeletal muscle independent of insulin action. Therefore, Rubiscolin-6 may be involved in the control of glucose metabolism. In the present study, we investigated the effect of rubiscolin-6 on glucose uptake in skeletal muscle. Rubiscolin-6-induced glucose uptake was measured using the fluorescent indicator 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) in L6 and C2C12 cell lines. The protein expressions of glucose transporter 4 (GLUT4) and AMP-activated protein kinase (AMPK) in L6 cells were observed by Western blotting. The *in vivo* effects of rubiscolin-6 were characterized in streptozotocin (STZ)-induced diabetic rats. Rubiscolin-6 induced a concentration-dependent increase in glucose uptake levels. The increase of phospho-AMPK (pAMPK) and GLUT4 expressions were also observed in L6 and C2C12 cells. Effects of rubiscolin-6 were blocked by opioid receptor antagonists and/or associated signals inhibitors. Moreover, Rubiscolin-6 produced a dose-dependent reduction of blood glucose and increased GLUT4 expression in STZ-induced diabetic rats. In conclusion, rubiscolin-6 increases glucose uptake, potentially via an activation of AMPK to enhance GLUT4 translocation after binding to opioid receptors in skeletal muscle.

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## 1. Introduction

Rubiscolin-6 is a bio-active peptide derived from the D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a major protein that is responsible for carbon dioxide fixation and photorespiration in the green leaves of plants [1,2]. Rubiscolin-6 is comprised of six amino acids, Tyr-Pro-Leu-Asp-Leu-Phe (YPLDLF). It has affinity to both  $\delta$ -opioid receptor (DOR) and  $\mu$ -opioid receptor (MOR), showing higher affinity to the DOR compared to the MOR [3–5]. Recent studies have shown that rubiscolin-6 can exhibit memory-enhancing [6], anxiolytic [7], and orexigenic activities in animal models [8].

The opioid receptors belong to the inhibitory G protein-coupled receptors (GPCRs) family, which is divided into four subtypes: MOR, DOR,  $\kappa$ -opioid receptor (KOR), and the least characterized, opioid receptor like-1 (ORL1) [9,10]. Each of these receptors is activated by its respective ligand(s), both endogenously produced opioid peptides and/or exogenously administered opiate compounds [11]. The opioid system is known to play a central role in pain management [12], and one of the key parts in hedonic homeostasis [13], mood and well-being [14]. Some studies also suggest the role of opioid receptors in regulation of glucose homeostasis. For example,  $\beta$ -endorphin, an endogenous opioid peptide, may regulate glucose homeostasis [15,16]. The activation of MOR may increase glucose uptake in C2C12 myoblast cells via the activation of protein kinase C (PKC) [17]. It also decreases plasma glucose levels in streptozotocin (STZ)-induced diabetic rats by increasing the glucose utilization and decreasing gluconeogenesis in the liver [18]. Similar to MOR, DOR also can stimulate glucose transport both in vitro and in vivo. DOR agonist [3H][D-pen(2), D-pen(5)] enkephalin (DPDPE), increased glucose uptake in skeletal muscle of lean and obese-diabetic mice [19]. In CHO cells, activation of DOR may stimulate glucose uptake mediated by glucose transporter 1 (GLUT1) [20]. Additionally, DOR may stimulate  $\text{Ca}^{2+}$  release and AMP-activated protein kinase (AMPK) phosphorylation on Thr172, through coincidence signaling with G protein,  $G_{\alpha_{q/11}}$ -coupled receptors that are associated with glucose uptake [21].

It is of special interest to understand whether rubiscolin-6 can enhance glucose uptake in skeletal muscle, both in vitro and in vivo. Therefore, in the present study, we used the rat myoblast cell line L6 and the mouse myoblast cell line C2C12 to investigate the cellular action of rubiscolin-6. Also, the in vivo effect of rubiscolin-6 was further characterized using STZ-induced diabetic rats.

## 2. Materials and methods

### 2.1. Rubiscolin-6 synthesis and purification

Rubiscolin-6 (YPLDLF) was synthesized by a solid-phase methodology with Fmoc-strategy using an automated peptide synthesizer (Model Pioneer; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The crude peptide was purified by a reverse-phase HPLC (Delta 600 HPLC System; Waters, Milford, Massachusetts, USA) on a column of Develosil ODS-HG-5 (2 × 25 cm; Nomura Chemical Co., Ltd, Seto, Japan).

High purity of the purified peptide was confirmed by analytical HPLC and MALDI-TOF MS analysis.

### 2.2. Cell culture

The L6 (CRL1458™) (ATCC, Manassas, Virginia, USA) rat myoblast cell line and C2C12 (CRL1772™) (ATCC, Manassas, Virginia, USA) mouse myoblast cell line were cultured in a humidified atmosphere of 5%  $\text{CO}_2$ –95% air at 37 °C, maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GE Life Sciences, Pittsburgh, Pennsylvania, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and supplemented with 1% penicillin and streptomycin (Biological Industries, Cromwell, Connecticut, USA) and 1% amphotericin B (Sigma–Aldrich, Saint Louis, Missouri, USA). Cells were reseeded at a density of  $1 \times 10^6$  cells/mL in 10 cm dish and incubated in DMEM until confluence.

### 2.3. Glucose uptake assay

Fluorescent indicator, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), was used to monitor the glucose uptake in L6 and C2C12 cells [22]. After the starvation for 4 h, cells were washed, harvested, and incubated in FBS-free medium in the presence of 2-NBDG (200  $\mu\text{M}$ ) or 2-NBDG (200  $\mu\text{M}$ ) together with rubiscolin-6 at 0.01, 0.1, or 1  $\mu\text{M}$ , and incubated in the dark at 37 °C with 5%  $\text{CO}_2$  for 30 min. Naltrindole (DOR antagonist) (Sigma–Aldrich, Saint Louis, Missouri, USA), naloxone (broad spectrum opioid antagonist) (Tocris Bioscience, Bristol, UK), naloxonazine (MOR antagonist) (Tocris Bioscience, Bristol, UK), YM-254890 ( $G_{\alpha_{q/11}}$  inhibitor) (Wako chemicals, Osaka, Japan), U73122 (phospholipase C (PLC) inhibitor) (Tocris Bioscience, Bristol, UK), or compound C (AMPK inhibitor) (Tocris Bioscience, Bristol, UK) pretreatment was conducted 30 min prior to the addition of 2-NBDG, at 1  $\mu\text{M}$ , respectively. After incubation in the same manner, the cells were washed with pre-cooled PBS three times using centrifugation, and glucose uptake was determined as previously described [22]. The fluorescence intensities were detected using Hitachi F-2000 fluorescence spectrofluorometer (Hitachi, Chiyoda, Tokyo, Japan), under the excitation wavelengths of 488 and emission of 520 nm. Protein concentration in cell lysates was determined using the bicinchoninic acid (BCA) protein assay methods (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's protocol.

### 2.4. Animals and treatment

Male wistar rats weighing 190–210 g (Japan SLC, Inc., Shizuoka, Japan) were housed in a pathogen-free facility in a temperature- and humidity-controlled room on a 12 h light dark cycle with free access to food and water at the animal center Kagoshima University (Kagoshima, Japan). All procedures in this study were approved by the Ethics Committee for Animal Care and Use of Kagoshima University (institutional review board (IRB) approval number MD18005) and followed the Japanese National Standardized Guidelines for Animal Experiments of Kagoshima University.

After adjustments period (2 weeks), the rats are randomly divided into two groups, the normal control group and STZ-treated group (STZ). STZ group was induced by intraperitoneal (IP) injection of 65 mg/kg STZ (Sigma–Aldrich, Saint Louis, Missouri, USA) dissolved in citrate buffer (10 mM, pH 4.5) after fasting 12 h [23]. On day 7, rats with blood glucose levels higher than 250 mg/dl were considered diabetic.

Each working group was intraperitoneally treated with saline (vehicle), rubiscolin-6 at 30 mg/kg or 100 mg/kg, or 10 mg/kg naloxone with 100 mg/kg of rubiscolin-6 daily once a day at 15:00 h for 28 consecutive days. The food intake and water intake in addition to the body weight were measured weekly. After the end of the treatment, all the animals were fasted for overnight, blood sample were collected from rat tail veins. Fasting blood glucose levels were measured using glucose meter (AlphaTRAK 2, Abbott Laboratories, Chicago, Illinois, USA). The animals were then anesthetized using an IP injection of the triple mixture of medetomidine, midazolam, and butorphanol with doses of 0.15, 2.0 and 2.5 mg/kg, respectively, exsanguinated with heart puncture, followed by cervical dislocation. Soleus muscle samples were carefully isolated and rapidly frozen using liquid nitrogen and stored in  $-80^{\circ}\text{C}$  for further checking.

### 2.5. Membrane protein preparation

For detecting the expression of glucose transporter 4 (GLUT4), membrane protein fractions were separated from cells and muscle tissue using plasma membrane protein extraction kit (Invent Biotechnologies, Inc., Plymouth, Minneapolis, USA). The procedures were following the manufacturer's protocol. Protein concentrations were determined using BCA protein assay methods as described above.

### 2.6. Western blot analysis

The phospho-AMPK (pAMPK) and GLUT4 levels were determined using Western blotting analysis [24]. Cells were treated with or without rubiscolin-6 (0.01, 0.1, 1  $\mu\text{M}$ ), in the absence or presence of naltrindole, naloxone, naloxonazine, YM-254890, U73122 or compound C pretreatment. The cells and the muscle tissues were homogenized with Ice-cold radio-immuno-precipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (Merck Millipore, Burlington, Massachusetts, USA). Insoluble materials were separate using centrifugation (12,000 rpm for 20 min at  $4^{\circ}\text{C}$ ). Samples were filtered and separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide gels) and transferred to polyvinylidene difluoride membranes using Bio-Rad Trans-Blot system (Bio-Rad laboratories Inc., Hercules, California, USA). The membranes were blocked for 1 h in 5% nonfat milk or bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). After blocking, membranes were washed three times in TBS-T and incubated overnight at  $4^{\circ}\text{C}$  with either pAMPK $\alpha$  (Thr 172) or AMPK antibodies (1:1.000 dilution; Cell Signaling Technology, Danvers, Massachusetts, USA) or GLUT4 (1:1.000 dilution; Abcam, Cambridge, UK) primary antibodies. Incubation with  $\beta$ -actin antibody (1:2000

dilution; Abcam, Cambridge, UK) was done as a positive control. All the membranes were then washed and re-incubated with appropriate secondary antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5000 dilution; Merck Millipore, Burlington, Massachusetts, USA) or anti-mouse IgG antibody (1:5.000 dilution; Merck Millipore, Burlington, Massachusetts, USA), for 1 h at room temperature. Chemiluminescent substrate (Luminata<sup>tm</sup> Forte, Merck Millipore, Burlington, Massachusetts, USA) was added. Chemiluminescence of signals were then captured using X-ray films (ECL<sup>tm</sup> Hyperfilm<sup>tm</sup>, GE healthcare life sciences, Buckinghamshire, UK) or a Charge-Coupled Device (CCD)-based camera imager (Chem X400, Avesene, New Taipei City, Taiwan). Band densities were compared to the positive control, results are shown as a relative intensity to the untreated control.

### 2.7. Statistical analysis

The data were statistically evaluated using SPSS software (SPSS, Inc., Chicago, Illinois, USA). Significant differences between each group were analyzed by analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. Results are presented as the mean  $\pm$  standard error of the mean (SEM) for cells or mean  $\pm$  standard deviation (SD) for rats, as appropriate. Statistical significance was set at  $p < 0.05$ .

## 3. Result

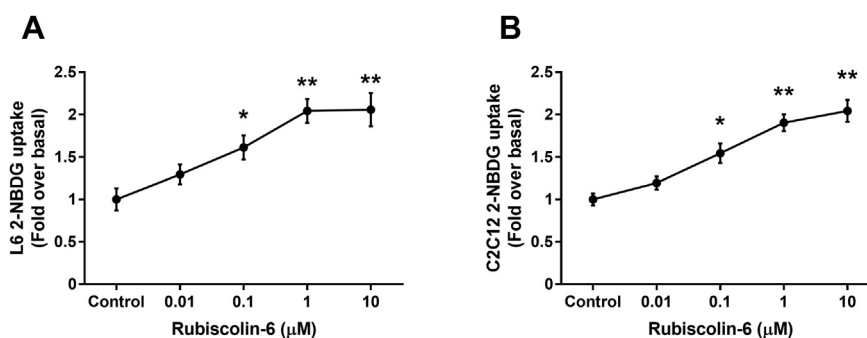
### 3.1. Rubiscolin-6 stimulates glucose uptake in L6 and C2C12 cells

In L6 cells, rubiscolin-6 displayed a concentration-dependent increase in glucose uptake using 2-NBDG as indicator, with a maximum response (2.04 fold) at 1  $\mu\text{M}$  as shown in Fig. 1A. Similarly, rubiscolin-6 also produced a concentration-dependent increase of glucose uptake in C2C12 cells, showing a 1.91-fold increase in glucose uptake at 1  $\mu\text{M}$  that reached the maximal (Fig. 1B).

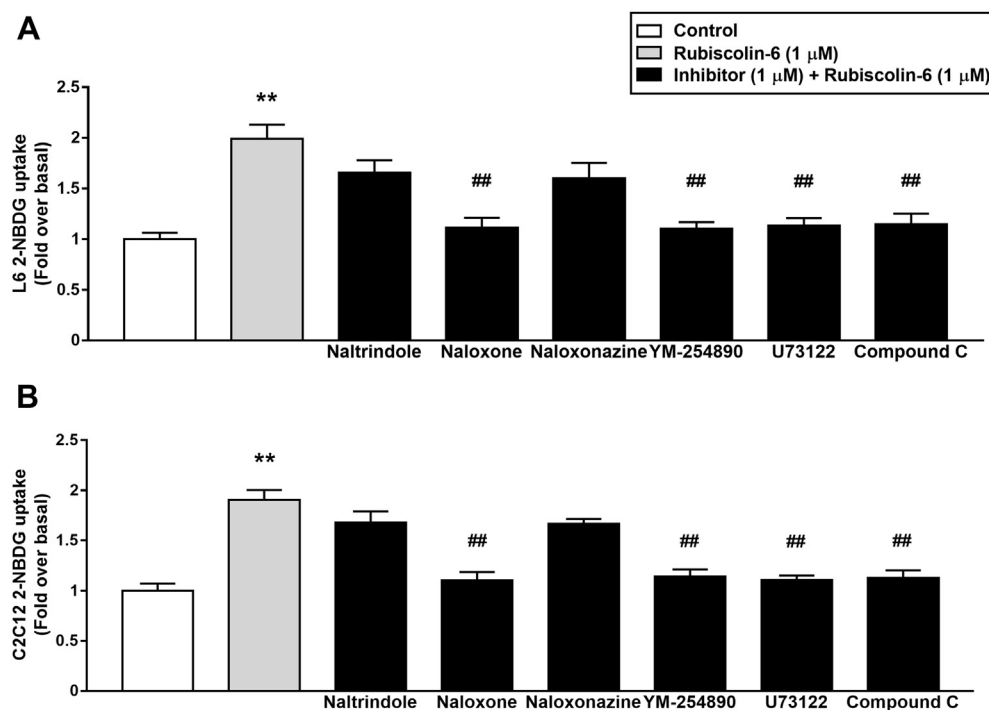
To examine the underlying mechanisms of rubiscolin-6, we used inhibitors specific to the opioid receptors,  $G\alpha_{q/11}$ , PLC, or AMPK to pre-treat with rubiscolin-6 for changes of glucose uptake in L6 or C2C12 cells. As shown in Fig. 2, naloxone (broad spectrum opioid receptor antagonist), YM-254890 ( $G\alpha_{q/11}$  blocker), U73122 (PLC inhibitor), and compound C (AMPK inhibitor) almost completely reversed the increase in glucose uptake by rubiscolin-6 both in L6 cells (Fig. 2A) and C2C12 cells (Fig. 2B). However, naltrindole (DOR antagonist) or naloxonazine (MOR antagonist) failed to show the same effect and partially inhibited the rubiscolin-6 induced glucose uptake (Fig. 2).

### 3.2. Rubiscolin-6 enhances GLUT4 expression through the AMPK pathway

To understand if the increased glucose uptake by rubiscolin-6 was due to an enhanced GLUT4 expression, the GLUT4 level in cell membrane was measured. After stimulation with different concentrations of rubiscolin-6, the GLUT4 level in



**Fig. 1** – Effect of rubinscolin-6 on glucose uptake in L6 and C2C12 cells. Cells were serum starved for 4 h and incubated with 2-NBDG or 2-NDBG together with varying concentrations of rubinscolin-6 for 30 min. (A) The increase in glucose uptake upon treatment with different concentrations of rubinscolin-6 in L6. (B) The increase in glucose uptake upon treatment with different concentrations of rubinscolin-6 in C2C12. Values are indicated as the mean  $\pm$  SEM (n = 8 per group). \*P < 0.05 and \*\*P < 0.01 compared to the control group.

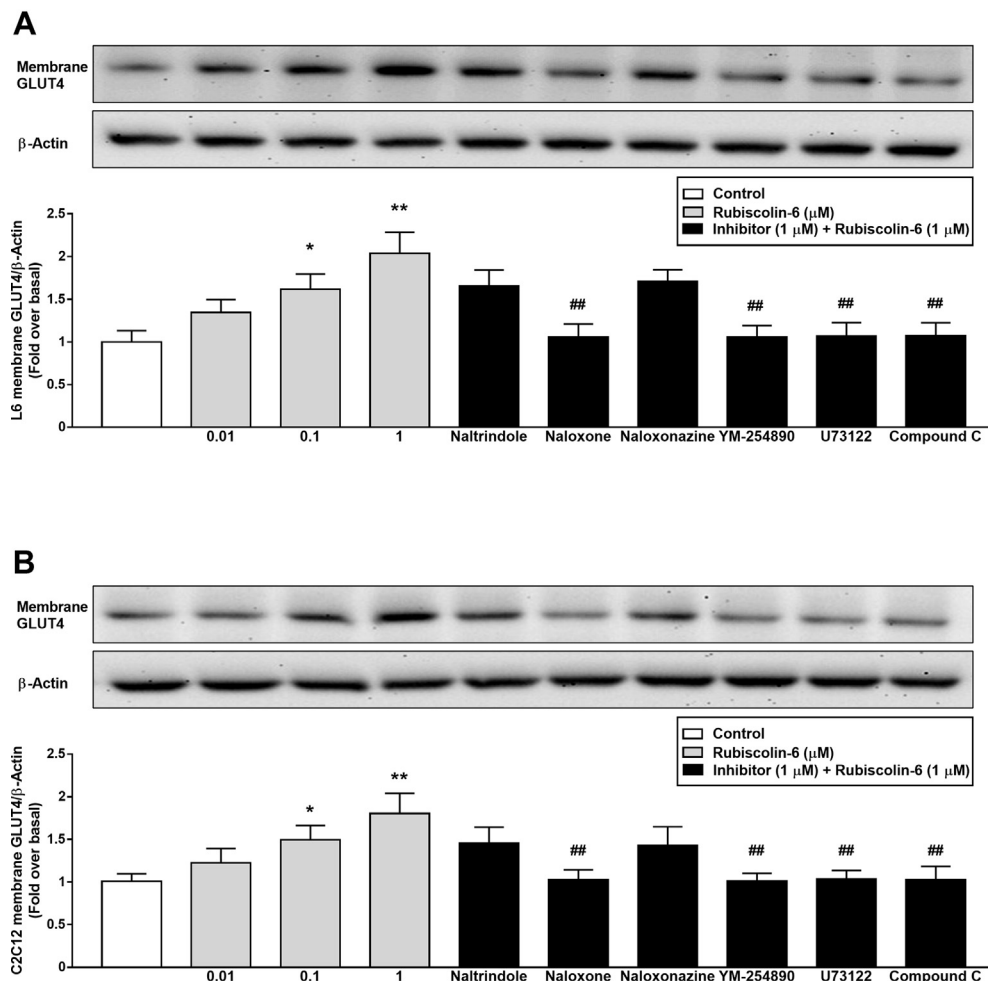


**Fig. 2** – Effect of inhibitors on rubinscolin-6 stimulated glucose uptake in L6 and C2C12 cells. Cells were serum-starved for 4 h and pretreated with naltrindole (DOR antagonist), naloxone (broad spectrum opioid antagonist), naloxonazine (MOR antagonist), YM-254890 ( $G\alpha_{q/11}$  inhibitor), U73122 (PLC inhibitor), or compound C (AMPK inhibitor), respectively, for 30 min. After inhibitor treatment, cells were stimulated with 1  $\mu$ M rubinscolin-6 for 30 min. Cells were stimulated with 1  $\mu$ M rubinscolin-6 in the absence of inhibitors as vehicle group. (A) The changes in rubinscolin-6 induced glucose uptake after pretreatment with different inhibitors in L6. (B) The changes in rubinscolin-6 induced in glucose uptake after pretreatment with different inhibitors in C2C12. Values are shown as the mean  $\pm$  SEM (n = 8 per group). \*\*P < 0.01 compared to the control group. ##P < 0.01 compared to 1  $\mu$ M rubinscolin-6-administered cells.

cell membrane was increased in a concentration-dependent manner (Fig. 3). Interestingly, the increase in GLUT4 translocation is correlated with the increase in glucose uptake at the same concentrations of rubinscolin-6.

The effect of rubinscolin-6 was abolished by Compound C at the concentration sufficient to block AMPK which is an intracellular regulator of glucose metabolism [25]. We also found that AMPK activation was concentration-dependently

raised by rubinscolin-6 and reached  $\sim$ 1.79-fold at 1  $\mu$ M of rubinscolin-6 both in L6 and 1.54-fold in C2C12 cells (Fig. 4). Additionally, pretreatment with compound C could abolish the rubinscolin-6-induced increase in membrane GLUT4 in both the L6 and C2C12 cells, which is consistent with the results in glucose uptake. Therefore, increase of GLUT4 translocation induced by rubinscolin-6 is associated with AMPK activation.



**Fig. 3** – Effect of rubinscolin-6 on GLUT4 translocation in L6 and C2C12 cells. After serum starvation, cells were treated with varying concentration of rubinscolin-6 for 2 h, respectively. To investigate the mechanism of rubinscolin-6, cells were pretreated with indicated inhibitors, respectively, for 30 min followed by treatment with 1 μM rubinscolin-6 for 30 min. The relative expression levels of GLUT4 in (A) L6 and (B) C2C12 cells. Images shown are representative of four independent experiments and the quantitative data are average of four experiments. \*P < 0.05 and \*\*P < 0.01 vs. control. ##P < 0.01 vs. 1 μM rubinscolin-6-administered cells.

Moreover, in the presence of PLC inhibitor, U73122, the rubinscolin-6-induced increase in AMPK activation or GLUT4 translocation was markedly reduced as compared to 1 μM rubinscolin-6-administered cells. Similarly, naloxone and YM-254890 inhibited the rubinscolin-6-induced increase in AMPK activation or GLUT4 translocation in the same manner. Therefore, activation of the opioid receptor that is coupled with second messenger  $G_{\alpha_{q/11}}$  in rubinscolin-6-induced increase in glucose uptake can thus be identified. However, DOR or MOR seem partially involved in the effects of rubinscolin-6 on glucose uptake in both cell lines because the specific antagonist, either naltrindole or naloxonazine, failed to fully abolish the effects of rubinscolin-6.

### 3.3. Rubinscolin-6 improves glucose homeostasis in STZ-Induced diabetic rats

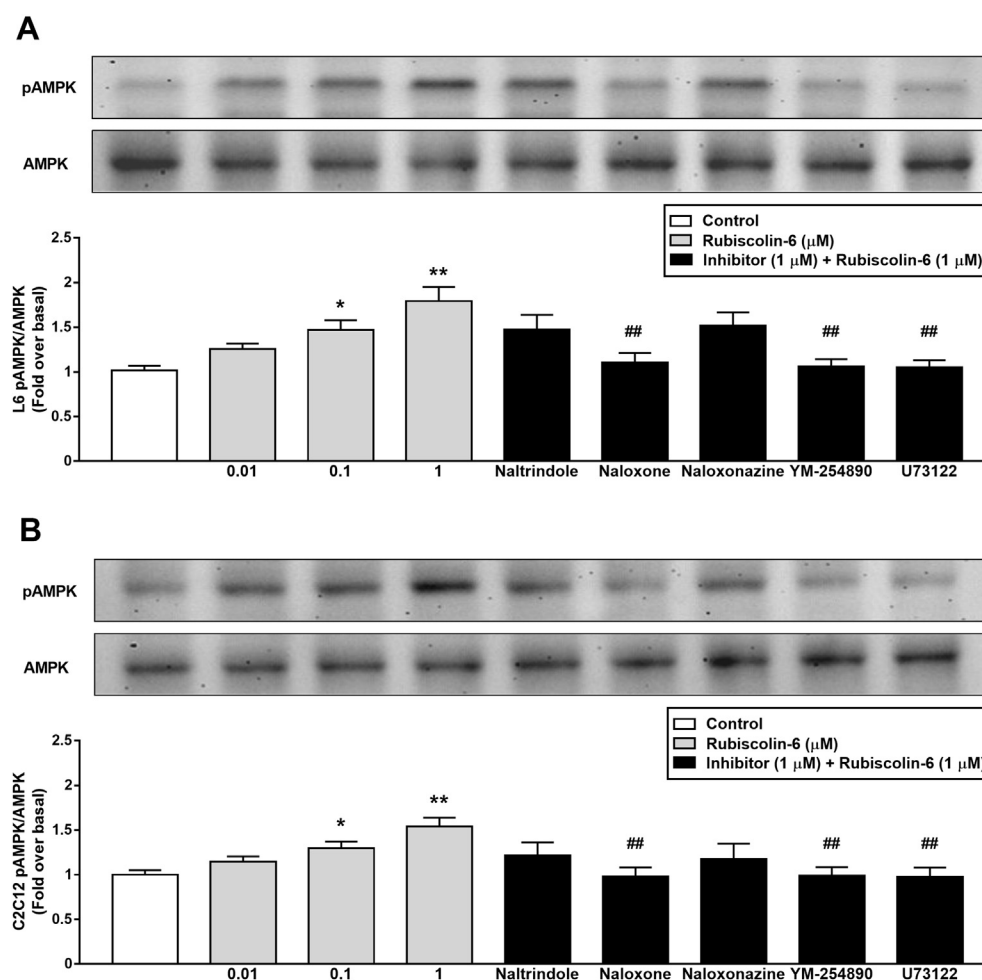
The blood glucose levels were significantly increased after an IP injection of 65 mg/kg STZ in rats. Administration of

rubinscolin-6 at 30 mg/kg and 100 mg/kg decreased the blood glucose levels to  $370 \pm 22.86$  mg/dl and  $341 \pm 32.79$  mg/dl, respectively, compared with vehicle-treated STZ group ( $418 \pm 30.4$  mg/dl) (Fig. 5A). Furthermore, Western blotting analysis showed a significant increase of GLUT4 expressions in skeletal muscle isolated from STZ group treated with rubinscolin-6 as compared to the vehicle-treated STZ group (Fig. 5B). Rubinscolin-6 also showed a preservation of body weight, and food intake (Fig. 5C,D). However, similar treatment with rubinscolin-6 failed to show an effect in normal rats. Effects of rubinscolin-6 were also abolished by naloxone in STZ-induced diabetic rats.

## 4. Discussion

In the present study, we found that rubinscolin-6 has an ability to enhance glucose uptake in the skeletal cell lines, L6 and C2C12. Rubinscolin-6 significantly increased 2-NBDG



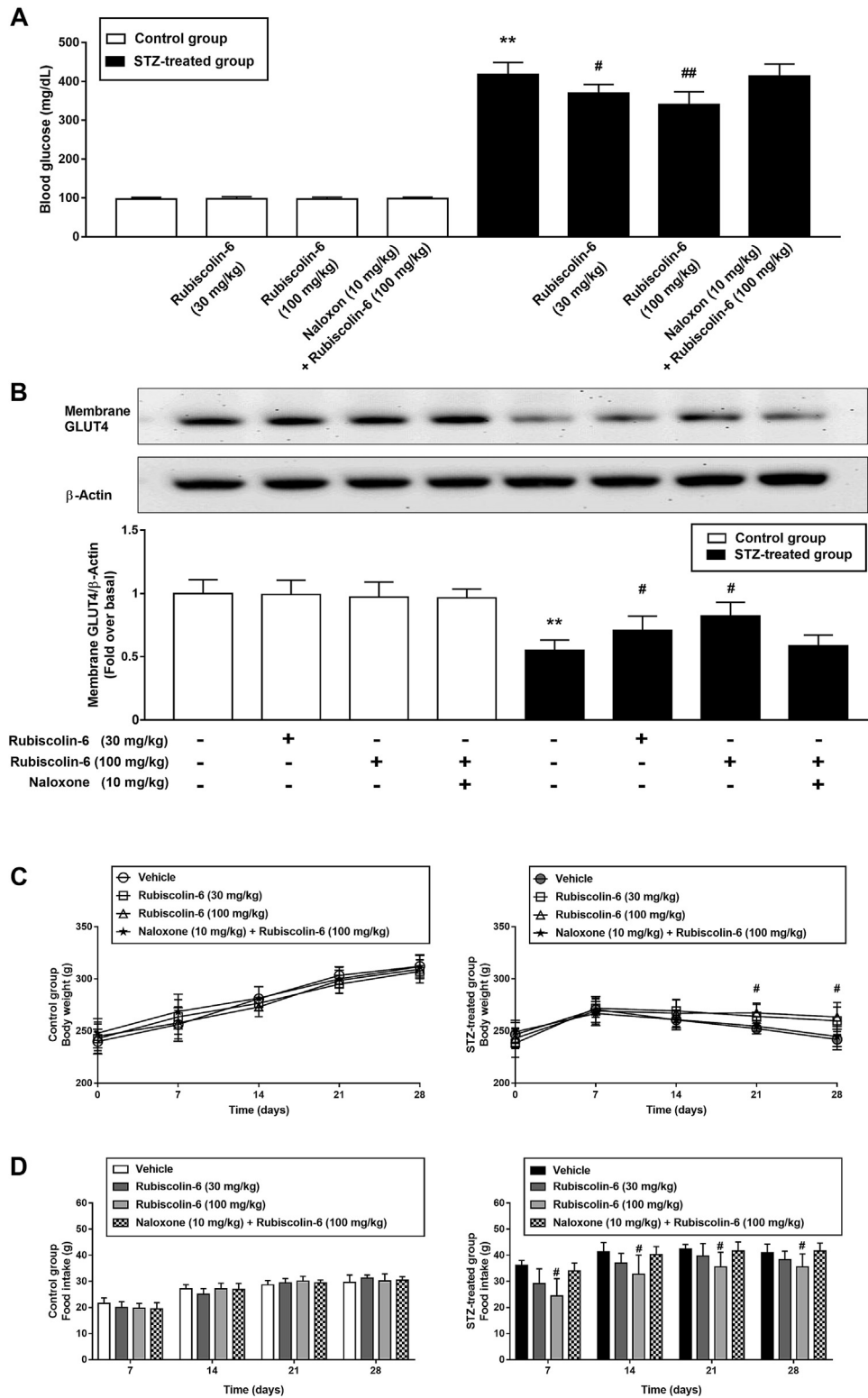


**Fig. 4** – Effect of rubinscolin-6 on AMPK activation in L6 and C2C12 cells. After serum starvation, cells were treated with varying concentration of rubinscolin-6 for 2 h respectively. To investigate the mechanism of rubinscolin-6, cells were pretreated with indicated inhibitors, respectively, for 30 min followed by treatment with  $1 \mu\text{M}$  rubinscolin-6 for 30 min. The relative expression levels of AMPK in (A) L6 and (B) C2C12 cells. Images shown are representative of four independent experiments and the quantitative data are average of four experiments. \* $P < 0.05$  and \*\* $P < 0.01$  vs. control. ## $P < 0.01$  vs.  $1 \mu\text{M}$  rubinscolin-6-administered cells.

uptakes in a dose-dependent manner in these two cell lines. Furthermore, rubinscolin-6 potentially modulates glucose uptake through opioid receptors. The effects of rubinscolin-6 were totally blocked by the broad-spectrum opioid antagonist, naloxone, but only partially inhibited by naltrindole, an antagonist selective for DOR, or naloxonazine, a selective antagonist for MOR. In the present study, the increase in glucose uptake by rubinscolin-6 was linked to  $G_{\alpha_{q/11}}$  subunit, as YM-254890 administration was sufficient to inhibit  $G_{\alpha_{q/11}}$  and abolished the effects. The receptor crosstalk between DOR and MOR or the existence of direct interaction between two receptors (dimerization) in cells [26,27], could be cited as a possible explanation. Heterodimerization between DOR and MOR have been shown to have unique functional properties that potentially share the same G protein messenger, and binding to one receptor could induce an allosteric influence on another receptor [28,29]. Moreover, previous studies demonstrated that in certain situations the opioid

receptors may change its functional response and interact with  $G_{\alpha_{q/11}}$ , and lead to the activation of the PLC pathway [30,31]. The PLC pathway is known to trigger an increase of cytoplasmic calcium mainly through inositol trisphosphate (IP3). In the present study, pretreatment with U73122, the specific inhibitor of PLC, suppressed glucose uptake increased by rubinscolin-6, demonstrating rubinscolin-6 dependence on the PLC pathway. Therefore, rubinscolin-6 could increase glucose uptake in muscle cells potentially by an interaction with the opioid receptors through the activation of PLC via  $G_{\alpha_{q/11}}$  messenger.

AMPK is a heteromeric protein complex composed of three subunits  $\alpha$ ,  $\beta$  and  $\gamma$  [32]. It has been documented that AMPK is an important enzyme in regulating glucose homeostasis in skeletal muscle [33]. Activation of AMPK has been viewed as an important aspect in insulin-independent glucose uptake [34]. The phosphorylation of threonine within the  $\alpha$ -subunit by an upstream kinase will result in an



**Fig. 5** – Rats were divided into control group and STZ-treated group. Each group was treated with saline (vehicle), 30 mg/kg rubiscolin-6, 100 mg/kg rubiscolin-6, or 10 mg/kg naloxone + 100 mg/kg of rubiscolin-6 every day for 28 consecutive days by IP injection, respectively. (A) The blood glucose levels were determined 1 h after administration of rubiscolin-6 on day 28. (B) The expression of membrane GLUT4, cytosol GLUT4 over β-actin in rat's skeletal muscle (C) The changes of body weight in experimental period. (D) The changes of food intake in experimental period. The values are expressed as the mean ± SD (n = 8). \*\*P < 0.01 compared with the vehicle-treated control group. #P < 0.05 and ##P < 0.01 compared with vehicle-treated STZ group.

activation of AMPK. Our results showed that AMPK inhibition by compound C markedly blocked glucose uptake, indicating the role of AMPK activation in glucose uptake. Western blotting showed that rubiscolin-6 caused a significant increase of pAMPK expression in L6 and C2C12 cells via a dose-dependent manner and was reversed by pretreatment with opioid antagonists,  $G\alpha_{q/11}$  blocker, and PLC inhibitor. Collectively, AMPK mediated the rubiscolin-6-stimulated glucose uptake in L6 and C2C12 cells independent with insulin signaling. Therefore, rubiscolin-6 may activate the opioid receptors coupled with  $G\alpha_{q/11}$  to enhance glucose uptake via AMPK activation.

Glucose uptake in skeletal muscle cells is facilitated by transport molecules, called glucose transporter [35], particularly, the GLUT4 glucose transporter [36]. GLUT4 transporter is mainly translocated from the cytoplasm to the plasma membrane and/or transverse tubules (T-tubules) by two major pathways, insulin-dependent pathway and insulin-independent pathway [37,38]. Rubiscolin-6 increased AMPK activity that could lead to the translocation of GLUT4 independent of insulin. Western blotting showed an increase in GLUT4 expression in the membrane induced by rubiscolin-6 in a dose-dependent manner, which was consistent with previous reports [39,40]. It was abolished by the pretreatment with opioid antagonists,  $G\alpha_{q/11}$  blocker, PLC inhibitor, and AMPK inhibitor, respectively. Therefore, rubiscolin-6 enhances glucose uptake due to an increased GLUT4 translocation in cells. Similarly, rubiscolin-6 noticeably induces same effects in animal models lacking insulin. Chronic administration of rubiscolin-6 markedly decreased the blood glucose in STZ-induced diabetic rats but not in control rats. Moreover, rubiscolin-6 also normalized the feeding behaviors in STZ-induced diabetic rats. The glucose lowering action of rubiscolin-6 in vivo was induced in the same manner as that in cells. All in all, our results showed a new finding that rubiscolin-6 can increase glucose uptake in both in vivo and in vitro through the activation of opioid receptor and modulate the GLUT4 translocation in skeletal muscle.

Some limitations remained in the present study. Such as the interaction between DOR and MOR for the reduction of rubiscolin-6-increased glucose uptake effect remaining unclear in both L6 and C2C12 cells. The effect of rubiscolin-6 on glucose uptake in the presence of insulin was not analyzed in the present study. Moreover, a type-2-like diabetes model was not used in our animal experiment. Further investigations are still required to evaluate the pharmacokinetics and pharmacodynamics of rubiscolin-6 in the future.

## 5. Conclusion

Rubiscolin-6, an opioid peptide derived from the plant Rubisco, has been demonstrated to enhance glucose uptake mainly via an activation of AMPK leading to the translocation of GLUT4 in skeletal muscle, independent of insulin. Additionally, opioid receptor-coupled  $G\alpha_{q/11}$  linked to PLC is identified as the upstream pathway. Therefore, rubiscolin-6 could be beneficial in impaired glucose uptake conditions/diseases including diabetes mellitus.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jfda.2018.06.012>.

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