Saffron (Crocus sativus L.) increases glucose uptake and insulin sensitivity in muscle cells via multipathway mechanisms

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ABSTRACT

Saffron (Crocus sativus Linn.) has been an important subject of research in the past two decades because of its various biological properties, including anti-cancer, anti-inflammatory, and anti-atherosclerotic activities. On the other hand, the molecular bases of its actions have been scarcely understood. Here, we elucidated the mechanism of the hypoglycemic actions of saffron through investigating its signaling pathways associated with glucose metabolism in C2C12 skeletal muscle cells. Saffron strongly enhanced glucose uptake and the phosphorylation of AMPK (AMP-activated protein kinase)/ACC (acetyl-CoA carboxylase) and MAPKs (mitogen-activated protein kinases), but not PI 3-kinase (Phosphatidylinositol 3-kinase)/Akt. Interestingly, the co-treatment of saffron and insulin further improved the insulin sensitivity via both insulin-independent (AMPK/ACC and MAPKs) and insulin-dependent (PI 3-kinase/Akt and mTOR) pathways. It also suggested that there is a crosstalk between the two signaling pathways of glucose metabolism in skeletal muscle cells. These results could be confirmed from the findings of GLUT4 translocation. Taken together, AMPK plays a major role in the effects of saffron on glucose uptake and insulin sensitivity in skeletal muscle cells. Our study provides important insights for the possible mechanism of action of saffron and its potential as a therapeutic agent in diabetic patients.

1. Introduction

Natural compounds with insulin-like activity (insulin mimetics) have been proposed as potential therapeutic agents in the prevention and/or treatment of diabetes (Lee et al., 2006; Pinet et al., 2008). They would act by promoting glucose transport and glucose metabolism (Alberti, Zimmet, & Shaw, 2006). As the incidence and prevalence of diabetes increase worldwide, there is a considerable demand for therapeutic reagents which have a high efficacy and low side effect profile. Therefore, screening for novel insulin mimetics from various natural sources, such as plants and herbs, are still attractive and some of them might be safely used on diabetes without causing serious side effects.

Saffron, the dry stigmas of the plant Crocus sativus Linn., belong to the Iridaceae family has been used as an important dietary ingredient in various parts of the world since ancient times. Furthermore, saffron is one of the highest priced and the most used spices around the world for flavoring and coloring food (Sampathu, Shivashankar, Lewis, & Wood, 1984). It has also been applied in traditional medicine in the treatment of cramps, asthma and bronchospasms, menstruation disorders, liver disease and pain (Schmidt, Betti, & Hensel, 2007). Among the estimated more than 150 volatile and several nonvolatile compounds of saffron, major biologically active compounds are crocin, picocrocin, crocetin, carotene and safranal (Rios, Recio, Giner, & Manez, 1996). Good quality saffron contains about 30% crocin, 5–15% picocrocin, and usually up to 2.5% volatile compounds including safranal (Schmidt et al., 2007). The major constituents of saffron have attracted a considerable amount of interest during the past two decades and shown both in vitro and in vivo to possess antioxidant, anti-cancer, anti-inflammatory, anti-atherosclerosis and memory-improving properties (Abdullaev & Espinosa-Aguirre, 2004; Abe & Saito, 2000; He et al., 2005; Hosseinzadeh & Younesi, 2002). Recently, accumulating evidence has suggested the possibility of saffron being used as a candidate drug for diabetes. The hypoglycemic effects of saffron were reported in a study involving diabetic rats (Mohajeri, Mousavi, & Doust, 2009), but the molecular mechanisms underlying this effect remain obscure.

The glucose transport in skeletal muscle is regulated by two distinct pathways including phosphatidylinositol-3 kinase (PI 3-kinase) and 5'-AMP-activated protein kinase (AMPK) (Fig. 1). The PI
3-kinase pathway includes activation of Akt leading to activation of glycogen synthesis and the other enzymes/proteins necessary for the acute metabolic effects of insulin, which promotes GLUT4 translocation from an intracellular pool to plasma membrane (Smith & Muscat, 2005). On the other hand, AMPK is a phylogenetically conserved intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism (Carling, 2004). Activation of AMPK leads to the phosphorylation and regulation of a number of downstream targets that are involved in diverse pathways, including acetyl-CoA carboxylase (ACC) in the body (Fryer & Carling, 2005). Both distinct pathways also increase the phosphorylation and activity of mitogen-activated protein kinase (MAPK) family components, which participates in the full activation of insulin-stimulated glucose uptake via GLUT4 translocation (Konrad et al., 2001).

In the present study, we showed that saffron activates AMPK/ACC and stimulate glucose uptake in C2C12 skeletal muscle cells. In addition, saffron enhanced insulin sensitivity in glucose metabolism via activation of AMPK/ACC, MAPKs, PI 3-kinase/Akt and GLUT4 translocation to the plasma membrane. We further showed that mTOR and its downstream pathway are also involved in this process. Taken together, our results provide new insights into possible mechanism of action by which saffron may contribute to the pharmacological intervention of diabetes.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco’s Modified Eagle’s Medium (DMEM), foetal bovine serum (FBS), bovine serum albumin (BSA), penicillin, streptomycin, trypsin were obtained from Gibco-BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Antibodies for Phospho-Acetyl-CoA Carboxylase (Ser79), Phospho-AMPKα (Thr172), Phospho-Akt (Ser473), Phospho-Erk (Thr202), Phospho-p38 (Thr180), Phospho-SAPK/JNK (Thr183), AMPK, Akt and GLUT4 were obtained from Cell Signaling Technology (Beverly, MA). Compound C and LY294002 were purchased from Calbiochem (Merck, Darmstadt, Germany). 2-Deoxy-D [3H] glucose was obtained from Perkin-Elmer Life Sciences (Boston, MA, USA). All other reagents used were of the purest grade available.

2.2. Plant material and extraction

The saffron was obtained from Saharkhiz Co. (Mashhad, Iran). After grinding the saffron stigmas, 2 g of dried stigma was extracted with 100 ml of methanol (80%) for 2 h in an ultrasonic bath, and then shaken (110 rpm) for 12 h at room temperature. After the extraction, the methanol was evaporated using rotary vacuum evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The final aqueous part was lyophilized and kept at −20 °C until use. The yield of extraction was around 47% (w/w).

2.3. Cell culture

C2C12 (mouse myoblasts cell line) were maintained in DMEM supplemented with 10% heat inactivated FBS and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), at 37 °C in a humidified atmosphere with 5% CO2. For differentiation into myotubes, cells were reseeded in 6-well plates (for immunoblotting), and 12-well plates (for glucose uptake) at a density of 2 × 10^4 cells/ml. After 48 h (over 80% confluence), the medium was switched to DMEM with 2% (v/v) FBS and was replaced after 2, 4 and 6 days of culture. Experiments were initiated on day 7 when myotube differentiation was complete.
2.4. Cytotoxicity assay

Cytotoxicity experiments were conducted to determine the maximal nontoxic dose of saffron. Cell morphology was assessed by using phase-contrast microscopy, and viability was evaluated by means of MTT assay (Mosmann, 1983). The cells were seeded in 24-well plates at a density of 0.5 × 10⁴ cells/well and cultured with or without saffron for 24 h. Briefly, 100 µl of MTT solution (5 mg/ml MTT in PBS) was added to each well and incubated for 3 h at 37 °C. After removing the supernatant, the formazan crystal generated was dissolved by adding 150 µl/well of dimethyl sulfoxide (DMSO) and the absorbance was detected at 540 nm using a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, USA). Relative cell viability of treatment was calculated as a percentage of vehicle-treated control ([OD of treated cells/OD of control cells] × 100).

2.5. Glucose uptake assay

Glucose uptake activity was analyzed by measuring the uptake of 2-Deoxy-D [3H] glucose in differentiated C2C12 myotubes. Cells were rinsed twice with warm PBS (37 °C), and then starved in serum free DMEM for 3 h. After saffron treatment, the cells were incubated in KRH buffer (20 mM HEPES, pH 7.4, 130 mM NaCl, 1.4 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄) containing 0.5 µCi of 2-Deoxy-D [3H] glucose for 10 min at 37 °C. The reaction was terminated by placing the plates on ice and washing twice with ice-cold PBS. The cells were lysed in 1% SDS and 400 µl of the lysate was mixed with 3.5 ml of scintillation cocktail and radioactivity was determined by scintillation counting. Non-specific uptake was determined in the presence of 20 µM cytochalasin B. The results were expressed as fold-increase.

2.6. Preparation of the cytosolic and the plasma membrane protein fractions

The subcellular fractionation of myotubes was carried out according to the method (Sato, Iemitsu, Aizawa, & Ajisaka, 2008) with slight modification. Briefly, the cells from 10-cm dishes were gently scraped in buffer A (20 mM Tris pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 0.25 M sucrose, 1 mM DTT, 50 mM NaF, 25 mM sodium pyrophosphate and 40 mM β-glycerophosphate). The resulting homogenates were clarified 400 g for 15 min. The supernatant was centrifuged at 50,000 rpm for 1 h. The resulting pellet was homogenized in buffer A and then sampled as a cytosol protein fraction. Proteins from different fractions were solubilized for 1 h at room temperature in buffer B (20 mM Tris pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2% Triton X-100, 50 mM NaF, 25 mM sodium pyrophosphate and 40 mM β-glycerophosphate). The homogenate was centrifuged, and the supernatant was spun for 1 h at 5000 rpm; it was then sampled as a plasma membrane fraction. The GLUT4 protein level was measured in both total and plasma membrane fractions. Translocation was evaluated by the difference in protein levels in the cytosol and plasma membrane fractions.

2.7. Protein extract and Western blotting

Cells were rinsed twice with ice-cold PBS, and then scraped with 100 µl of lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM NaVO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin). The plate was rocked on ice for 3 min, each sample was allowed to lyse for additional 30 min on ice with periodic vortexing. Cell debris was removed by centrifugation (22,000g, at 4 °C for 30 min) and the resulting supernatants were collected for immunoblot analyses. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay reagent (Bio-Rad, CA, USA). Proteins in cultured cell lysates (30–40 µg) were separated on 12% SDS–polyacrylamide gel, transferred to PVDF membranes (Bio-Rad, CA, USA), and subsequently subjected to immunoblot analysis using specific primary antibodies. After incubation overnight at 4 °C with the primary antibody, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. The blots were visualized by using the enhanced chemiluminescene method (ECL, Amersham Biosciences, Buckinghamshire, UK) on blue light-sensitive film (Fujiﬁlm Corporation, Tokyo, Japan). In some cases, the blotted membranes were stripped and reprobed using other antibodies. Densitometric analysis was performed with a Hewlett-Packard scanner and NIH Image software (Image J).

2.8. Statistical analysis

The results are expressed as a mean ± standard deviation (SD). A paired Student’s t-test was used to assess the significance of difference between two mean values. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Saffron stimulates glucose uptake and AMPK/ACC pathways in differentiated C2C12 cells

In order to assess the non-cytotoxic concentration of saffron, the viability of C2C12 cells was evaluated at the dose ranges between 0 and 200 µg/ml using 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) assay. Within the tested concentrations, saffron only showed negligible cytotoxicity (data not shown), and the concentrations up to 2.5 µg/ml of saffron were used in subsequent experiments. For determining the role of saffron in glucose metabolism of muscle cells, differentiated C2C12 myotube cells were treated with saffron and their glucose uptakes were measured by 2-Deoxy-D [3H] glucose uptake assay. Interestingly, the glucose uptake of myotubes was considerably stimulated by the treatment of saffron in a concentration-dependent manner with a maximal increase occurring at 2.5 µg/ml (Fig. 2A) for the present study. This result suggests that saffron may act on the proteins being associated with glucose uptake signaling pathways in muscle cells. To further elucidate the possible mechanism of action of saffron, the signaling of AMPK and ACC were examined from C2C12 myotubes that were exposed to saffron at 2.5 µg/ml for the indicated time periods (Fig. 2B and C) or alternatively at the various concentrations for 1 h (Fig. 2D and E). From this, it was found that Saffron can induce a time- and dose-dependent increase of AMPK phosphorylation in C2C12 cells. The phosphorylation of AMPK-α (Thr172) significantly increased from 1 h of saffron treatment and the phosphorylation of ACC-Ser75, which is the best-characterized downstream molecule of AMPK, also showed a similar activation response. More surprisingly, the stimulation of AMPK and ACC could be observed even at the lowest concentration (0.125 µg/ml) tested in the present study (Fig 2C and E).

3.2. Saffron-induced glucose uptake is mediated by AMPK/ACC and MAPKs pathway, but not by PI 3-kinase/Akt pathway

Akt is a serine/threonine protein kinase that leads to a translocation of insulin-sensitive glucose transporter (GLUT4) to plasma membrane via the activation of signal transduction cascade involving insulin, insulin receptor substrate (IRS) family, and PI 3-kinase (Alessi & Cohen, 1998). To determine whether the basal activity of PI 3-kinase/Akt is also involved in the effect of saffron on glucose
metabolism, we investigated the effects of saffron on PI 3-kinase/Akt pathway in comparison with those of insulin. As shown in Fig. 3, Insulin caused a robust phosphorylation of Akt, while saffron treatment had no stimulatory effect on Akt. These results indicate that mechanism of action underlying the saffron-induced glucose uptake in muscle cells is linked to the activation of AMPK/ACC but not to the PI 3-kinase/Akt pathway. We next corroborated the roles of AMPK/ACC and MAPKs in the saffron-induced glucose metabolism of muscle cells. For this, the effect of saffron on AMPK/ACC and MAPKs pathways was investigated using the cell signal specific inhibitors (compound C, an AMPK inhibitor, and LY294002, a PI 3-kinase inhibitor). The results showed that compound C, but not LY294002, a PI 3-kinase inhibitor. The results showed that compound C, but not LY294002, potently suppressed saffron-stimulated AMPK/ACC and MAPKs phosphorylation (Fig. 4A–D). Similarly, the saffron-induced glucose uptake, from 2-deoxyglucose uptake assay, was also significantly inhibited in muscle cells by the pretreatment of compound C, but not by LY294002 (Fig. 4E). From these findings, it could be concluded that saffron by itself can induce glucose uptake in skeletal muscle cells via the mechanisms of AMPK/ACC and MAPKs, but not through PI-3K/Akt signaling pathway.

Fig. 3. Effect of saffron on insulin signaling pathway in C2C12 cells. Differentiated C2C12 cells were treated with either 2.5 μg/ml of saffron for different time periods (5, 15, 30 and 60 min) or 100 nM of insulin for 30 min. Protein extracts were prepared and subjected to Western blot assay using the primary antibodies for phospho-Akt (Ser473). GAPDH protein levels were used as a control for equal loading. The results are representative of three independent experiments.

3.3. Saffron improves insulin sensitivity via activation of AMPK/ACC, MAPKs, PI 3-kinase/Akt and mTOR pathway

Insulin has a major regulatory function for glucose metabolism in liver, adipocyte and skeletal muscle by redistributing GLUT4
from intracellular vesicles to the cell surface. Hence, it was utmost important for the present study to investigate the interaction between saffron and insulin on glucose metabolism-associated cell signaling. We first attempted to study whether or not saffron has an insulin-sensitizing potential. To this end, C2C12 cells were treated with 100 nM insulin in the presence or the absence of saffron. The treatment of saffron alone did increase both AMPK/ACC and MAPKs phosphorylations without any effect on Akt (Fig. 4). Surprisingly, when saffron and insulin were co-treated to C2C12 cells, the phosphorylation levels of AMPK/ACC, MAPKs and Akt were further increased to the levels, which were much higher than the individual effects of each reagent (Fig. 5A–D). To further develop this concept, we performed a GLUT4 Western blotting for the cytosol and membrane fractions of the C2C12 cells. The co-treatment of saffron and insulin further increased the translocation of and the plasma membrane abundance of GLUT4, without much changing GLUT4 content in the cytosol (Fig. 5E and F). On the other hand, the co-treatment of saffron and insulin slightly inhibited the phosphorylations of mammalian target of rapamycin (mTOR) and its downstream targets (p70S6K and 4EBP1) (Fig. 6). Insulin-stimulation of PI 3-kinase/Akt is a key step in insulin signaling mechanism and negatively regulated by mTOR pathway (Tzatsos & Kandror, 2012).
2006), which also inhibited the activation of AMPK (Inoki, Zhu, & Guan, 2003). These results suggest that saffron can increase the sensitivity of muscle cells to insulin via the glucose metabolism-associated cell signaling pathways, such as AMPK/ACC, MAPKs, PI-3/Akt pathway, and mTOR pathway.

The present findings were further confirmed by investigating the effects of saffron and insulin co-treatment in the presence or absence of pharmacological inhibitors for AMPK or PI 3-kinase/Akt pathway. Saffron-enhanced insulin sensitivity on AMPK/ACC, JNK and p38 were almost completely obliterated by compound C (an AMPK inhibitor), but mostly not by LY294002 (a PI 3-kinase inhibitor) (Fig. 7A–D). On the other hand, the activation of Akt under the co-treatment was significantly modulated only by LY294002 but not by compound C (Fig. 7A and B). In contrary to other MAPks, the Erk stimulation was only slightly attenuated by compound C but not by LY294002 (Fig. 7C and D). Consistent with other results, the induction of glucose uptake could be effectively attenuated by either compound C or LY294002 (Fig. 7E). These results provide another evidence for a crosstalk between AMPK/ACC and MAPks in the glucose metabolism, which eventually leads to activation of PI-3/Akt signal pathway.

4. Discussion

Diabetes mellitus is a chronic metabolic disorder affecting about 6% of population worldwide. Chronic hyperglycemia is a main contributor of the associated disorders, which can exacerbate defective glucose disposal by interfering with insulin action in insulin-target tissues such as skeletal muscle, liver and adipose tis-
dose-dependent manner in differentiated C2C12 cells (Fig. 2A), indi-
cating its possible regulatory role in the glucose metabolism of skeletal muscle cells. The activation of insulin stimulated glucose uptake occurs independently of insulin, and promotes the translocation of GLUT4 from an intracellular pool to the cell surface, which in turn can lead to the improvement of insulin stimulated glucose uptake (Zheng et al., 2001). The GLUT4 protein levels in skeletal muscle cells showed that saffron can increase insulin sensitivity by promoting the translocation of GLUT4 protein to the plasma membrane (Fig 5E and F). In addition to the insulin signaling pathway, mammalian target of rapamycin (mTOR) was also well-known to be activated by insulin and exert the physiological feedback regulation on insulin signaling through increasing the phosphorylation of serine residue on IRS-1 (Um et al., 2004). Moreover, activation of AMPK by treatment with AI-CAR (an AMPK activator) is associated with enhanced phosphorylation of MAPKs and decreased phosphorylation of mTOR, suggesting that the repressive action of AMPK on mTOR signaling is dominant to the stimulatory action of MAPKs pathway (Williamson, Bolster, Kimball, & Jefferson, 2006a, 2006b). In the present study, saffron slightly inhibited the phosphorylations of mTOR and its downstream targets of p70S6K and 4EBP1 (Fig. 6). The augmentation of insulin sensitivity by saffron was further confirmed using compound C and LY294002, which abrogated saffron-induced glucose uptake and AMPK/ACC, MAPKs and PI 3-kinase/Akt activation (Fig. 7). Therefore, it can be concluded that saffron enhances insulin sensitivity via multiple mechanisms. Consistent with our study, recent evidences provide that insulin-like natural compounds can improve insulin sensitivity by activating AMPK and PI 3-kinase/Akt pathways (Huang et al., 2010; Kang & Kim, 2010; Li, Ge, Zheng, & Zhang, 2008).

In conclusion, saffron plays a beneficial role in glucose metabolism of differentiated C2C12 skeletal muscle cells. The activation of

**Fig. 6.** Inhibition of mTOR pathway is involved in the effect of saffron on insulin signaling. Differentiated C2C12 cells were treated with saffron (2.5 μg/ml) for 1 h in the presence of absence of insulin (100 nM). Protein extracts were prepared and subjected to Western blot assay (A) using the primary antibodies for phospho-mTOR, phospho-p70S6K (Thr423), and phospho-4EBP1 (Thr37/46), then they were quantified (B). GAPDH protein levels were used as a control for equal loading. The results are expressed as mean ± S.D. for three independent experiments. *P < 0.05, **P < 0.01, as compared with the control value.

sue (Schenk, Saberi, & Olefsky, 2008). Among the insulin-target tissues, skeletal muscle is responsible for more than 75% of glucose disposal in response to insulin in the post-prandial state (Defronzo et al., 1981). Regulation of glucose metabolism of skeletal muscle is therefore quantitatively most important in energy balance, and is the primary tissue of insulin-stimulated glucose uptake, disposal and storage (Kang et al., 2010). It demonstrated that a brown alga of polyphenol-rich, Ecklonia cava possesses insulinoimmitic properties, since it decrease hyperglycemia in streptozotocin-diabetic rats. Furthermore, it stimulates glucose uptake in skeletal muscle cell line mediated by the activation of both AMPK and PI 3-kinase/Akt pathways. From the reports on the potential effectiveness of traditional remedies against diabetes, it is believed that the natural products may play a major role in the management of diabetes (Srinivasan, 2005).

The principal finding of present study is that saffron stimulates glucose uptake and increase insulin sensitivity in skeletal muscle cells via multiple mechanisms. These findings indicate that the hypoglycemic effect of saffron is attributable to the metabolic activity of saffron in skeletal muscle. Results of the glucose uptake assay demonstrated that saffron can enhance glucose uptake in a dose-dependent manner in differentiated C2C12 cells, (Fig 2A), indicating its possible regulatory role in the glucose metabolism of skeletal muscle cells. In an effort to understand the signaling pathways involved in saffron-mediated glucose uptake, we carried out Western blot analyses using specific antibodies for the signaling molecules associated with glucose metabolism. Saffron was shown to activate AMPK in a time- (Fig. 2B and C) and dose- (Fig. 2D and E) dependent manner. Accordingly, ACC-Ser79, the best-characterized phosphorylation site by AMPK, was also significantly phosphorylated by the treatment of saffron. In insulin signaling pathway, PI 3-kinase is a key molecule, and its main downstream target is Akt, that is important in insulin-stimulated glucose transport and metabolism (Ueki et al., 1998). Concurrently, we examine whether PI 3-kinase/Akt pathway is involved in this stimulatory effect of saffron. From this, insulin caused a robust phosphorylation of Akt while saffron treatment had no significant effect (Fig. 3), suggesting saffron alone does not stimulate PI 3-kinase/Akt pathway.

Recent studies have demonstrated a correlation between the AMPK and MAPKs signaling pathways; for example, p38 MAPK activation was shown to have been completely abolished in skeletal muscle cells expressing the dominant-negative AMPK mutant (Kim et al., 2010; Pelletier, Joly, Prentki, & Codere, 2005). Therefore, MAPKs is likely a downstream molecule of AMPK and a possible target in glucose metabolism. In order to confirm the relationship between AMPK and MAPKs in the skeletal muscle cells, the C2C12 cells were pre-incubated with either compound C or LY294002. Our results demonstrated that compound C potently attenuated the phosphorylation of AMPK/ACC, MAPKs and glucose uptake stimulated by saffron, whereas LY294002 did not significantly inhibit saffron-induced activation of glucose metabolism (Fig. 4). These facts revealed that AMPK/ACC and MAPKs could be the key factor in saffron-activated glucose uptake.

Several findings indicated there is a crosstalk between AMPK and insulin signaling pathways (Kang & Kim, 2010; Kovacic et al., 2003). Consistent with these previous reports, when the cells were exposed to saffron and insulin simultaneously, both of the AMPK and PI 3-kinase/Akt pathways were more greatly stimulated compared with those of their individual treatments (Fig. 5A and B). Concurrently, the MAPKs were also further activated by the co-treatment of saffron and insulin from those observed by the each treatment (Fig. 5C and D), which is probably due to the internalization of insulin signaling is also required to phosphorylate and activate the MAPKs pathway (Montagut et al., 2010). Additionally, the activation of AMPK on glucose uptake occurs independently of insulin, and promotes the translocation of GLUT4 from an intracellular pool to the cell surface, which in turn can lead to the improvement of insulin stimulated glucose uptake (Zheng et al., 2001).
AMPK/ACC and MAPKs pathways is directly associated with saffron-induced glucose uptake. Saffron also increases insulin sensitivity which is coupled to basal glucose translocation of GLUT4 through both of insulin-independent (AMPK/ACC and MAPKs) and insulin-dependent (PI 3-kinase/Akt and mTOR) pathways. These findings support a therapeutic potential of saffron in the treatment of diabetes and its complications.

Fig. 7. Effect of saffron on insulin sensitivity is down-regulated by specific inhibitors. Differentiated C2C12 cells were pretreated with either compound C (20 μM) or LY294002 (25 μM) for 30 min, then treated with saffron (2.5 μg/ml) for 1 h in the presence or absence of insulin (100 nM). (A–D) Protein extracts of cytosol and/or subcellular membrane fractions were prepared and subjected to Western blot assay (A and B) using the primary antibodies for phospho-acetyl-CoA carboxylase (Ser79), phospho-AMPKα (Thr172), phospho-Akt (Ser473), phospho-JNK, phospho-p38, and phospho-ERK, then they were quantified (B and D). (E) Glucose uptake was measured as described in Materials and methods. GAPDH protein levels were used as a control for equal loading. The results are expressed as mean ± S.D. for three independent experiments, *P < 0.05, **P < 0.01, as compared with the control value.

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