Biochimica et Biophysica Acta 1850 (2015) 51–61

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

The mechanism of action of ursolic acid as insulin secretagogue and insulinomimetic is mediated by cross-talk between calcium and kinases to regulate glucose balance

Allisson Jhonatan Gomes Castro [a], Marisa Jádna Silva Frederico [a], Luisa Helena Cazarolli [b], Camila Pires Mendes [a], Lizandra Czermainski Bretanha [c], Éder Carlos Schmidt [d], Zenilda Laurita Bouzon [d], Veronica Aiceles de Medeiros Pinto [e], Cristiane da Fonte Ramos [e], Moacir Geraldo Pizzolatti [c], Fátima Regina Mena Barreto Silva [a,*]

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[b] Universidade Federal da Fronteira Sul, Campus Universitário Laranjeiras do Sul, Laranjeiras do Sul, PR, Brazil
[c] Departamento de Química, Centro de Ciências Físicas e Matemáticas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[d] Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[e] Departamento de Anatomia, Universidade Estadual do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 23 September 2014
Accepted 3 October 2014
Available online 13 October 2014

Keywords:
Calcium
Secretagogue
GLUT4
Hyperglycemia
Insulin
Triterpene

ABSTRACT

Background: The effect of *in vivo* treatment with ursolic acid (UA) on glycemia in hyperglycemic rats and its mechanism of action on muscle were studied.
Methods: The UA effects on glycemia, glycogen, LDH, calcium and on insulin levels were evaluated after glucose tolerance curve. The β-cells were evaluated through the transmission electron microscopy. UA mechanism of action was studied on muscles through the glucose uptake with/without specific insulin signaling inhibitors. The nuclear effect of UA and the GLUT4 expression on muscle were studied using thymidine, GLUT4 immunocontent, immunofluorescence and RT-PCR.
Results: UA presented a potent antihyperglycemic effect, increased insulin vesicle translocation, insulin secretion and augmented glycogen content. Also, UA stimulates the glucose uptake through the involvement of the classical insulin signaling related to the GLUT4 translocation to the plasma membrane as well as the GLUT4 synthesis. These were characterized by increasing the GLUT4 mRNA expression, the activation of DNA transcription, the expression of GLUT4 and its presence at plasma membrane. Also, the modulation of calcium, phospholipase C, protein kinase C and PKCaM II is mandatory for the full stimulatory effect of UA on glucose uptake. UA did not change the serum LDH and serum calcium balance.
Conclusions: The antihyperglycemic role of UA is mediated through insulin secretion and insulinomimetic effect on glucose uptake, synthesis and translocation of GLUT4 by a mechanism of cross-talk between calcium and protein kinases.
General significance: UA is a potential anti-diabetic agent with pharmacological properties for insulin resistance and diabetes therapy.

1. Introduction

D-Glucose is the major fuel for most cells and its homeostasis requires an integrated control by the whole organism. Insulin is, by far, the chief regulating element of glycemia and is secreted from pancreatic β-cells induced by glucose [1]. Through binding to its receptors in different tissues, insulin activates signaling pathways that involve a complex cascade of protein kinases and regulatory proteins that conduce to the metabolic effects of insulin [2].

Skeletal muscle composes a large percentage of total body mass and is the major site for insulin-dependent glucose disposal. In this tissue, insulin induces a redistribution of glucose transporter protein (GLUT4) from the cell interior to the surface of plasma membrane, which ultimately leads to a greater glucose uptake rate in the muscle cells [1]. The increase in GLUT4 exocytosis in response to insulin is triggered through signaling *via* the insulin receptor that induces the downstream activation of signaling pathways such as PI3K/AKT/PKB, PKCs and CAP/CBL/TC10 [2]. Also, insulin mediates a wide spectrum of biological responses acting through the MAPK pathways, including activation of

* Corresponding author at: Departamento de Bioquímica, Centro de Ciências Biológicas, UFSC. Campus Universitário, Bairro Trindade, Cx Postal 5069, CEP: 88040-970, Florianópolis, SC, Brazil. Tel.: +55 48 3721 69 12; fax: +55 48 3721 96 72.
E-mail address: mena.barreto@ufsc.br (F.R.M.B. Silva).

http://dx.doi.org/10.1016/j.bbagen.2014.10.001
0304-4165/

the transcription of specific genes and modulation of cellular growth and differentiation [2].

The GLUT4 translocation and glucose uptake can also be activated by muscle contraction through signaling pathways that cross talk with the classical insulin regulatory mechanisms of glucose uptake. Muscle contraction involves increase in the intracellular calcium levels which regulate several signaling pathways and metabolic events. The increase in myocellular calcium concentrations has been proposed to be a signal in the initiation of contraction-stimulated glucose transport and GLUT4 translocation. In addition, one or more of the calcium regulated intracellular proteins may be involved in these processes and potential candidates include calmodulin and the calmodulin-dependent protein kinase II (CaMKII). Ca^{2+}–calmodulin-dependent kinase II (CaMKII) has been proposed to act as an intermediate component of the contraction as well as insulin-induced signaling cascades that result in the translocation of GLUT4 vesicles to the plasma membrane, increased GLUT4 expression and increased glucose uptake in the skeletal muscle [3].

Several investigations have demonstrated that natural compounds can potentiate insulin release and/or stimulate GLUT4 translocation and glucose uptake [4–7]. The pentacyclic triterpenes are isoprenoids derived from plant secondary metabolism and represent the major constituents of several medicinal plants [8]. Ursolic acid (3-beta-hydroxyurs-12-en-28-oic acid; UA), the most representative ursane-type triterpenoid is being described as an anti-inflammatory, hepatoprotective, antitumoral and antidiabetic agent [9]. It is known that ursolic acid has significant action in glucose homeostasis, reduces blood glucose levels in diabetic individuals, and improves the glucose and insulin tolerance, increases insulin synthesis and release from pancreas and inhibits protein glycation [10]. However, the mechanism by which the UA regulates glucose uptake at the level of the main tissues responsible for the balance of blood glucose, such as skeletal muscle and adipose tissue, is not fully described. Therefore, the aim of the present study was to investigate the role of UA on glucose homeostasis and the calcium involvement on the mechanism of action of this triterpene on glucose uptake in soleus muscle.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059), colchicine, wortmannin, KN93, cycloheximide, mouse anti-β-actin, acrylamide and bis-acrylamide, mouse anti-β-actin, and anti-mouse conjugated to Cy3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). GLUT4 (sc-53566) antibody was obtained from Santa Cruz Biotechnology (California, USA). The peroxidase conjugated goat anti-mouse IgG, the Immobilon™ Western chemiluminescent horseradish peroxidase (HRP) substrate and the enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of rat insulin (catalogue no. EZRMI-13K) were obtained from Millipore (St Charles, MO; Temecula, California, USA). [U-^{14}C]-2-deoxy-D-glucose (^{14}C-DG), specific activity 9.25 GBq/mmol, thymidine [methyl-^{14}C], specific activity 1.7464 GBq/mmol and biodegradable liquid scintillation fluid were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). Chlorhydrate of lidocaine was purchased from Neo Quimica (Anápolis, Goiás, Brazil). All other chemicals were of analytical grade.

2.2. Isolation of UA and chemical characterization

Leaves of *Rosmarinus officinalis* (1500 g) purchased from Quimer Natural Products Industry were submitted to maceration in ethanol (96%) for fifteen days at room temperature (25 ± 2 °C). Thereafter, the extract was filtered and then concentrated under reduced pressure at approximately 60 °C. This procedure was repeated three times. After removing the solvent, the ethanol extract was dried by liophilization to yielding 153 g of a greenish brown solid. The crude extract was subjected to passage on a short silica gel 60 (Vetec-63-230 mesh) column with hexane, ethyl acetate and ethanol in order of polarity, to give three fractions: hexane (HEX), ethyl acetate (EtOAc) and ethanol (EtOH). Part of the EtOAc fraction (8.83 g) was submitted to the silica gel column eluted with hexane–ethyl acetate gradient in increasing polarity, to give 33 fractions. Fractions 20–33 eluted with hexane–acetone solution (8:2 v/v) were combined and purified by flash chromatography (silica gel 60 column; Vetec-230–400 mesh) with isocratic elution (hexane: acetone 4:1 v/v) to give ursolic acid (87 mg). Ursolic acid was identified by analysis of IR and NMR data and comparison with literature [11].

2.3. Experimental animals

Male albino Wistar rats, 50–55 days old (180–210 g), were used. Rats were bred in the animal facility and housed in an air-conditioned room (approximately 21 ± 2 °C) with controlled lighting (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available *ad libitum*. Fasted rats were deprived of food for 16 h but allowed free access to water. All the animals were carefully monitored and maintained in accordance with the local Ethical Committee for Animal Use (Protocol CEUA-UFSC PP00414).

2.4. Oral glucose tolerance curve

Fasted rats were divided into three groups of six animals. Group I, hyperglycemic rats that received glucose (4 g/kg; 8.9 M); Group II, hyperglycemic rats that received the vehicle, 2.5% Tween 80; and Group III, hyperglycemic rats that received the UA (0.1, 1 and 10 mg/kg). Glycemia was measured before any treatment (zero time). Immediately, rats received the treatment (vehicle or UA) and after 30 min they were overloaded with glucose. The glucose tolerance curve was initiated just after the glucose overload and then glycemia was measured at 15, 30, 60 and 180 min. All treatments were administered by oral gavage. A local anesthesia (chlorhydrate of lidocaine) was used and blood was collected from the tail vein to determine glycemia by the glucose oxidase method [12] and the serum insulin levels.

2.5. Insulin serum measurements

The insulin levels were measured by ELISA according to the manufacturer's instructions. The range of values detected by this assay was 0.2–10 ng/mL. The intra- and inter-assay coefficients of variation for insulin were 3.22 and 6.95, respectively, with a sensitivity value of 0.2 ng/mL. Insulin levels were estimated by means of colorimetric measurement at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) through interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng of insulin serum mL^{-1} [13].

2.6. Glycogen content measurements

The soleus muscle was harvested from hyperglycemic rats and hyperglycemic rats treated with UA (0.1, 1 and 10 mg/kg) and the glycogen content was determined after 180 min of glucose overload (4 g/kg). The glycogen was isolated from the tissue as described by [14] and the results were expressed as mg of glycogen/g of tissue [15].

2.7. Studies on ^{14}C-glucose uptake in rat soleus muscle

For the [U-^{14}C]-2-deoxy-D-glucose (^{14}C-DG) uptake, the muscles of euglycemic fasted rats were used. Slices of muscle were distributed (alternately left and right) between the basal and treated groups. The muscles were dissected, pre-incubated (30 min) and then incubated

(60 min) at 37 °C in Krebs Ringer-bicarbonate (KRb) buffer with a composition of 3 mM glucose, 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, and 25 mM $NaHCO_3$ and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4. UA (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-12} M) was added to the pre-incubation (30 min) and incubation medium (60 min) in the presence or absence of 100 μM HNMPA-$(AM)_3$, 100 nM wortmannin, 1 μM colchicine, 2 mM EGTA, 50 μM BAPTA-AM, 1 μM nifedipine, 1 μM flunarizine, 1 μM U73122, 10 μM KN93, 40 μM RO 318220, 350 μM cycloheximide or 50 μM PD 98059. ^{14}C-DG (0.1 μCi/mL; 0.12 nM) was added to each sample during the incubation. Samples were processed according to [16]. For total protein quantification the method of Lowry et al. [17] was used. The glucose uptake results were expressed as nmol glucose units/mg of protein.

2.8. Studies on thymidine incorporation into DNA

For the [methyl-^{14}C]-thymidine incorporation into DNA, slices of soleus muscle were distributed (alternately left and right) between control and treated groups. The muscles were dissected, and pre-incubated (30 min) and incubated (60 min) at 37 °C in KRb buffer with O_2/CO_2 (95%:5%, v/v), pH 7.4. UA (1 nM) was added to the pre-incubation (30 min) and incubation (60 min) medium. ^{14}C-thymidine (0.5 μCi/mL) was added into all samples during the incubation period. After incubation, muscles were rinsed in cold KRb and processed according to [15]. The results were expressed as cpm/μg of protein.

2.9. Polyacrylamide gel electrophoresis and immunoblotting analysis

For the whole muscle homogenates, soleus muscles were incubated (105 min) with/without 1 nM UA and in the presence of 10 μM KN93, homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl pH 6.8, 4% (w/v) SDS and the total protein concentration determined. For the electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations were loaded onto 12% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli [18] and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were incubated for 2 h in blocking solution (TBS; 0.5 M NaCl, 20 mM Trizma, plus 5% defatted dried milk) and then incubated overnight at 4 °C with anti-GLUT4 diluted to a ratio of 1:500. Membranes were incubated for 2 h with anti-rabbit IgG (1:1000) and immunoreactive bands were visualized using the Immobilon™ Western chemiluminescence HRP substrate kit [19]. Autoradiograms were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining the optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

2.10. Immunofluorescence analysis

Soleus muscles of *in vitro* incubation (105 min) with/without 1 nM UA or *in vivo* treatment (harvested after 180 min of oral treatment with 1 mg/kg UA) were fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin, as described by [20]. Representative blocks of paraffin-embedded tissues were cut into 5 μm lengths, deparaffinized, rehydrated and placed in 10 mM citrate buffer for 8 min at 37 °C for antigen retrieval. Sections were submitted to a block solution of 3% BSA and 1% Igepal in PBS for 1 h at 37 °C and incubated overnight at 7 °C with mouse anti-GLUT4 (1:500) or mouse anti-β-actin (1:7500). After several washes, sections were incubated for 1 h at 37 °C with anti-mouse conjugated to Cy3 (1:500, Sigma) in the same solution and then counterstained with DAPI. Sections were then mounted in a FluorSave reagent (Calbiochem). The analysis of GLUT4 distribution in the tissue was merely qualitative (adapted from [21]).

2.11. Transmission electron microscopy (TEM)

For the observation under the transmission electron microscope (TEM), pieces of rat pancreas were incubated in the presence of UA (1 nM) for 5 or 20 min and were fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose [22]. The material was post-fixed with 1% osmium tetroxide for 3 h, dehydrated in a graded acetone series and embedded in Spurr's resin. Then, thin sections were stained with aqueous uranyl acetate followed by lead citrate according to [23]. Four replicates were prepared for each experimental group; two samples per replicate were examined under TEM (Jeol JEM1011 at 80 kV) at the Central Laboratory of Electron Microscopy (LCME, UFSC, Brazil).

In order to evaluate the ratio of cytoplasmic insulin vesicles per β-cell [24], an isolated islet was sectioned and the vesicles of β-cells were counted. So, the total number of vesicles was divided by the number of β-cells in the islet. Similarities based on the comparison of individual treatments with replicates suggested that the ultra-structural analyses were reliable.

2.12. Real time PCR

Total ribonucleic acid was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All RNA samples were rid of contaminating DNA by using DNA-free reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One μg of RNA was reversed transcripted with Superscript III (Invitrogen). The real time PCR amplification was performed using 2 μL of cDNA, specific primers for each gene and SyBR Green reagent (Invitrogen), in a final volume of 10 μL. The $2^{-(\Delta\Delta Ct)}$ method [25] was used to calculate the $\Delta\Delta C_t$ values. Moreover, β-actin was used as internal control. The primer sequences used are as follows: GLUT4: Forward: 5′-CGCGGCCTCCTATGAGATAC-3′; reverse: 5′-CCTGAGTAGGCGCCAA TGA-3′ β-actin; forward: 5′-TGTTACCAACTGGGACGA-3′; reverse: 5′-GGGGTGTTGAAGGTCTCA-3′

2.13. Calcium serum measurements and lactate dehydrogenase

The calcium serum levels were determined after zero, 15, 30, 60 and 180 min. For serum extracellular lactate dehydrogenase (LDH) it was measured after 180 min of oral UA treatment (0.1, 1 and 10 mg/kg). Blood samples and the serum were used to determine extracellular LDH activity. Also, the calcium levels were determined according to the manufacturer's instructions [15].

2.14. Data and statistical analysis

Data were expressed as mean ± S.E.M. One and two-way analysis of variance (ANOVA) followed by Bonferroni post-test or unpaired Student's *t*-test were used to determine whether there were significant differences between groups. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Effect of UA on glycemia and insulin secretion

Fig. 1A and B shows the structure and the serum glucose levels of hyperglycemic rats after oral treatment with UA (0.1, 1 and 10 mg/kg). This triterpene was effective in reducing glycemia in all doses tested during the period studied when compared with the hyperglycemic control group. The treatment with 0.1, 1 and 10 mg/kg showed a sustained effect from 15 to 180 min, improving significantly the glucose tolerance curve. In percentage terms, the compound reduced about 28, 27, 17 and 16% of serum glucose levels when compared to the respective time on hyperglycemic group. Also, the presence of vehicle 2.5% Tween 80 did

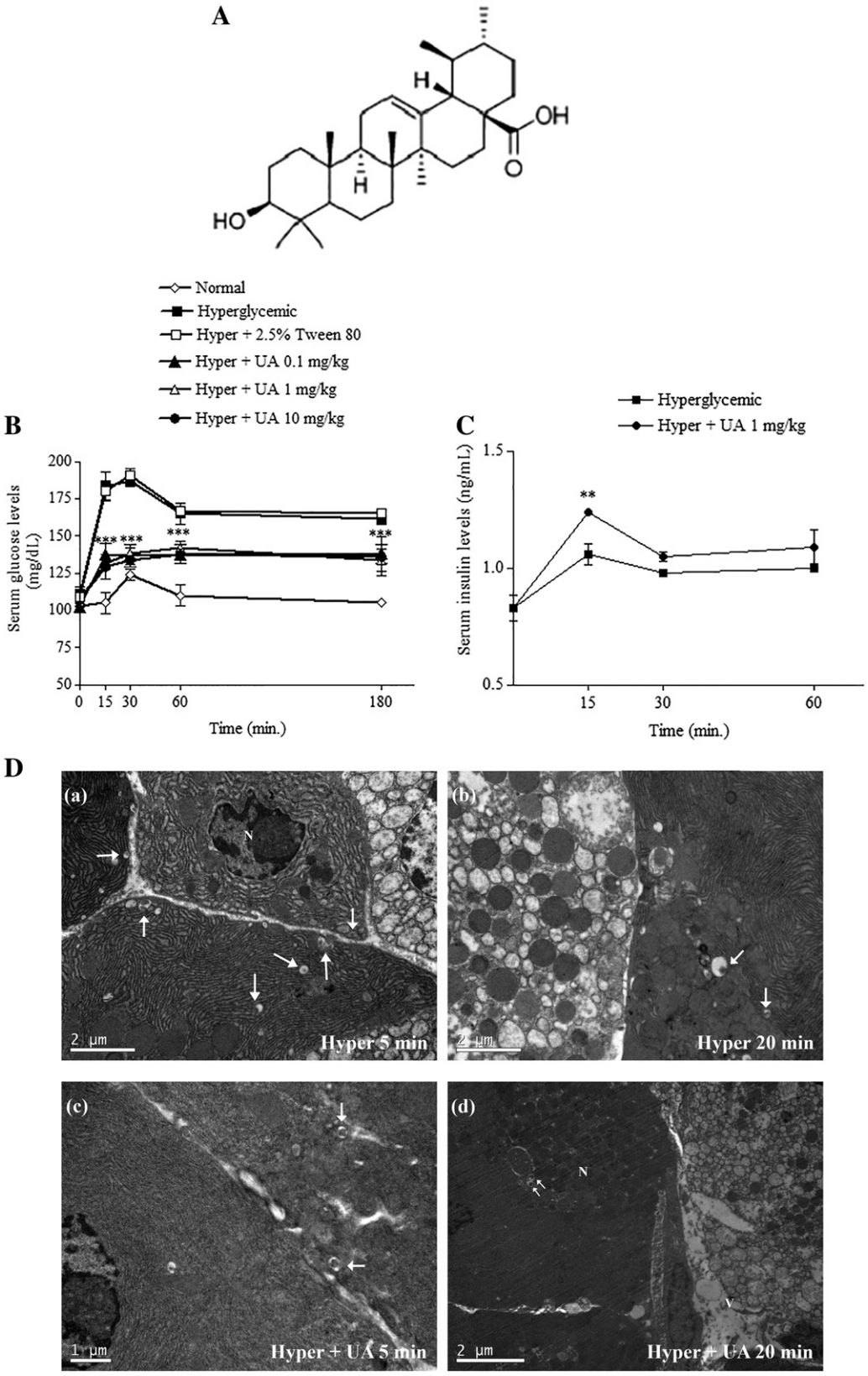

Fig. 1. UA structure (A). Effect of UA on oral glucose tolerance curve (B) and on serum insulin secretion (C) in hyperglycemic rats. Transmission electron microscopy of control and stimulated insulin granules (D). Values are expressed as mean ± S.E.M. with n = 5. Significant at $^{***}p < 0.001$ and $^{**}p < 0.01$ compared to the respective value for hyperglycemic control group.

not modify the glycemic profile over the period studied when compared to the hyperglycemic group. As it was expected, the euglycemic control group did not show a significant change on the profile of glycemia over the time studied. Taking into account the antihyperglycemic effect of UA after *in vivo* treatment, the serum insulin levels were determined in fasted rats after an oral glucose overload. As it was expected, glucose

induced-insulin secretion reinforced the classical secretagogue effect of glucose. In addition, the treatment with UA significantly potentiated the insulin secretion induced by glucose at 15 min in hyperglycemic rats (Fig. 1C). Moreover, as shown in Fig. 1D, insulin granules are present in β-cells and accumulate around the plasma membrane. Also, after pancreas incubation with UA 1 nM for 5 min, a reduction in the insulin granules of about 24% was observed when compared with the respective hyperglycemic control. Additionally, after 20 min of incubation UA was not able to modify the amount of insulin vesicles on β-cells compared with the respective hyperglycemic control group. Also, Table 1 shows the ratio of cytoplasmic insulin vesicles per β-cell from the control and treated islets as represented on Fig. 1D.

3.2. Effect of UA on muscle glycogen content

Fig. 2 shows the effect of UA after *in vivo* treatment on muscle glycogen content. The treatment of hyperglycemic rats with 0.1, 1 and 10 mg/kg UA increased significantly the glycogen content in soleus muscle 3 h after the oral treatment compared to the hyperglycemic control group. In addition, the effect of UA by oral gavage potently increased glycogen content (around 2 fold), when compared with the hyperglycemic control group.

3.3. Mechanism of action of UA on glucose uptake on soleus muscle and its effect on thymidine incorporation into DNA

Following up these studies, the *in vitro* effect of UA (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-12} M) on glucose uptake in the rat soleus muscle at 60 min of incubation was carried out. The stimulatory effect of UA on glucose uptake was significant at 100, 10, 1, 0.1 and 0.001 nM and represented around 2.0, 2.5, 3.0, 3.0 and 3.1 fold compared to the basal value at 60 min, respectively (Fig. 3). Considering that there were no statistical significant differences among the lower concentrations of UA, the concentration of 1 nM was chosen to study the mechanisms of action of this triterpene.

To determine the mechanism by which UA induces the glucose uptake in soleus muscle, the glucose uptake assay was performed with 100 nM wortmannin, a specific inhibitor of PI3K, 1 μM colchicine, a microtubule-depolymerizing agent or 100 μM of $HNMPA(AM)_3$, an inhibitor of insulin receptor tyrosine kinase activity. In all cases, the concentrations used were those previously assayed and published using similar approach [16,26]. Fig. 4 shows that the increase on glucose uptake by 1 nM UA was completely inhibited by wortmannin and colchicine pretreatment, whereas no change was observed in the presence of $HNMPA(AM)_3$. When only the inhibitors were added to the muscle samples, any significant change on glucose uptake was observed compared with the control group.

In order to characterize more precisely the metabolic effects of UA, 50 μM BAPTA-AM, an intracellular calcium chelator and 2 mM EGTA, an extracellular calcium chelator were used to investigate the calcium involvement on glucose uptake. As it can be seen on Fig. 5A, in the presence of each chelator, EGTA and BAPTA-AM, the stimulatory action of UA on glucose uptake was abrogated. Also, in order to verify the participation of T- and L-type voltage-dependent calcium channels (L-VDCC)

Table 1
The ratio of cytoplasmic insulin vesicles per β-cell in the presence of 1 nM UA (treated) or absence (control) in the first and second phases of insulin secretion. Incubation time = 5 and 20 min with/without UA. n = 29 β-cells of one islet for hyperglycemic control group at 5 min; n = 29 β-cells of one islet for hyper + UA group at 5 min; n = 16 β-cells of one islet for hyperglycemic control group at 20 min and n = 23 β-cells of one islet for hyper + UA at 20 min. Triplicates of each group were analyzed.

Time	5 min		20 min	
	Hyper	Hyper + UA	Hyper	Hyper + UA
Cytoplasmic vesicles/cell	4.03	0.96	1.62	1.65

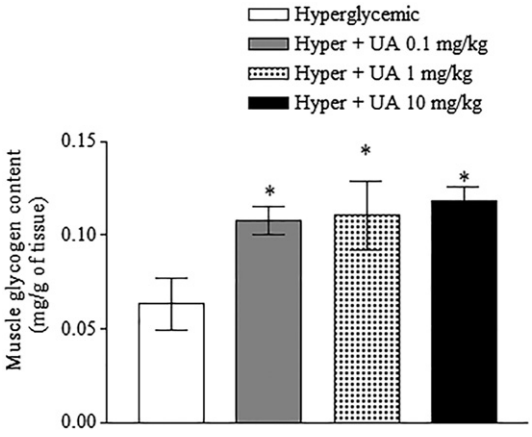

Fig. 2. Effect of UA on glycogen content from soleus muscle in hyperglycemic rats 180 min after treatment by oral gavage. Values are expressed as mean ± S.E.M. with n = 5 for each group. Statistically significant at $^{*}p < 0.05$ compared to the hyperglycemic control group.

on the mechanism involved in the UA action on glucose uptake, 1 mM flunarizine and 1 μM nifedipine were used to block these channels, respectively. The results showed that nifedipine and flunarizine completely blocked the UA-stimulated glucose uptake (Fig. 5B). Since the results indicated that the intracellular calcium levels and extracellular calcium influx through T- and L-type voltage-dependent calcium channels (L-VDCC) are involved in the glucose uptake, it was also sought to determine whether other intracellular pathways could play a role on the stimulatory effect of UA on glucose uptake. The use of 1 μM U73122, 10 μM KN93, 40 μM RO318220 allowed us to corroborate the participation of PLC, PKCaM II (a calcium-dependent kinase) and atypical protein kinase C (aPKC) in the mechanism of action of UA (Fig. 5C).

To determine whether the UA signal transduction leads to nuclear actions, such as the MAPK pathways, genic expression and/or general protein synthesis we examined the effect of PD 98059, an inhibitor of MEK/ERK pathway, and the effect of cycloheximide on glucose uptake stimulated by UA as well as the action of UA on GLUT4 mRNA expression (Fig. 6A and B). The stimulatory effect of UA was completely blocked in the presence of PD98059 while cycloheximide was not able to alter the UA-induced glucose uptake in muscle. Also, by RT-PCR, we observed that UA stimulated the increase of GLUT4 mRNA expression in this period of incubation. Moreover, the thymidine incorporation in rat soleus muscle also was increased in the presence of UA when compared to the control group (Fig. 6C). These results indicate that UA may act through the nuclear activity and the MAPK pathways, both of them are also involved in the insulin phosphorylation cascades.

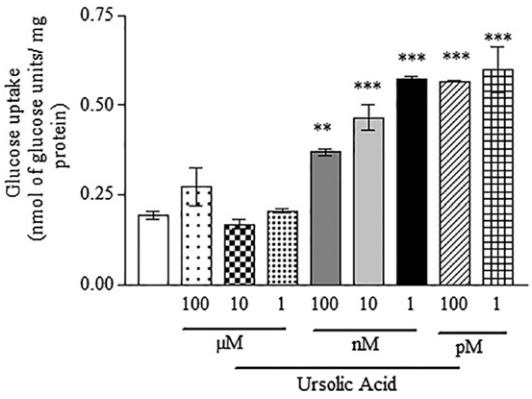

Fig. 3. Concentration–response curve of UA on [^{14}C]-glucose uptake in rat soleus muscle. Pre-incubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant at $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ in relation to control group.

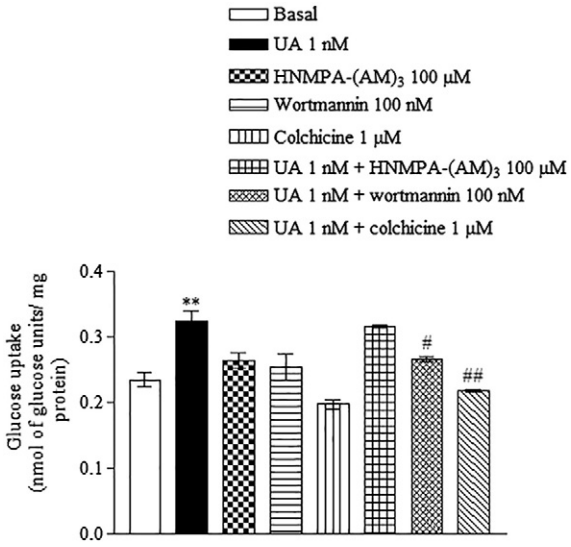

Fig. 4. Effect of HNMPA-$(AM)_3$, colchicine and wortmannin on the stimulatory action of UA on [^{14}C]-glucose uptake in rat soleus muscle. Control group = no treatment. Pre-incubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; n = 6 in duplicate for each group. Significant to $^{**}p \leq 0.01$ in relation to control group. Significant to $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ in relation to UA group.

3.4. Effect of ursolic acid on GLUT4

Fig. 7A shows the whole GLUT4 immunocontent on muscle. A significant increase in the whole GLUT4 in the presence of UA (about 30%) was observed after 105 min of *in vitro* incubation. 10 μM KN93 was not able to modify the basal GLUT4. However, in the presence of the triterpene it nullified the stimulatory effect of UA on GLUT4 immunocontent. The acute effect of the positive control, insulin, presented the most prominent effect on the GLUT4 content (increased 76%) (data not shown). In addition, the localization of GLUT4 on muscle after *in vitro* treatment (105 min) with UA (panel B) and also for UA after *in vivo* treatment (180 min (panel D)) was investigated by fluorescence (Fig. 7B). The presence of GLUT4 at plasma membrane was markedly increased by 1 nM UA at 105 min (*in vitro*) incubation as well as by 1 mg/kg UA at 180 min (*in vivo*) treatment compared with respective basal group (panel A and C). The negative control, performed by incubating the slices without primary anti-GLUT4 antibody, demonstrates GLUT4 immuno negative reaction (panel E). This qualitative analysis is in line with the effect of UA on whole GLUT4 immunocontent, reinforcing the results showed in Fig. 7A.

3.5. Effect of UA on calcium and LDH serum levels

In order to verify a potential toxicity of this compound, the LDH and calcium serum levels were evaluated after the oral treatment with UA. The doses 0.1, 1 and 10 mg/kg orally administrated were not able to change neither extracellular LDH (Fig. 8A) nor calcium serum levels (Fig. 8B), indicating that in this experimental condition the UA by oral gavage was not able to exhibit toxic effects.

4. Discussion

Ursolic acid, an ursane pentacyclic triterpene has been described to influence carbohydrate metabolism [9,27,28,10]. In the present study, the antihyperglycemic effect of UA indicated that this compound can control hyperglycemia since it significantly reduced serum glucose concentrations, potentiated the glucose effect on insulin releasing and maintained high insulin levels throughout the period studied. This is in line with the results observed by Jang et al. [9] and Jayaprakasam et al. [29] who showed significant increases on insulin levels with the preservation of pancreatic β-cells and modulation of blood glucose levels in diabetic mice and C57BL/6 obese mice after a dietary UA supplementation. Additionally, other triterpenes have also been shown to

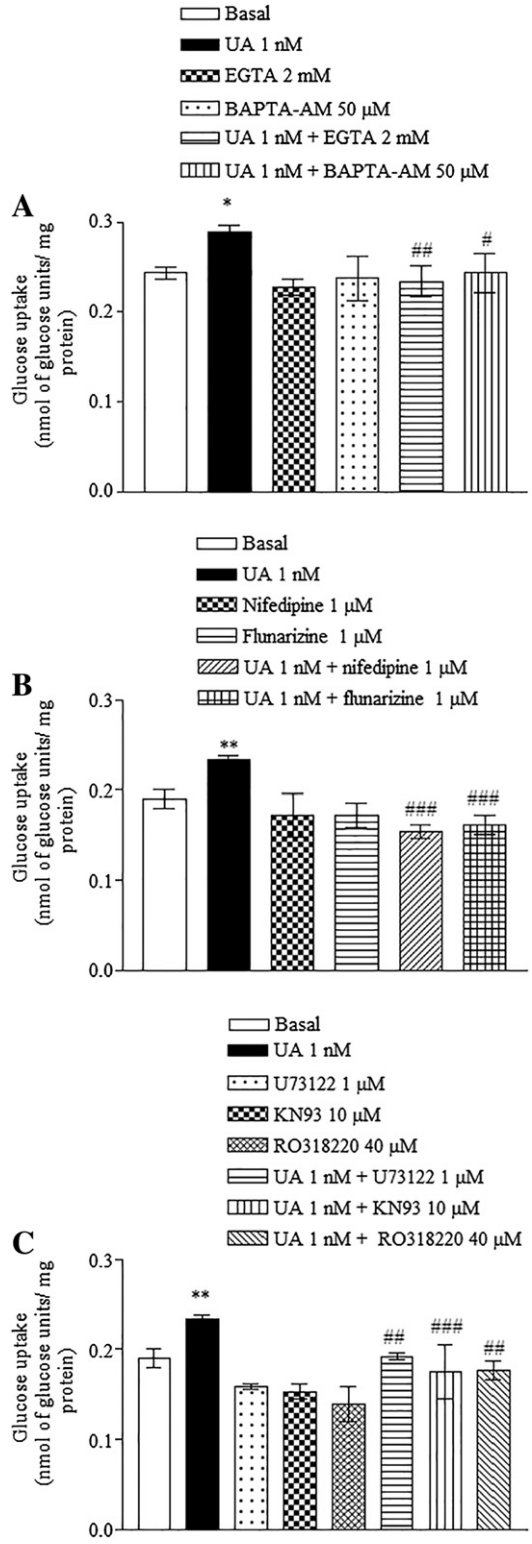

Fig. 5. Effect of EGTA, BAPTA-AM (A), nifedipine, flunarizine (B), U73122, KN93, RO318220 (C) on the stimulatory action of UA on [^{14}C]-glucose uptake in rat soleus muscle. Control group = no treatment. Pre-incubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M.; $n = 6$ in duplicate for each group. Significant to $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ in relation to basal group. Significant to $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$ in relation to UA group.

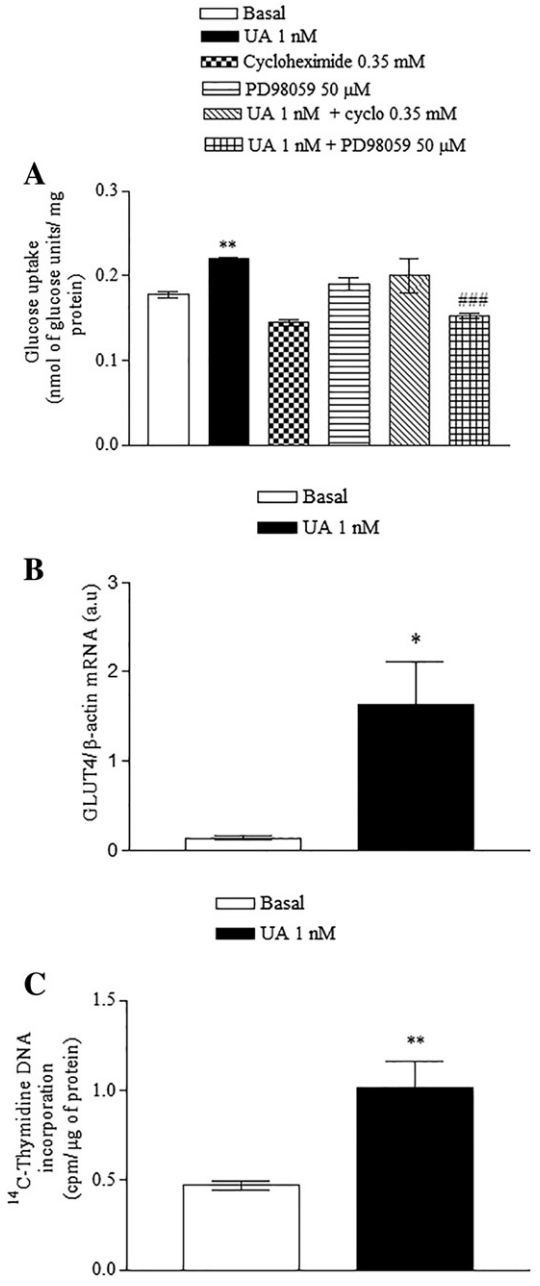

Fig. 6. Effect of cycloheximide and PD98059 on stimulatory effect of UA on ^{14}C-glucose uptake in muscle (A), effect of UA on GLUT4 mRNA expression (B) and effect of UA on ^{14}C-thymidine incorporation into soleus muscle (C). Pre-incubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± S.E.M. with n = 6 in triplicate for each group. Significant at $^{**}p < 0.01$ compared to the control group. Significant at $^{###}p < 0.001$ compared to UA group.

improve glycemia and insulin secretion such as betulinic acid, α and β-amyrin and oleanolic acid [9,30–32,15]. All together, our data suggest that UA acts by increasing the insulin secretion stored at vesicles and induces translocation of granules pointing to an immediate effect of the triterpene on the first phase of insulin secretion. It's worthwhile to mention that the doses assayed on oral gavage treatment with UA were supported by the oral bioavailability of UA nanoparticles using supercritical anti-solvent process reported by Yang et al. [33]. These authors described that after 1 h of 100 mg/kg UA by gavage around 63.7 nM was bioavailable in the plasma (very close to the dose that stimulates the glucose uptake 100 nM in our conditions). Furthermore, UA potentiates the glucose-induced insulin secretion as characterized in the profile of serum insulin levels in this approach.

In tissues such as skeletal muscle and liver, insulin promotes glucose uptake and glycogen synthesis, constituting an important mechanism of glycemic control [1]. The UA significantly increased the glycogen content in the skeletal muscle which is in line with its effect on liver glycogen content after an *in vivo* treatment [27]. As previously demonstrated, several triterpenes show to influence glycogen metabolism, especially through the inhibition of muscle glycogen phosphorylase activity or increase on AMPK and GSK-3β phosphorylation [34–38]. Therefore, the increase in muscle glycogen deposition associated with higher serum insulin levels induced by UA contributes to the serum glucose lowering observed in hyperglycemic rats.

The results presented herein imply UA as an anti-diabetic agent, however the mechanisms by which this compound ameliorates the glycemia remain to be completely elucidated. UA significantly stimulated glucose uptake in skeletal muscle and its effect was inhibited by wortmannin and colchicine pointing to an involvement of PI3K and microtubules integrity for the UA-induced glucose uptake. It has been demonstrated that the insulin induced-glucose uptake in muscle is powered through signaling *via* insulin receptor, PI3K and cytoskeleton integrity ending up to the translocation of GLUT4 and glucose uptake [2,39]. Several triterpenes and its derivatives such as UA, pachymic acid, betulinic acid, oleanolic acid and corosolic acid were demonstrated to stimulate glucose uptake through the increase of the auto-phosphorylation and/or activation of IRβ, PI3K, IRS1 and AKT, components of the classical insulin signaling. Additionally, these compounds were able to stimulate GLUT4 translocation in 3 T3-L1 adipocytes, CHO/HIR cells, skeletal muscle and in *in vivo* models [40–44,5,15]. From our data, the IR tyrosine kinase activity on the stimulatory effect of UA on glucose uptake seems not to be essential to the complete activation of glucose uptake induced by UA, studied in this experimental approach. This is in line with the results showing that UA had no effect on IRβ auto-phosphorylation and on IR tyrosine kinase activity at concentrations up to 10 μg/mL (approximately 20 μM). The significant activation of IRβ auto-phosphorylation by UA was observed just at concentrations above 50 μg/mL (approximately 0.1 μM) [41]. Although UA seemed to not directly activate the IR at low concentrations but significantly stimulated the glucose transport, it is supposed that UA can act through the inhibition of protein tyrosine phosphatases that cause the dephosphorylation of signaling molecules which helps to terminate insulin signaling [45]. Several triterpenes and their derivatives were described as potent inhibitors of the PTPases, especially, PTP1B [44,46,47]. Taking this into account, we consider that UA may act like an insulin sensitizer, to elevate insulin receptor β phosphorylation indirectly (PTP1B inhibition?) or as an insulin mimetic agent acting through the insulin cascade.

In addition to the role of the classical PI3K pathway on the stimulatory effect of UA on glucose uptake, the involvement of intracellular second messengers potentially associated with insulin pathways that enhance glucose uptake was investigated. As an important intracellular signaling molecule, calcium is necessary for muscle contraction and acts as a second messenger in insulin dependent and independent pathways that lead to glucose uptake [48,49]. In the skeletal muscle, the free cytosolic calcium levels are tightly controlled by the influx from the extracellular medium or the release from the sarcoplasmic reticulum [50]. It has been demonstrated that insulin increases intracellular Ca^{2+} levels involving both, extracellular and intracellular calcium mobilizations and this effect is positively correlated with the insulin-induced glucose uptake in muscle [51,49,52]. Also, insulin induces an increase at near-membrane calcium concentration through an L-type calcium channel dependent mechanism and the induced glucose uptake is attenuated by L-type Ca^{2+} channel blockers [53,54,51]. Our findings indicated that the UA-induced glucose uptake can result from extracellular calcium as well as from cytosolic calcium mobilization since the calcium chelation with BAPTA-AM and EGTA completely blocked its stimulatory effect on glucose uptake. This result agrees with those reported for oleanolic acid, asiatic acid and maslinic acid that increased cellular

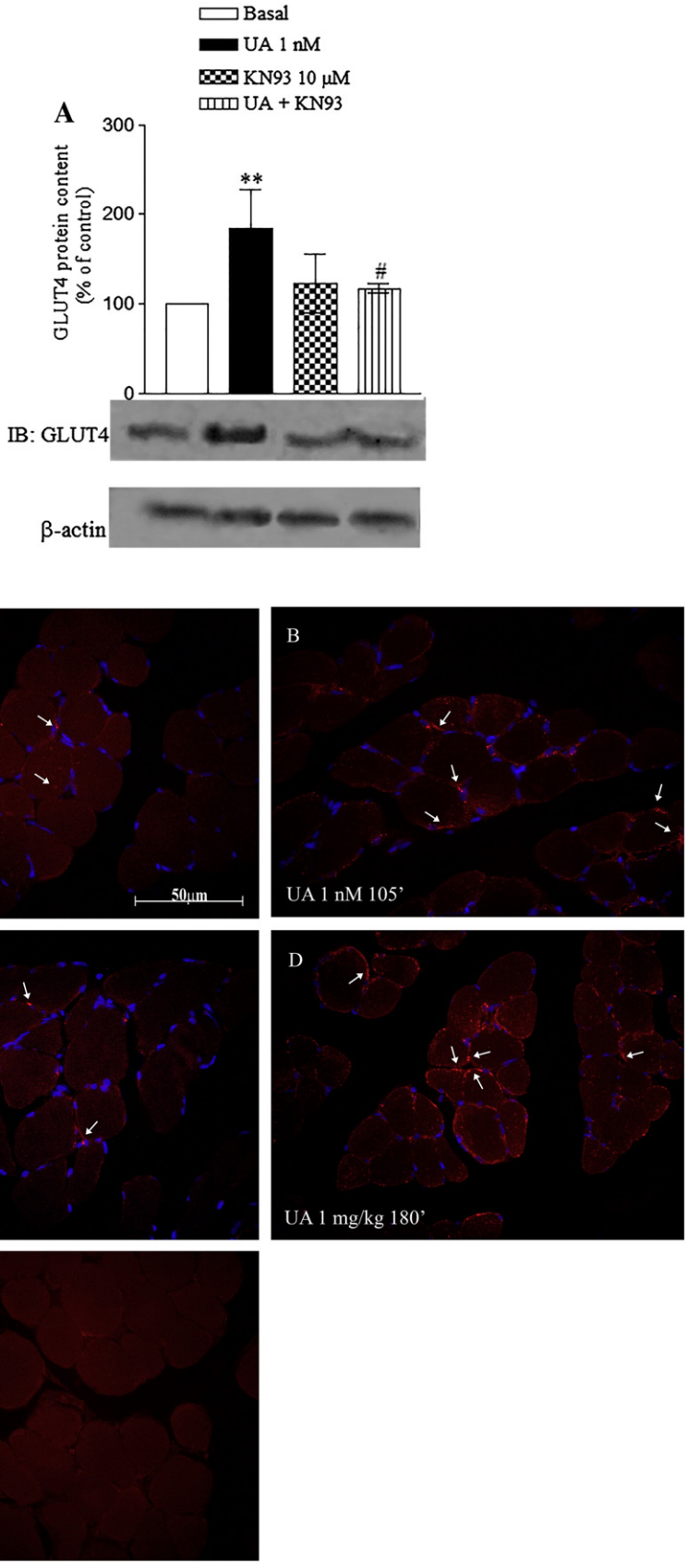

Fig. 7. Effect of UA on whole GLUT4 immunocontent (whole tissue extract) in soleus muscle. KN93 10 μM was present during incubation (105 min) medium, in the presence or absence of UA 1 nM. β-actin was used as loading control (A). Representative immunoblot is shown. Values are expressed as mean ± S.E.M. n = 4 for three independent experiments. Significant at $^{**}p < 0.001$ compared with control group. Significant at $^{\#}p < 0.05$ compared to UA group. Immunolocalization of GLUT4 on soleus muscle (B). At 105 min, *in vitro*: panel A (basal), panel B (1 nM UA). At 180 min, *in vivo* treatment: panel C (basal), panel D (1 mg/kg UA) and panel E (immunonegative reaction for GLUT4). imunocontrol-NC). GLUT4 was stained with Cy3, and immunofluorescence revealed the presence of GLUT4 at plasma membrane of muscles treated 1 nM or 1 mg/kg UA (arrows). Nuclei were stained with DAPI (blue).

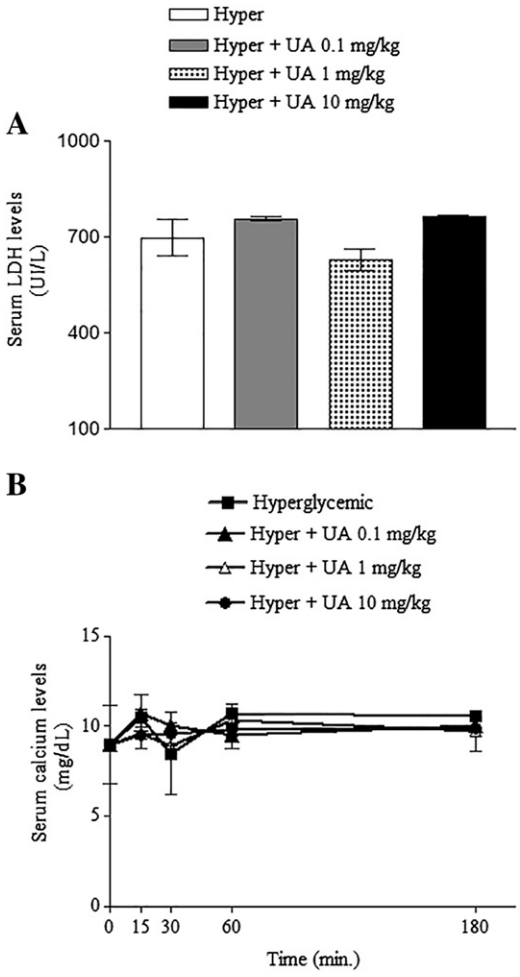

Fig. 8. Effect of UA on serum lactase dehydrogenase (A) and on serum calcium (B). Values are expressed as mean ± S.E.M. with n = 5 for each group.

calcium content by stimulating extracellular Ca^{2+} influx or intracellular Ca^{2+} release [55–57]. Also, the data reported herein suggest that UA-induced glucose uptake depends on the influx of calcium through T- and L-type voltage-dependent calcium channels (L and T-VDCC) as already described for UA on cardiac muscle cells and as well as for rutin in skeletal muscle [58,59].

Besides the opening of calcium channels at plasma membrane, the release of Ca^{2+} from the internal store – usually the endoplasmic reticulum (ER) – represents another important mechanism involved in increased cytosolic calcium levels to change cell behavior. This process can be induced by Ca^{2+} itself (calcium-induced calcium release) and/or by an expanding group of messengers, such as inositol-1,4,5-trisphospate (IP_3), that either stimulate or modulate the releasing of calcium from internal stores [50]. The synthesis of inositol-1,4,5-trisphospate (IP_3) is driven by a family of phospholipase C enzymes which are activated through different mechanisms and stimuli [60]. There is evidence that insulin activates phospholipase C (PLC) in a PI3K-dependent manner, which generates the second messengers diacylglycerol (DAG) and IP_3 from phosphatidylinositol-4,5-bisphosphate (PIP_2). The former functions to activate PKCs and the latter stimulates the Ca^{2+} release from stores [61–63]. Several studies suggest that PLC plays a role in insulin-stimulated GLUT4 translocation and glucose uptake probably acting through the generation of DAG and IP_3 which leads to the subsequent activation of PKCζ and to the increase on cytosolic [Ca^{2+}] from endoplasmic reticulum [61–66]. Our results indicate that the stimulatory effect of UA on glucose uptake involves the PLC and aPKCs activities probably through PI3K and calcium-dependent mechanisms. These are in line with those above-proposed mechanisms by which insulin and contraction stimulate glucose uptake. Additionally, triterpenes were shown to stimulate PLC and PKC activities as well as calcium mobilization in platelet aggregation [56,67,68]. As far as we know, this is the first report on triterpenes, especially UA, acting through PLC, aPKCs and cytosolic calcium to stimulate glucose uptake in skeletal muscle.

Considering that the increase on calcium concentration inside the cells can lead to calcium-calmodulin complex formation with subsequent CaMKII activation [69] the involvement of this kinase was also investigated on UA induced-glucose uptake in soleus muscle. CaMKII is the predominant isoform expressed in skeletal muscle it is activated and autophosphorylated by calcium-calmodulin complex [69,48,3]. The Ca^{2+}–CaMKII signaling network can be established as a convergence point for contraction and insulin-mediated glucose uptake. The activation of CaMKII has been reported to stimulate cell surface GLUT4 translocation and the glucose uptake [70–72,52,73,74]. Additionally, the activated CaMKII also regulates GLUT4 expression. The CaMKII has been described to phosphorylate and influence the MAPK–MEK–ERK1/2 activation [75,3] and also to modulate increases in the binding of myocyte enhancer factor 2 (MEF2) and GLUT4 enhancer factor (GEF) to their binding sites on the Glut4 gene leading to increased GLUT4 synthesis. Smith et al. [76,77] demonstrated that the GLUT4 mRNA and the GLUT4 protein levels were increased after exercise in *in vivo* experiments and these effects were inhibited in the presence of KN93 indicating that CaMKII activity during exercise is required for full expression of GLUT4. Our findings demonstrated the participation of CaMKII in glucose uptake induced by UA. This protein is a potential mediator for nuclear activity altered by UA that significantly stimulated the GLUT4 mRNA expression, thymidine incorporation into DNA and also blocked the stimulatory effect of UA on GLUT4 immunocontent. Additionally, the stimulatory effect of UA on glucose uptake involves the MAPK pathway which is in accordance to the increased GLUT4 content in muscle after the *in vitro* treatments. Also, Although UA oral treatment was carried out in a short-term it did not exhibit cell toxicity or calcium unbalance showing additional physiological advantage for this nutraceutical compound.

The influence of triterpenes on GLUT4 expression was previously reported by our group for betulinic acid, which was able to up regulate GLUT4 immunocontent in the skeletal muscle through the involvement of MAPK pathway and influencing the overall protein synthesis in muscle [15]. Similar results were also described for another triterpene, pachymic acid, which increased the GLUT4 expression in 3 T3-L1 adipocytes after an *in vitro* treatment [42]. Based on the present results, UA seems to have a multitude of cellular targets considering glucose metabolism. It's important to note that from this study there is a long research that will come from the relevant data described herein. It involves a series of research tools such as RT-PCR and immunoblotting of some selected kinases, immunofluorescence of some proteins engaged specifically in GLUT4 translocation and anchoring at plasma membrane as well as a strong effort on ionic involvement through electrophysiology and patch clamp studies in order to investigate more deeply the details of UA on glucose homeostasis."

5. Conclusions

In summary, UA regulates glucose homeostasis involving insulin secretion and insulin sensitizing with calcium participation in its mechanism of action. UA stimulates glucose uptake through a cross talk between different signaling pathways, linking the PI3K and MAPK pathways with Ca^{2+}–CaMKII network in intracellular translocation steps as well as in GLUT4 expression in skeletal muscle.

Acknowledgments

This study was supported by CNPq (Grant # 472071/2013-0), CAPES (Grant # 554/07); FAPESC (Grant # 745/000) and PPG-Biochemistry

(PROAP/2013). The authors express their appreciation to Gilberto Domingos Marloch and Eliana de Medeiros Oliveira (LAMEB II/CCB-UFSC) for their technical support and special thanks to the Central Laboratory of Electronic Microscopy facilities at CCB/UFSC. AJGC, MJSF and CPM are registered on the Biochemistry Postgraduate Program of CCB/UFSC.

References

[1] D.G. Hardie, Organismal carbohydrate and lipid homeostasis, Cold. Spring. Harb. Perspect. Biol. 4 (2012) a006031.
[2] M. Kanzaki, Insulin receptor signals regulating GLUT4 translocation and actin dynamics, Endocr. J. 53 (2006) 267–293.
[3] E.O. Ojuka, V. Goyaram, J.A. Smith, The role of CaMKII in regulating GLUT4 expression in skeletal muscle, Am. J. Physiol. Endocrinol. Metab. 303 (2012) E322–E331.
[4] L.H. Cazarolli, L. Zanatta, E.H. Alberton, M.S. Figueiredo, P. Folador, R.G. Damazio, M.G. Pizzolatti, F.R. Silva, Flavonoids: cellular and molecular mechanism of action in glucose homeostasis, Mini. Rev. Med. Chem. 8 (2008) 1032–1038.
[5] J.M. Castellano, A. Guinda, T. Delgado, M. Rada, J.A. Cayuela, Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes, Diabetes 62 (2013) 1791–1799.
[6] L.H. Cazarolli, D.F. Pereira, V.D. Kappel, P. Folador, M.O.S. Figueiredo, M.G. Pizzolatti, F.R. Silva, Insulin signaling: a potential signaling pathway for the stimulatory effect of kaempferitrin on glucose uptake in skeletal muscle, Eur. J. Pharmacol. 712 (2013) 1–7.
[7] F.R. Silva, L. Zanatta, M.J.S. Frederico, M.G. Pizzolatti, A.M. Campos, Kaempferol and kaempferitrin: nutraceutical compounds contribute to glucose homeostasis by acting at multiple biological sites, in: Garion Villers, Yves Fougere (Eds.), (Org.). Kaempferol: Chemistry, Natural Occurrences and Health Benefits, 1 ed.Nova science publishers, Inc, New York, 2013, pp. 1–45.
[8] J.A. Salvador, V.M. Moreira, B.M. Gonçalves, A.S. Leal, Y. Jing, Ursane-type pentacyclic triterpenoids as useful platforms to discover anticancer drugs, Nat. Prod. Rep. 29 (2012) 1463–1479.
[9] S.M. Jang, S.T. Yee, J. Choi, M.S. Choi, G.M. Do, S.M. Jeon, J. Yeo, M.J. Kim, K.I. Seo, M.K. Lee, Ursolic acid enhances the cellular immune system and pancreatic beta-cell function in streptozotocin-induced diabetic mice fed a high-fat diet, Int. Immunopharmacol. 9 (2009) 113–119.
[10] Z.H. Wang, C.C. Hsu, C.N. Huang, M.C. Yin, Anti-glycative effects of oleanolic acid and ursolic acid in kidney of diabetic mice, Eur. J. Pharmacol. 628 (2010) 255–260.
[11] S.B. Mahato, A. Kundu, ^{13}C NMR spectra of pentacyclic triterpenoids a compilation and some salient features, Phytochemistry 37 (1994) 1517–1575.
[12] H. Varley, A.H. Gowenlock, M. Bell, Practical Clinical Biochemistry, in: I.W. Heinemann (Ed.), fifth ed.Medical Books Ltd, London, 1976.
[13] R.G. Damazio, A.P. Zanatta, L.H. Cazarolli, L.D. Chiaradia, A. Mascarello, R.J. Nunes, R.A. Yunes, F.R. Barreto Silva, Antihyperglycemic activity of naphthylchalcones, Eur. J. Med. Chem. 45 (2010) 1332–1337.
[14] C.R. Krisman, A method for the colorimetric estimation of glycogen with iodine, Anal. Biochem. 4 (1962) 17–23.
[15] A.J. Castro, M.J. Frederico, L.H. Cazarolli, L.C. Bretanha, L.E.C. Tavares, Z.E.S. Buss, M.F. Dutra, A.Z. de Souza, M.G. Pizzolatti, F.R. Silva, Betulinic acid and 1,25$(OH)_2$ vitamin D_3 share intracellular signal transduction in glucose homeostasis in soleus muscle, Int. J. Biochem. Cell Biol. 48 (2014) 18–27.
[16] L.H. Cazarolli, P. Folador, H.H. Moresco, I.M. Brighente, M.G. Pizzolatti, F.R. Silva, Mechanism of action of the stimulatory effect of apigenin-6-C-(2″-O-alpha-l-rhamnopyranosyl)-beta-L-fucopyranoside on ^{14}C-glucose uptake, Chem. Biol. Interact. 179 (2009) 407–412.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[19] L. Zanatta, A. Zamoner, R. Gonçalves, A.P. Zanatta, H. Bouraïma-Lelong, C. Bois, S. Carreau, F.R. Silva, Effect of 1α,25-dihydroxyvitamin D3 in plasma membrane targets in immature rat testis: ionic channels and gamma-glutamyl transpeptidase activity, Arch. Biochem. Biophys. 515 (2011) 46–53.
[20] J.M. dos Santos, S.A. Benite-Ribeiro, G. Queiroz, J.A. Duarte, The effect of age on glucose uptake and GLUT1 and GLUT4 expression in rat skeletal muscle, Cell Biochem. Funct. 30 (2012) 191–197.
[21] R.P. Barros, U.F. Machado, M. Warner, J.A. Gustafsson, Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 1605–1608.
[22] E.C. Schmidt, L.A. Scariot, T. Rover, Z.L. Bouzon, Changes in ultrastructure and histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales), as a consequence of ultraviolet B radiation exposure, Micron 40 (2009) 860–869.
[23] E.S. Reynolds, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, J. Cell Biol. 17 (1963) 208–212.
[24] C. Annerén, M. Welsh, L. Jansson, Glucose intolerance and reduced islet blood flow in transgenic mice expressing the FRK tyrosine kinase under the control of the rat insulin promoter, Am. J. Physiol.-Endocrinol. Metab. 292 (2007) E1183–E1190.
[25] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method, Methods 25 (2001) 402–408.
[26] L.H. Cazarolli, V.D. Kappel, A.P. Zanatta, D.O.H. Suzuki, R.A. Yunes, R.J. Nunes, M.G. Pizzolatti, F.R.M.B. Silva, Natural and synthetic chalcones: tools for the study of targets of action — insulin secretagogue or insulin mimetic? in: Atta-ur Rahman (Ed.), (Org.). Studies in Natural Products Chemistry, 1st ed.Elsevier, 2012, pp. 47–91.
[27] M.F. Azevedo, C. Camsari, C.M. Sá, C.F. Lima, M. Fernandes-Ferreira, C. Pereira-Wilson, Ursolic acid and luteolin-7-glucoside improve lipid profiles and increase liver glycogen content through glycogen synthase kinase-3, Phytother. Res. 24 (Suppl. 2) (2010) S220–S224.
[28] S.M. Jang, M.J. Kim, M.S. Choi, E.Y. Kwon, M.K. Lee, Inhibitory effects of ursolic acid on hepatic polyol pathway and glucose production in streptozotocin-induced diabetic mice, Metabolism 59 (2010) 512–519.
[29] B. Jayaprakasam, L.K. Olson, R.E. Schutzki, M.H. Tai, M.G. Nair, Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (*Cornus mas*), J. Agric. Food Chem. 54 (2006) 243–248.
[30] T. Teodoro, L. Zhang, T. Alexander, J. Yue, M. Vranic, A. Volchuk, Oleanolic acid enhances insulin secretion in pancreatic beta-cells, FEBS Lett. 582 (2008) 1375–1380.
[31] C.L. de Melo, M.G. Queiroz, A.C. Arruda Filho, A.M. Rodrigues, D.F. de Sousa, J.G. Almeida, O.D. Pessoa, E.R. Silveira, D.B. Menezes, T.S. Melo, F.A. Santos, V.S. Rao, Betulinic acid, a natural pentacyclic triterpenoid, prevents abdominal fat accumulation in mice fed a high-fat diet, J. Agric. Food Chem. 57 (2009) 8776–8781.
[32] A.B. Singh, D.K. Yadav, R. Maurya, A.K. Srivastava, Antihyperglycaemic activity of alpha-amyrin acetate in rats and db/db mice, Nat. Prod. Res. 23 (2009) 876–882.
[33] L. Yang, Z. Sun, Y. Zu, C. Zhao, X. Sun, Z. Zhang, L. Zhang, Physicochemical properties and oral bioavailability of ursolic acid nanoparticles using supercritical anti-solvent (SAS) process, Food Chem. 132 (2012) 319–325.
[34] L. Zhang, J. Chen, Y. Gong, J. Liu, W. Hua, H. Sun, Synthesis and biological evaluation of asiatic acid derivatives as inhibitors of glycogen phosphorylases, Chem. Biodivers. 6 (2009) 864–874.
[35] X. Wen, H. Sun, J. Liu, K. Cheng, P. Zhang, L. Zhang, J. Hao, P. Ni, S.E. Zographos, D.D. Leonidas, K.M. Alexacou, T. Gimisis, J.M. Hayes, N.G. Oikonomakos, Naturally occurring pentacyclic triterpenes as inhibitors of glycogen phosphorylase: synthesis, structure-activity relationships, and X-ray crystallographic studies, J. Med. Chem. 51 (2008) 3540–3554.
[36] P. Zhu, Y. Bi, J. Xu, Z. Li, J. Liu, L. Zhang, W. Ye, X. Wu, Terpenoids. III: synthesis and biological evaluation of 23-hydroxybetulinic acid derivatives as novel inhibitors of glycogen phosphorylase, Bioorg. Med. Chem. Lett. 19 (2009) 6966–6969.
[37] D.T. Ha, D.T. Tuan, N.B. Thu, N.X. Nhiem, T.M. Ngoc, N. Yim, K. Bae, Palbinone and triterpenes from Moutan Cortex (*Paeonia suffruticosa*, Paeoniaceae) stimulate glucose uptake and glycogen synthesis via activation of AMPK in insulin-resistant human HepG2 Cells, Bioorg. Med. Chem. Lett. 19 (2009) 5556–5559.
[38] Z. Liang, L. Zhang, L. Li, J. Liu, H. Li, L. Chen, K. Cheng, M. Zheng, X. Wen, P. Zhang, J. Hao, Y. Gong, X. Zhang, X. Zhu, J. Chen, H. Liu, H. Jiang, C. Luo, H. Sun, Identification of pentacyclic triterpenes derivatives as potent inhibitors against glycogen phosphorylase based on 3D-QSAR studies, Eur. J. Med. Chem. 46 (2011) 2011–2021.
[39] Y. Chen, Y. Wang, W. Ji, P. Xu, T. Xu, A pre-docking role for microtubules in insulin-stimulated glucose transporter 4 translocation, FEBS J. 275 (2008) 705–712.
[40] T. Miura, Y. Itoh, T. Kaneko, N. Ueda, T. Ishida, M. Fukushima, F. Matsuyama, Y. Seino, Corosolic acid induces GLUT4 translocation in genetically type 2 diabetic mice, Biol. Pharm. Bull. 27 (2004) 1103–1105.
[41] S.H. Jung, Y.J. Ha, E.K. Shim, S.Y. Choi, J.L. Jin, H.S. Yun-Choi, J.R. Lee, Insulin-mimetic and insulin-sensitizing activities of a pentacyclic triterpenoid insulin receptor activator, Biochem. J. 403 (2007) 243–250.
[42] Y.C. Huang, W.L. Chang, S.F. Huang, C.Y. Lin, H.C. Lin, T.C. Chang, Pachymic acid stimulates glucose uptake through enhanced GLUT4 expression and translocation, Eur. J. Pharmacol. 648 (2010) 39–49.
[43] M.S. Lee, P.T. Thuong, Stimulation of glucose uptake by triterpenoids from Weigela subsessilis, Phytother. Res. 24 (2010) 49–53.
[44] L. Shi, W. Zhang, Y.Y. Zhou, Y.N. Zhang, J.Y. Li, L.H. Hu, J. Li, Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation, Eur. J. Pharmacol. 584 (2008) 21–29.
[45] W. Zhang, D. Hong, Y. Zhou, Y. Zhang, Q. Shen, J.Y. Li, L.H. Hu, J. Li, Ursolic acid and its derivative inhibit protein tyrosine phosphatase 1B, enhancing insulin receptor phosphorylation and stimulating glucose uptake, Biochim. Biophys. Acta 1760 (2006) 1505–1512.
[46] Y.N. Zhang, W. Zhang, D. Hong, L. Shi, Q. Shen, J.Y. Li, J. Li, L.H. Hu, Oleanolic acid and its derivatives: new inhibitor of protein tyrosine phosphatase 1B with cellular activities, Bioorg. Med. Chem. 16 (2008) 8697–8705.
[47] J.Y. Choi, M. Na, I.H. Hwang, S.H. Lee, E.Y. Bae, B.Y. Kim, J.S. Ahn, Isolation of betulinic acid, its methyl ester and guaiane sesquiterpenoids with protein tyrosine phosphatase 1B inhibitory activity from the roots of Saussurea lappa C.B.Clarke, Molecules 14 (2009) 266–272.
[48] N. Wijesekara, F.S.L. Thong, C.N. Antonescu, A. Klip, Diverse signals regulate glucose uptake into skeletal muscle, Can. J. Diab. 30 (2006) 80–88.
[49] J.T. Lanner, J.D. Bruton, A. Katz, H. Westerblad, Ca(2+) and insulin-mediated glucose uptake, Curr. Opin. Pharmacol. 8 (2008) 339–345.
[50] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529.
[51] J.T. Lanner, A. Katz, P. Tavi, M.E. Sandström, S.J. Zhang, C. Wretman, S. James, J. Fauconnier, J. Lännergren, J.D. Bruton, H. Westerblad, The role of Ca^{2+} influx for insulin-mediated glucose uptake in skeletal muscle, Diabetes 55 (2006) 2077–2083.
[52] S.K. Mohankumar, C.G. Taylor, P. Zahradka, Domain-dependent modulation of insulin-induced AS160 phosphorylation and glucose uptake by Ca^{2+}/calmodulin-dependent protein kinase II in L6 myotubes, Cell. Signal. 24 (2012) 302–308.
[53] G.D. Cartee, C. Briggs-Tung, J.O. Holloszy, Diverse effects of calcium channel blockers on skeletal muscle glucose transport, Am. J. Physiol. 263 (1992) R70–R75.

[54] J.D. Bruton, A. Katz, H. Westerblad, Insulin increases near-membrane but not global Ca^{2+} in isolated skeletal muscle, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 3281–3286.
[55] Y.S. Lee, D.Q. Jin, E.J. Kwon, S.H. Park, E.S. Lee, T.C. Jeong, D.H. Nam, K. Huh, J.A. Kim, Asiatic acid, a triterpene, induces apoptosis through intracellular Ca^{2+} release and enhanced expression of p53 in HepG2 human hepatoma cells, Cancer Lett. 186 (2002) 83–91.
[56] J.J. Lee, Y.R. Jin, Y. Lim, J.Y. Yu, T.J. Kim, H.S. Yoo, H.S. Shin, Y.P. Yun, Oleanolic acid, a pentacyclic triterpenoid, induces rabbit platelet aggregation through a phospholipase C-calcium dependent signaling pathway, Arch. Pharm. Res. 30 (2007) 210–214.
[57] D.M. Wu, D. Zhao, D.Z. Li, D.Y. Xu, W.F. Chu, X.F. Wang, Maslinic acid induces apoptosis in salivary gland adenoid cystic carcinoma cells by Ca^{2+}-evoked p38 signaling pathway, Naunyn Schmiedeberg's Arch. Pharmacol. 383 (2011) 321–330.
[58] H.Z. Cui, J.F. Wen, H.R. Choi, X. Li, K.W. Cho, D.G. Kang, H.S. Lee, Ursolic acid increases the secretion of atrial natriuretic peptide in isolated perfused beating rabbit atria, Eur. J. Pharmacol. 653 (2011) 63–69.
[59] V.D. Kappel, L. Zanatta, B.G. Postal, F.R. Silva, Rutin potentiates calcium uptake via voltage-dependent calcium channel associated with stimulation of glucose uptake in skeletal muscle, Arch. Biochem. Biophys. 532 (2013) 55–60.
[60] J.T. Brozinick, B.A. Berkemeier, J.S. Elmendorf, "Acting" on GLUT4: membrane & cytoskeletal components of insulin action, Curr. Diabetes. Rev. 3 (2007) 111–122.
[61] M. Lorenzo, T. Teruel, R. Hernandez, A.G. Kayali, N.J.G. Webster, PLCγ participates in insulin stimulation of glucose uptake through activation of PKCζ in brown adipocytes, Exp. Cell Res. 278 (2002) 146–157.
[62] J. Eichhorn, A.G. Kayali, D.A. Austin, N.J. Webster, Insulin activates phospholipase C-gamma1 via a PI-3 kinase dependent mechanism in 3T3-L1 adipocytes, Biochem. Biophys. Res. Commun. 282 (2001) 615–620.
[63] M. Van Epps-Fung, K. Gupta, R.W. Hardy, A. Wells, A role for phospholipase C activity in GLUT4-mediated glucose transport, Endocrinology 138 (1997) 5170–5175.
[64] E.J. Henriksen, K.J. Rodnick, J.O. Holloszy, Activation of glucose transport in skeletal muscle by phospholipase C and phorbol ester. Evaluation of the regulatory roles of protein kinase C and calcium, J. Biol. Chem. 264 (1989) 21536–21543.
[65] D.C. Wright, B.W. Craig, C.A. Fick, K.I. Lim, The effects of phospholipase C inhibition on insulin-stimulated glucose transport in skeletal muscle, Metabolism 51 (2002) 271–273.
[66] D.C. Wright, C.A. Fick, J.B. Olesen, B.W. Craig, Evidence for the involvement of a phospholipase C-protein kinase C signaling pathway in insulin stimulated glucose transport in skeletal muscle, Life Sci. 73 (2003) 61–71.
[67] C.N. Wang, J.S. Chen, M.S. Shiao, C.T. Wang, Activation of human platelet phospholipases C and A2 by various oxygenated triterpenes, Eur. J. Pharmacol. 267 (1994) 33–42.
[68] K. Takahashi, S. Suzuki, Y. Hano, T. Nomura, Protein kinase C activation by iridal type triterpenoids, Biol. Pharm. Bull. 25 (2002) 432–436.
[69] L.S. Maier, D.M. Bers, Calcium, calmodulin, and calcium–calmodulin kinase II: heartbeat to heartbeat and beyond, J. Mol. Cell. Cardiol. 34 (2002) 919–939.
[70] D.C. Wright, P.C. Geiger, J.O. Holloszy, D.H. Han, Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca^{2+}-dependent mechanism in slow-twitch rat soleus muscle, Am. J. Physiol. Endocrinol. Metab. 288 (2005) E1062–E1066.
[71] E.R. Chin, The role of calcium and calcium/calmodulin-dependent kinases in skeletal muscle plasticity and mitochondrial biogenesis, Proc. Nutr. Soc. 63 (2004) 279–286.
[72] C.A. Witczak, N. Jessen, D.M. Warro, T. Toyoda, N. Fujii, M.E. Anderson, M.F. Hirshman, L.J. Goodyear, CaMKII regulates contraction- but not insulin-induced glucose uptake in mouse skeletal muscle, Am. J. Physiol. Endocrinol. Metab. 298 (2010) E1150–E1160.
[73] D.C. Wright, C.A. Fick, J.B. Olesen, K. Lim, B.R. Barnes, B.W. Craig, A role for calcium/calmodulin kinase in insulin stimulated glucose transport, Life Sci. 74 (2004) 815–825.
[74] M. Murgia, T.E. Jensen, M. Cusinato, M. Garcia, E.A. Richter, S. Schiaffino, Multiple signalling pathways redundantly control glucose transporter GLUT4 gene transcription in skeletal muscle, J. Physiol. 587 (2009) 4319–4327.
[75] M. Illario, S. Monaco, A.L. Cavallo, I. Esposito, P. Formisano, L. D'Andrea, E. Cipolletta, B. Trimarco, G. Fenzi, G. Rossi, M. Vitale, Calcium-calmodulin-dependent kinase II (CaMKII) mediates insulin-stimulated proliferation and glucose uptake, Cell. Signal. 21 (2009) 786–792.
[76] J.A. Smith, M. Collins, L.A. Grobler, C.J. Magee, E.O. Ojuka, Exercise and CaMK activation both increase the binding of MEF2A to the Glut4 promoter in skeletal muscle in vivo, Am. J. Physiol. Endocrinol. Metab. 292 (2007) E413–E420.
[77] J.A. Smith, T.A. Kohn, A.K. Chetty, E.O. Ojuka, CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the Glut4 gene, Am. J. Physiol. Endocrinol. Metab. 295 (2008) E698–E704.