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


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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.

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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...
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). Ca2+ in the cytosol regulates several signaling pathways and metabolic events. The increase in intracellular calcium levels which activates an increase in the contraction-stimulated glucose transport and GLUT4 translocation. The latter involves as well 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), is the most representative ursane-type triterpenoid is being described as an anti-inflammatory, hepatoprotective, antitumoral and anti-diabetic 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-α-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-14C]-2-deoxy-D-glucose (14C-DG), specific activity 9.25 GBq/mmol, thymidine [methyl-14C], 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 lyophilization 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 ursoic 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 14C-glucose uptake in rat soleus muscle

For the [U-14C]-2-deoxy-D-glucose (14C-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 MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, and 25 mM NaHCO₃ and bubbled with O₂/CO₂ (95%/5%, v/v) until pH 7.4. UA (10⁻⁴, 10⁻³, 10⁻², 10⁻¹, 10⁻⁰, 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 mM wortmannin, 1 mM colchicine, 2 mM EGTA, 50 μM BAPTA-AM, 1 μM nifedipine, 1 μM flurazimine, 1 μM U73122, 10 μM KN93, 40 μM RO 318220, 350 μM cycloheximide or 50 μM PD 98059. 14C-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-14C]-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 O₂/CO₂ (95%/5%, v/v), pH 7.4. UA (1 nM) was added to the pre-incubation (30 min) and incubation (60 min) medium. 14C-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 4 °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]).
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

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.
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 mM 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 M 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 tripterase.

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 nM of HNMPA(AM)₃, an inhibitor of insulin receptor tyrosine kinase activity. In all cases, the regulatory effect of UA on glucose uptake was significant at 100, 10, 1, 0.1 and 0.001 nM wortmannin, a specific inhibitor of PI3K, 1 μM colchicine, 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) 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.

Table 1

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Fig. 3. Concentration−response curve of UA on [14C]-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 ≤ 0.01 and ***p ≤ 0.001 in relation to control 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
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 Ca2+ 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 Ca2+ 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 immunoccontent (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). 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).
which generates the second messengers diacylglycerol (DAG) and IP3. Insulin activates phospholipase C (PLC) in a PI3K-dependent manner, through different mechanisms and stimuli [60]. There is evidence that in the reticulum [61], calcium release [55] to activate PKCs and the latter stimulates the calcium release from stores (IP3), that either stimulate or modulate the releasing of calcium from intracellular stores. This process is mediated by an expanding group of messengers, such as inositol-1,4,5-trisphosphate (IP3) which leads to the subsequent activation of PKCζ and PKCδ by the generation of DAG and IP3 which leads to the subsequent activation of PKCζ and PKCδ.

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 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 stimulates the GLUT4 mRNA expression, thymidine incorporation into DNA and also blocked the stimulatory effect of UA on GLUT4 immunoccontent. 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 immunoccontent 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 Ca2⁺–CaMKII network in intracellular translocation steps as well as in GLUT4 expression in skeletal muscle.

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