FEBS Letters 584 (2010) 531–536

FEBS Letters

journal homepage: www.FEBSLetters.org

Gallic acid induces GLUT4 translocation and glucose uptake activity in 3T3-L1 cells

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ARTICLE INFO

Article history:
Received 15 October 2009
Revised 16 November 2009
Accepted 28 November 2009
Available online 3 December 2009

Edited by Robert Barouki

Keywords:
Gallic acid
GLUT4 translocation
Glucose uptake
Atypical protein kinase
Akt inhibitor

ABSTRACT

GLUT4, a 12 transmembrane protein, plays a major role in insulin mediated glucose transport in muscle and adipocytes. For glucose transport, the GLUT4 protein needs to be translocated to the plasma membrane from the intracellular pool and it is possible that certain compounds may be able to enhance this process. In the present work, we have shown that gallic acid can increase GLUT4 translocation and glucose uptake activity in an Akt-independent but wortmannin-sensitive manner. Further analysis suggested the role of atypical protein kinase Cζ/λ in gallic acid mediated GLUT4 translocation and glucose uptake.

1. Introduction

Insulin stimulates glucose transport in target tissues (muscles and adipocytes) by inducing the translocation of glucose transporter 4 (GLUT4) to the plasma membrane [1]. GLUT4 protein resides in the intracellular vesicles, and is predominantly localized to the perinuclear compartment in the basal state [2]. Phosphorylation of insulin receptor by insulin binding activates phosphatidylinositol 3-kinase (PI3K), and induces the activation of downstream signaling molecules like protein kinase B (PKB/Akt) and atypical PKCs (aPKCs), leading to GLUT4 translocation and glucose uptake [3–6]. Studies have shown that insulin stimulates glucose uptake primarily by inducing the GLUT4 translocation to the plasma membrane rather than increasing the intrinsic activity of the transporter protein [7]. Lack of insulin or defects in insulin signaling pathway lead to a concomitant rise in blood glucose level and hence to diabetes [8]. Among the different types of diabetes, type 2 diabetes is characterized by insulin resistance, a condition in which normal insulin level is inadequate to produce desired response in fat, muscle and liver cells [9–13]. Since insulin resistance is the major abnormality in type 2 diabetes, there has been considerable interest in identifying insulin sensitizing agents to counteract insulin resistance for the treatment of type 2 diabetes. Any compound that induces the exocytosis and/or decreases the endocytosis will result in increased GLUT4 expression on the plasma membrane, and ultimately enhance glucose absorption.

The study reported here is a bioassay directed identification of the active principle from seabuckthorn leaves (*Hippophae rhamnoides* L. Elaeagnaceae), that induces GLUT4 translocation and glucose uptake in 3T3-L1 cells. Seabuckthorn is widely distributed in the mountainous regions of Asia and Europe. Berries and other parts are widely used as medicine in China and middle Asia [14]. This plant is an important resource of natural products with antioxidant, anti-tumor, hepatoprotective and immuno-modulatory properties [15,16]. There are several reports suggesting the therapeutic applications of seabuckthorn in treating various ailments like cancer, gastrointestinal, cardiovascular, liver and skin diseases [17]. In the present study, we have identified and functionally characterized gallic acid (GA) as the active principle from seabuckthorn leaf extract that increases glucose uptake in 3T3-L1 adipocytes. GA stimulates glucose uptake by inducing GLUT4 translocation in a wortmannin-sensitive but Akt-independent manner. Further analysis suggests that GA induces glucose uptake via atypical protein kinase Cζ/λ.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media and supplements were obtained from Sigma (St. Louis, MO, USA). 3T3-L1 cells were obtained from National Centre for Cell Sciences, Pune, Maharashtra, India. The myc-GLUT4-GFP construct was kindly provided by Prof. Jeffrey E. Pessin, (Stony Brook University, NY, USA). Primary antibodies against myc epitope, phospho-PKCζ/λ$^{Thr410/403}$, phospho-Akt^{Ser473},

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doi:10.1016/j.febslet.2009.11.092

phospho-AMPKα^{Thr172}, β-actin, alexa-conjugated, and HRP-conjugated anti-mouse, anti-rabbit secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). Akt inhibitor III (Akti) was from Calbiochem (Los Angeles, CA, USA). Authentic sample of GA was obtained from Nice Chemicals, India. 2-Deoxy-D-(^{3}H)-glucose was obtained from Amersham Life Sciences (Buckinghamshire, UK). Organic solvents and other chemicals used for extraction and purification were of the highest analytical grade.

2.2. Plant extraction and column purification

The plant material was collected from Leh (Ladakh, Jammu and Kashmir, India) and was identified as *H. rhamnoides* ssp. *Turkestanica* by Dr. O.P. Chaurasia, Scientist, Field Research Laboratory (FRL), Defense Research and Development Organization, Leh, India. Dried seabuckthorn leaves (50 g) were extracted repeatedly with methanol at room temperature. The solvent was removed in a rotary evaporator at 50 °C and the concentrate was analyzed. The crude extract was concentrated and dissolved in DMSO (20 mg/ml) for the assay. A portion of the crude extract was subjected to acid hydrolysis and separation of individual components was carried out by column chromatography and gel-permeation chromatography. The progress of separation of compounds was monitored by thin-layer chromatography (TLC), circular paper chromatography and high-performance liquid chromatography (HPLC, Shimadzu, CTO-104SUP). Confirmatory chemical tests, optical spectroscopy (infra red, ultra violet), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (Bruker ADVANCE DPX$_{300}$ series), and mass spectroscopy (JEOL JMS 600 and Shimazdu GC–MS 5050 series) were used for molecular characterization.

2.3. Cell culture and transfection of 3T3-L1 preadipocytes

3T3-L1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 0.1% amphotericin B. Cells were maintained and passaged as preconfluent cultures at 37 °C in a 5% CO_2 humidified incubator. Preadipocytes were transfected with myc-GLUT4-GFP construct by electroporation. For this purpose, 10^6–10^7 cells were resuspended in 400 μl DMEM and subjected to a single pulse at 250 mV with the plasmid construct. The transfected cells were cultured in DMEM containing 600 μg/ml of G418 to select stable clones. Each clone was subcultured and screened by fluorescent microscopy. A stable clone expressing GLUT4, with proper intracellular localization was used for further studies. Differentiation of 3T3-L1 fibroblasts was carried out following standard protocols with slight modifications. Completely confluent plates were incubated in DMEM containing 10% FBS with 500 μM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 100 nM insulin. Two days after incubation, the medium was replaced with DMEM containing 100 nM insulin. Fresh culture medium was added after 2 days and then every other day till the cells attained adipocyte morphology.

2.4. GLUT4 translocation assay

3T3-L1 cells stably transfected with myc-GLUT4-GFP chimera were grown to 70% confluency on cover slips. After serum starvation for 2 h, cells were induced with insulin, different concentrations of plant extract or GA for 30 min. Cells were then washed twice with phosphate buffered saline (PBS), and fixed with 2% paraformaldehyde. These were incubated with anti-myc antibody for 1 h at room temperature, washed, and counterstained with alexa-conjugated rabbit anti-mouse secondary antibody for 30 min and membrane GLUT4 was visualized by fluorescence microscopy. Cells treated with insulin and DMSO vehicle were used as positive and negative control, respectively. The images were obtained with a 40× objective (Olympus-X-71, Olympus America Inc., USA) and the total surface fluorescence of the secondary antibody was taken into consideration. Fluorescence intensity was calculated by Image-Pro Plus software (version 5.1.2).

2.5. Glucose uptake assay

Differentiated cells were induced with various concentrations of plant extract or GA for 30 min. Adipocytes were pretreated either with 100 nM wortmannin for 10 min or with 1 μM Akti for 1 h followed by treatment with GA for 30 min. After induction, cells were washed with Krebs–Ringer-Phosphate (KRP) buffer, and glucose uptake was initiated by the addition of 0.5 ml KRP buffer containing 10 μM 2-deoxy-D-glucose and 0.1 μCi 2-deoxy-D-(^{3}H)-glucose. After 5 min, glucose uptake was terminated by washing the cells with ice cold KRP buffer for three times. Cells were lysed with 0.1% sodium dodecyl sulfate (SDS) and the radioactivity retained by the cell lysate was measured by a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA, USA). Insulin was used as a positive control and cells treated with DMSO were used as a negative control for the uptake assay.

2.6. Western blot analysis

Adipocytes were serum starved for 2 h and induced with DMSO, 50 nM insulin or 10 μM GA for 30 min. Following induction, the cells were washed twice with ice cold PBS and incubated in a lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) for 5 min. The lysates were briefly sonicated for 5 s and centrifuged at 14 000×g for 10 min. Total protein was estimated and equal amount of protein (50 μg) were subjected to 10%-SDS–PAGE and transferred to PVDF membrane. The membrane was blocked and incubated with desired primary antibody for 2 h, washed, and incubated with HRP-conjugated secondary antibody for 1 h. After washing the membrane, bands were detected by Phototope-HRP Western Blot detection system (Cell Signaling).

3. Results

3.1. Extraction, fractionation and purification of the active constituent from seabuckthorn leaves

Analysis of the concentrated crude extract (henceforth referred to as SBL) revealed the presence of significant amounts of phenolics based upon positive ferric reactions. An attempt to isolate the active principle by chromatographic technique was not successful. Hence, to resolve this problem, hydrolysis of SBL was carried out. Aqueous sulfuric acid (7%) for 1 h at 70 °C was found to be optimum for complete hydrolysis of SBL. The products of hydrolysis (henceforth referred to as SBL-H$^+$) were exhaustively extracted with ethyl acetate. Initial extracts were yellow in color (green ferric reaction) while the later fractions were less colored (blue black ferric reaction). The concentrate obtained from the extraction was subjected to extensive adsorption chromatography followed by gel-permeation chromatography. Three compounds A, B and C were isolated. Compound C isolated from the extract was a white amorphous powder, soluble in methanol and water. This compound gave a prominent color reaction with ferric chloride (bluish black color) and was soluble in alkali solution giving pink color. Based on the results of ^{1}H and ^{13}C NMR spectroscopy, compound C appeared to be a small molecule with prominent UV absorption

at 274 nm in methanol. These properties suggested that compound C could be GA. The argument was supported by direct comparison of UV, NMR and paper chromatography data with commercially obtained GA. Compounds A and B were identified as quercetin and isorhamnetin, respectively, which are known to inhibit glucose uptake. Hence our subsequent studies were focused on GA using commercially available GA.

3.2. Seabuckthorn leaf extract and GA induces GLUT4 translocation in transfected 3T3-L1 preadipocytes

Insulin is known to mobilize GLUT4 from specialized compartment to the plasma membrane. This process can be studied in vitro by using 3T3-L1 fibroblasts expressing tagged GLUT4 [18]. 3T3-L1 preadipocytes stably expressing myc-GLUT4-GFP chimera were incubated with various concentrations of SBL, SBL-H^+ and GA for 30 min. Treatment with 50 nM insulin was used as a positive control. The amount of GLUT4 on the membrane was measured as the intensity of GLUT4 fluorescence after non-permeabilized fixation. Surface expression of GLUT4 increased with increasing concentration of SBL (data not shown) and SBL-H^+. It was observed that the maximum concentration of SBL-H^+ (5 μg/ml) resulted in 65% increase in the levels of GLUT4 on the plasma membrane (Fig. 1A and B) compared to untreated control cells. Similar to SBL-H^+, treatment with GA also induced membrane translocation of GLUT4 in a concentration dependent manner. A maximum of 90% induction of GLUT4 surface expression was observed with 10 μM GA. However, further increase in the GA concentration reduced the expression of GLUT4 on the plasma membrane (Fig. 1C and D).

3.3. Increased glucose uptake activity by seabuckthorn leaf extract and GA

To determine if induction of GLUT4 translocation by SBL, SBL-H^+ or GA led to increased glucose uptake in differentiated 3T3-L1 cells, glucose transport assay was utilized. Treatment with 100 μg/ml of crude SBL extract resulted in 35% increase in glucose uptake compared to untreated cells (Fig. 2A). Subsequent assays with the fractionated SBL-H^+ showed an increase in glucose uptake in a concentration dependent manner. At a maximum concentration of 5 μg/ml, SBL-H^+ showed a 58% increase in glucose transport (Fig. 2A). This increase in glucose uptake activity correlates with the increase in GLUT4 translocation using same concentration of SBL-H^+ as shown in Fig. 1. Treatment with GA also induced glucose uptake in a concentration dependent manner. Ten micromolars of GA showed 90% increase in glucose uptake activity compared to untreated cells (Fig. 2B). Further increase in GA concentration reduced the rate of glucose transport. Since 10 μM GA and 50 nM insulin showed similar induction of glucose uptake, it was interesting to determine if simultaneous treatment of GA and insulin could cause a synergistic increase in glucose transport. Addition of GA along with insulin neither increased nor inhibited the glucose uptake activity in 3T3-L1 adipocytes (data not shown).

3.4. The involvement of insulin signaling proteins in the cellular phosphorylation induced by GA

GLUT4 translocation and glucose uptake in muscle cells and adipocytes is mediated by two major signaling pathways: AMPK-mediated insulin independent pathway and PI3K dependent insu-

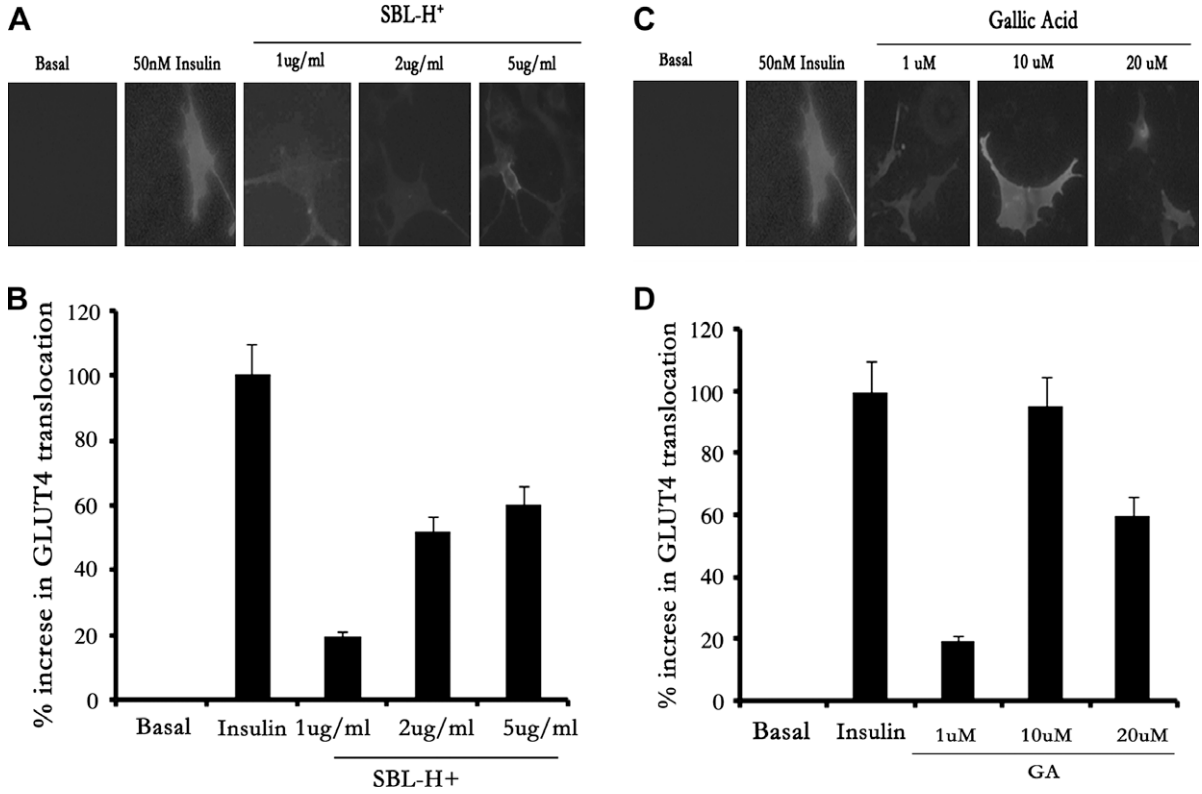

Fig. 1. Effect of SBL-H^+ and GA on GLUT4 translocation. 3T3-L1 preadipocytes were transfected with myc-GLUT4-GFP chimera, serum starved for 2 h, and incubated with indicated concentrations of SBL-H^+ or insulin for 30 min. Cells were fixed and surface myc-GLUT4-GFP was labeled with alexa by indirect immunofluorescence. (A and C) Images show the membrane GLUT4 (*surface*) upon treatment with varying concentration of SBL-H^+ or GA as indicated. Cells treated with vehicle (0.1% DMSO) were used to measure non-specific fluorescence. (B and D) Graph showing the percentage increase in fluorescence intensity of secondary antibody upon treatment with varying concentration of SBL-H^+ and GA compared to the basal condition. Values are shown as the mean ± S.D. of three different focal planes of the same experiment.

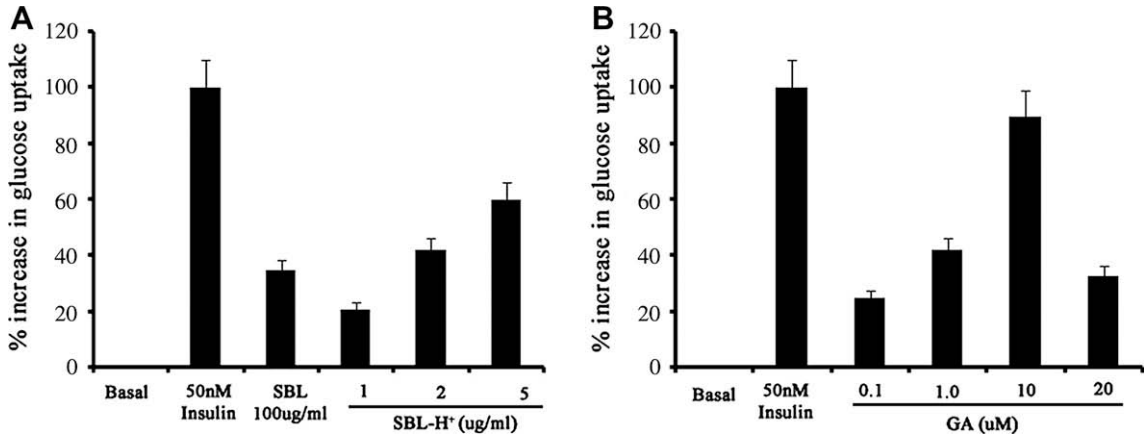

Fig. 2. Effect of SBL, SBL-H^+ and GA on glucose uptake. Glucose uptake activity was analyzed by measuring the uptake of radiolabelled 2-deoxy glucose in differentiated 3T3-L1 cells. Cells were serum starved for 2 h and incubated with the indicated concentration of insulin, SBL or SBL-H^+ for 30 min. Cells incubated with vehicle (0.1% DMSO) alone were used to measure the basal rate of glucose uptake. Radiolabelled 2-deoxy glucose uptake was measured using liquid scintillation counter. Data shown are percentage increase in glucose uptake compared to the basal condition. (A) Increase in glucose uptake upon treatment with SBL or SBL-H^+ or (B) varying concentration of GA. Values are shown as the mean ± S.D. of three different experiments carried out in duplicates.

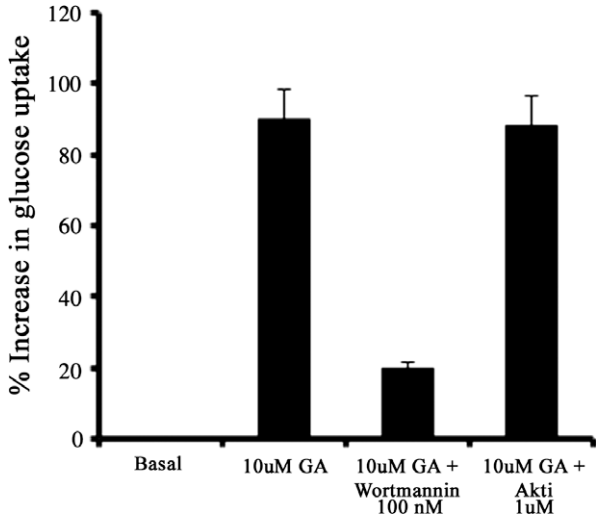

Fig. 3. Effect of wortmannin or Akti on GA stimulated glucose uptake. 3T3-L1 adipocytes were serum starved for 2 h, and incubated with 10 μM GA in the presence or absence of wortmannin or Akti for 30 min. Cells incubated with vehicle (0.1% DMSO) were used to measure the basal rate of glucose uptake. Radiolabelled 2-deoxy glucose uptake was measured using liquid scintillation counter. Data shown are percentage increase in glucose uptake compared to the basal condition. Values are shown as the mean ± S.D. of three different experiments carried out in duplicates.

lin signaling pathway [19]. To determine the role of AMPK-dependent pathway in GA stimulated glucose uptake, differentiated cells were treated with 10 μM GA for 30 min and the phosphorylation of AMPK was analyzed. GA did not affect the phosphorylation of AMPK (Fig. 4A). Next, to investigate the role of PI3K in GA induced GLUT4 translocation and glucose uptake, 3T3-L1 cells were treated with wortmannin, an inhibitor of PI3K signaling pathway. GA mediated glucose uptake was abolished by wortmannin (Fig. 3), suggesting that the stimulation of glucose uptake following GA treatment was PI3K dependent. Furthermore, we analyzed the activation status of Akt, a key downstream effector of PI3K. GA did not promote Akt phosphorylation at Ser^{473} (Fig. 4A), suggesting that activation of Akt may not be involved in GA mediated glucose uptake. Hoehn et al. have shown a non-linear relationship between the levels of activated Akt and glucose uptake and it was surprising to see that only 5% of the total activated Akt is sufficient to achieve maximum GLUT4 translocation [20]. To rule out the involvement of activated Akt in the GA induced glucose uptake, we have treated 3T3-L1 adipocytes with Akt inhibitor. Adipocytes treated with Akt inhibitor did not affect the GA stimulated glucose uptake (Fig. 3), confirming that the GA induced glucose uptake is Akt independent. Taken together, our observations suggest that the stimulatory effect of GA on glucose uptake is mediated via PI3K signaling pathway, and is independent of Akt or AMPK.

3.5. GA stimulates the activation of PKCζ/λ in differentiated 3T3-L1 adipocytes

Since GA showed a PI3K dependent increase in glucose uptake without activating Akt, it is possible that one of the products of PI3K; phosphatidylinositol 3,4,5-trisphosphate, can activate atypi-

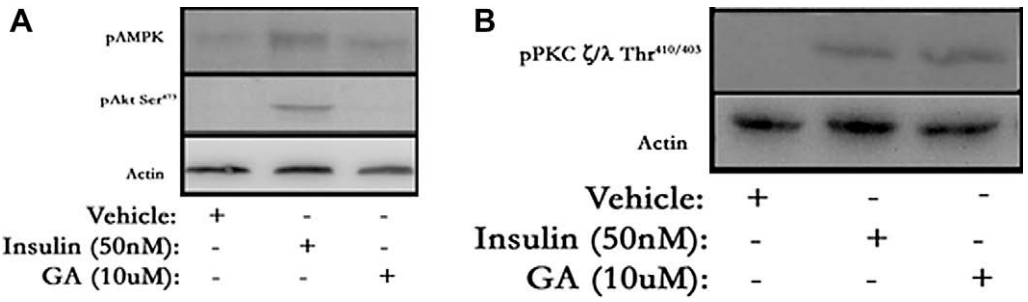

Fig. 4. Effect of GA on protein phosphorylation. Differentiated adipocytes were treated with 50 nM insulin or 10 μM GA for 30 min. Equal amount of protein was resolved by SDS–PAGE. After transferring to PVDF membrane, Akt, AMPK and aPKCζ/λ phosphorylation was detected by western blot analysis. Treatment with GA did not stimulate Akt as well as phosphorylation (A), whereas GA stimulated aPKCζ/λ phosphorylation similar to insulin (B).

cal protein kinase C, PKCζ/λ [21,22]. To test this possibility, we analyzed the phosphorylation status of PKCζ/λ. Treatment with 10 μM GA resulted in 1.4-fold increase in aPKCζ/λ phosphorylation (Fig. 4B), suggesting the involvement of PKCζ/λ in GA-mediated GLUT4 translocation and glucose uptake.

4. Discussion

Seabuckthorn is a deciduous plant widely distributed in the mountainous regions of China, Russia and India. It is a rich source of carotenoids, tocopherols, sterols, flavonoids, lipids, ascorbic acid, and tannins. Several of these compounds are known to have various medicinal properties [23]. Considering the fact that seabuckthorn leaves contain large amount of polyphenols, which are known to stimulate glucose uptake activity, we ventured to identify the active constituent from this plant that induces glucose transport activity [24–26].

In this study, a systematic analysis was performed in order to identify compounds from seabuckthorn leaves with glucose uptake enhancing activity. Crude SBL extract showed a significant stimulation of glucose transport compared to the basal level. In order to isolate the active principle, we tried various approaches including gel-permeation chromatography, but with limited success. This is because the phytochemicals in seabuckthorn exist as glycosides or conjugates. To overcome this problem, the crude extract was subjected to acid hydrolysis and further extracted with ethyl acetate. The hydrolyzed product (SBL-H^+) was found to be a mixture of three major compounds viz. quercetin, isorhamnetin and GA. Even though polyphenolic compounds are reported to have in vitro and in vivo antidiabetic activity, many of the flavones like apigenin, luteolin, kaempferol, myrcetin, quercetin, isorhamnetin, fisetin, genistein, silybin and epigallocatechin gallate (EGCG) were shown to inhibit glucose uptake in mice and rat adipocytes [27–29]. Among these, apigenin, luteolin, kaempferol, quercetin, and fisetin were found to inhibit insulin stimulated activation of Akt, and suppress insulin dependent translocation of GLUT4 to the plasma membrane [30,31]. Many of the glycoside varieties of flavones are also known to inhibit insulin stimulated glucose uptake. Previous studies from our laboratory have shown that kaempferitrin can inhibit insulin stimulated glucose uptake by directly interfering with glucose transport channel and also by inhibiting insulin mediated signal transduction [32,33]. Based on these data, we speculated that quercetin and isorhamnetin might not have any antidiabetic activity. Therefore we focused on GA as a potential compound in the SBL-H^+ possessing increased glucose uptake activity in 3T3-L1 cells.

We observed that GA treatment stimulated GLUT4 translocation and glucose transport in a concentration dependent manner with maximum stimulation at 10 μM concentration. GA, a small phenolic compound, is a major component in several plants. Seabuckthorn leaf is also reported to have large amounts of GA [34]. It has been demonstrated that GA possesses anti-allergic, anti-inflammatory, anti-mutagenic and anti-carcinogenic effects [35–37]. Although some of the reported antidiabetic plants possess GA as a major chemical compound [38,39], studies so far have not demonstrated GA as a compound with antidiabetic or anti-hyperglycemic properties. Here, we have clearly shown the antidiabetic property of GA in an in vitro cell-based assay system wherein GA was found to induce GLUT4 translocation with subsequent stimulation of glucose uptake. Increased GLUT4 expression on the plasma membrane is important for the uptake of glucose into the adipose and muscle tissues, and plays a key role in maintaining normal blood glucose level.

To investigate the mechanism of stimulation of GLUT4 translocation and glucose uptake by GA, we examined the effect of GA on cellular signaling pathways known to modulate these processes. It has been shown that GLUT4 translocation and glucose uptake are mediated by two major pathways; an insulin independent AMPK pathway and an insulin mediated PI3K pathway. Recently, Lee et al. have reported that berberine stimulates glucose uptake in 3T3-L1 adipocytes and L6 myotubes in an AMPK-dependent pathway [40]. To identify whether GA also increases GLUT4 translocation in a similar manner, we determined the level of phosphorylated AMPK in GA treated 3T3-L1 cells. However, we did not see any AMPK activation (Fig. 4A), suggesting that AMPK does not play a role in GA mediated glucose uptake.

Activation of PI3K is a major event in the early phases of insulin signaling cascade. Further, this insulin stimulated PI3K signaling diverges into two pathways, an Akt dependent pathway and a protein kinase C mediated pathway [41]. Even though Akt activation is important for insulin mediated glucose uptake, studies have shown that insulin can increase glucose uptake in cells expressing dominant negative mutant of Akt [42]. Furthermore, it is reported that the ED50 for insulin stimulated GLUT4 translocation in adipocytes is less than that of insulin stimulated Akt activation [43]. It is known that insulin activates aPKCζ/λ as a downstream effector of PI3K activation [44]. Our findings demonstrate that GA induces glucose uptake in a PI3K dependent manner and not through the activation of AMPK. This is confirmed by the inhibitory effect of wortmannin on GA induced glucose uptake. Further, to identify the downstream activators of PI3K which are involved in GA stimulated glucose uptake, we analyzed the phosphorylation status of Akt and aPKCζ/λ. Unlike insulin, GA did not stimulate Akt phosphorylation in differentiated adipocytes. Also Akt inhibitor did not inhibit GA mediated glucose uptake suggesting the absence of Akt involvement in GA mediated glucose uptake. However, GA treatment resulted in the activation of PKCζ/λ similar to insulin.

The participation of PKCζ in insulin stimulated glucose uptake has been documented in muscles and adipocytes [45]. Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), the product of insulin mediated PI3K activation, recruits PDK-1 to the plasma membrane by direct binding to its pleckstrin homology (PH) domain. When translocated to the membrane, PDK-1 activates PKCζ by phosphorylating Thr410 [46]. In adipocytes, Cb1-TC10 pathway is also activated in parallel to insulin signaling pathway, and PKCζ/λ serves as a convergent downstream target for IRS-PI3K and Cb1-TC10 pathway in 3T3-L1 adipocytes. Studies have shown that TC10 activation leads to actin remodeling through PKCζ and is important for insulin stimulated GLUT4 translocation [47,48]. In adipocytes, activated TC10 recruits PKCζ/λ to the plasma membrane through Par6–Par3 complex. Also it has been shown that PKCζ interacts with protein kinase C substrate 80-KH and releases the clamp action of Munc-18c to facilitate the docking of GLUT4 vesicle [49]. Our results suggest that GA stimulates GLUT4 translocation and glucose uptake in an aPKCζ/λ dependent manner. Further studies need to be carried out to understand how GA induced aPKCζ/λ activation mediates GLUT4 translocation and vesicle sorting.

Acknowledgements

The research was supported by a grant from Council of Scientific and Industrial Research, Govt. of India [EMR No. 38(1216)/09]. The authors wish to thank Prof. Jeffrey Pessin, Stony Brook University, New York, USA, for the kind gift of the myc-GLUT4-GFP construct. We thank Dr. Sonali Barwe for the critical reading of the manuscript. We also thank Mr. Anup Kumar Nair for his valuable comments.

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