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Ellagic Acid Stimulates Glucose Transport in Adipocytes and Muscles through AMPK Mediated Pathway

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Abstract

Glucose transporter 4 (GLUT4) plays a pivotal role in maintaining whole body glucose homeostasis by mediating insulin responsive glucose transport into adipocytes and skeletal muscles. This is achieved through translocation of GLUT4 from an intracellular pool to the cell surface and certain compounds may enhance this process. In the present study we have shown that ellagic acid, a plant polyphenol, can stimulate glucose uptake activity in both 3T3-L1 adipocytes and C2C12 myotubes by inducing GLUT4 translocation. Unlike insulin, ellagic acid did not stimulate the Ser473 phosphorylation and activation of Akt. However, it was found to induce AMP-activated protein kinase (AMPK) activation in both cell lines. Analyzing the downstream mechanism suggested an absence of involvement of Rab GAP AS160 in ellagic acid induced GLUT4 translocation. Further studies revealed that ellagic acid stimulated glucose transport occurs though a mechanism involving extracellular signal-regulated kinase (ERK1/2) and atypical PKC ζ/λ (aPKC ζ/λ) activation.

Keywords: Ellagic acid; GLUT4 translocation; Glucose uptake; AMP activated Protein Kinase; Extracellular signal regulated kinase; Atypical protein kinase C

Abbreviations: GLUT4: Glucose Transporter 4; EA: Ellagic Acid; AMPK: AMP Activated Protein Kinase; AS160: Akt Substrate of 160 kDa; ERK: Extracellular Signal Regulated Kinase; a PKC: atypical Protein Kinase C

Introduction

Glucose transport in to adipocytes and skeletal muscles is a major mechanism by which the body disposes excess glucose from the blood stream after a meal. These tissues express a unique glucose transporter isoform known as glucose transporter 4 (GLUT4) [1]. The majority of the GLUT4 expressed are retained in intracellular compartments in an unstimulated state and undergo rapid redistribution to the plasma membrane in the presence of the hormone, insulin, to carry out the massive uptake of glucose [2,3]. The insulin stimulated GLUT4 translocation is mainly mediated through a phosphatidylinositol 3-kinase (PI3K) dependent pathway [4]. Studies have shown that activation of AMP-activated protein kinase (AMPK) pathway can also induce GLUT4 translocation [5]. Defects in the insulin signal transduction machinery which in turn results in impaired GLUT4 translocation is one of the leading causes for insulin resistance and type 2 diabetes [6]. In this scenario, there is considerable interest in finding molecules that enhance GLUT4 translocation and glucose transport. Given the role of AMPK in regulating glucose transport, compounds that activate AMPK would have significant therapeutic implications.

Several plant extracts and/or small molecules have been shown to demonstrate hypoglycemic activity in animal model systems [7-9] as well as in cell based assay systems [10-13]. A study from our laboratory has identified gallic acid as the active principle from seabuckthorn leaf extract that induces glucose uptake in 3T3-L1 adipocytes [14]. It was later proved to be antihyperglycemic in streptozotocin induced diabetic rats [8]. Another example is berberine, an isoquinoline alkaloid that has gained substantial attention due to its blood glucose lowering effects in diabetic and insulin resistant states [15,16]. The widely used oral hypoglycemic agent metformin itself is a derivative of galegine from the plant *Galega officinalis* [17]. In an attempt to identify novel modulators of glucose transport from medicinal plant extracts, the methanol extract prepared from the leaves of *Terminalia arjuna* was found to stimulate glucose transport in 3T3-L1 adipocytes. Fractionation of the extract led to the identification of ellagic acid (EA) as an active component present in the extract. Further studies revealed that EA stimulated GLUT4 translocation occurs in an AMPK dependent manner and involves extracellular signal-regulated kinase 1/2 (ERK1/2) and atypical protein kinase C ζ/λ (aPKC ζ/λ) activation.

Materials and Methods

Chemicals and reagents

Tissue culture media and supplements were purchased from Sigma Aldrich (St Louis, MO, USA). 3T3-L1 fibroblasts and C2C12 myoblasts were obtained from National Centre for Cell Sciences, Pune, Maharashtra, India. 2-deoxy-D-[^{3}H]-glucose was purchased from Amersham Life sciences (Buckinghamshire, UK). The myc-GLUT4-GFP construct was a kind gift from Prof. Jeffrey E. Pessin, (Albert Einstein College of Medicine, NY, USA). The primary antibodies against myc epitope, phospho-Akt (Ser^{473}), Akt, phospho-AMPK alpha (Thr^{172}), AMPK, phospho-PKC ζ/ λ (Thr$^{410/403}$), phospho-ERK (Thr^{202}/ Tyr^{204}), ERK ½, phospho-As160 (Thr^{642}) and β-actin, alexa-conjugated, and HRP-conjugated anti-mouse, anti-rabbit secondary antibodies and Enhanced chemical luminescence (ECL) were purchased from Cell Signaling (Beverly, MA, USA). Wortmannin, Compound C, PD98059 and the authentic sample of EA were obtained from Sigma. Berberine was kindly provided by Dr Asoke Banerji (Amrita Vishwa Vidyapeetham, India). Organic solvents for extraction and other chemicals used were of the highest analytical grade.

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Plant material, extraction and fractionation

Terminalia arjuna leaves were collected from Wayanad, Kerala, India. The authenticity of the plant was confirmed by Dr M.K. Ratheesh Narayanan, Senior Scientist, M S Swaminathan Research Foundation (MSSRF), Wayanad. Leaves (485g) were dried in hot air oven at 50^0C, powdered and successively extracted with petroleum ether, chloroform, ethyl methyl ketone and methanol in a soxhlet apparatus. A portion of the methanol extract was subjected to acid hydrolysis (8% H_2SO_4, 2h at 70°C) and the individual components were separated by column chromatography. The progress of separation of compounds in column was monitored by thin-layer chromatography (TLC); circular paper chromatography and high-performance liquid chromatography (HPLC, Shimadzu, CTO-104SUP) were used for the direct comparison of isolated compound with authentic sample. Confirmatory chemical tests, melting point determination, optical spectroscopy (infra red, ultra violet) were used for molecular characterization.

Cell culture, differentiation and transfection

3T3-L1 fibroblasts and C2C12 myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 0.1% amphotericin B in an atmosphere of 5% CO_2 at 37°C. Differentiation of 3T3-L1 fibroblasts was induced by incubating confluent plates in differentiation induction medium containing 500 µM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 100 nM insulin. Two days after induction, the medium was replaced with DMEM containing 100 nM insulin. The medium was subsequently replaced with fresh culture medium every alternative day until the cells attained adipocyte morphology. Differentiation of C2C12 myoblasts was induced by switching confluent cells to DMEM supplemented with 2% FBS and allowing formation of myotubes with medium changes every 24 h. Cells were used for experiments 4-5 days after differentiation. 3T3-L1 preadipocytes were transfected with the myc-GLUT4-GFP construct by electroporation at 250 mV. The transfected cells were maintained in DMEM containing 600 µg/ml G418 to allow the growth of stable clones. Each clone was subcultured and monitored by fluorescent microscopy to detect GFP expression. One of these clones with the proper intracellular localization of GFP was selected for further studies.

Glucose uptake assay

Differentiated 3T3-L1 adipocytes and C2C12 myotubes grown on 24-well plates were serum starved for two hours and treated with 0.1% dimethyl sulphoxide (basal), 50 nM insulin (positive control) or various concentrations of EA for 30 min. After induction, cells were washed with Krebs-Ringer-Phosphate (KRP) buffer (pH 7.4) containing 130 mM NaCl, 5 mM KCl, 0.8 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM Na_2HPO_4. Glucose uptake was initiated by the addition of 0.5 ml KRP buffer containing 10 µM 2-deoxy D-glucose and 0.25 µCi/ml 2-deoxy-D-[^{3}H]-glucose at 37°C. Glucose uptake was terminated after 5 min by three rapid washes with ice-cold KRP buffer. Cells were lysed with 0.1% sodium dodecyl sulfate (SDS) and the cell associated radioactivity was measured in liquid scintillation counter (Beckman Coulter, LS 6500, Fullerton, CA, USA). Glucose uptake assay was also conducted in cells prior treated with inhibitors; wortmannin, compound C or PD98059.

GLUT4 translocation assay

3T3-L1 cells stably expressing a myc-GLUT4-GFP chimera were grown to 70% confluency on glass cover slips. After serum starvation for 2 h, cells were incubated with varying concentrations of EA for 30 min. Treatment with 50 nM insulin was used as the positive control. The cells were washed twice with phosphate buffered saline (PBS), and fixed with 2% paraformaldehyde for 30 minutes in dark. The cells were again washed with PBS and quenched with 50 mM NH_4Cl for 10 minutes. The cells were then incubated with anti-myc antibody for 1h 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. Fluorescence intensity of the cells attached to cover slips was quantified and plotted. Fluorescence intensity was calculated by Image-Pro Plus software (version 5.1.2).

Immunoblotting

Differentiated adipocytes and muscles were serum starved for 2 h. Following treatment with insulin and EA for 30 min, cells were washed with PBS and lysed with 1× SDS loading buffer. Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked for 1 h with 5% (w/v) BSA, incubated with primary antibodies overnight at 4°C and the secondary antibodies for 1 h at room temperature. The bands were detected by the ECL kit.

Statistical analysis

The data are expressed as mean ± s.d. Statistical comparisons were made using Student's unpaired t-test using Microsoft excel and P-values < 0.05 were considered significant.

Results

Extraction and isolation of ellagic acid from *Terminalia arjuna* leaves

Each of the sequential extracts was analyzed for their effect on glucose transport in 3T3-L1 adipocytes, among which the methanol extract (hence forth referred to as TM) stimulated glucose uptake in a dose dependent manner (Figure 1A). The compounds in the extract, due to their possible complex nature, did not resolve well in the TLC. Hence an acid hydrolysis was carried out with aqueous sulfuric acid followed by extraction with ethyl acetate. In order to isolate the active compound, the products of hydrolysis (henceforth referred to as TMH) were subjected to silica column chromatography. Several fractions were collected and pooled together based on their similarity in TLC. The active fraction was a light beige crystalline powder which was slightly soluble in water and was found to be ferric chloride positive. HPLC analysis of this fraction suggested the presence of a single peak with a characteristic UV absorption at 253 and 367 nm. The preliminary observations suggest that the isolated compound could be EA and it was confirmed by direct comparison of UV, IR, melting point, TLC and paper chromatography data of the compound with an authentic sample of EA. The subsequent studies were carried out with commercially obtained EA.

Ellagic acid stimulates glucose transport in 3T3-L1 adipocytes and C2C12 myotubes

In the present study, TM displayed a concentration dependent increase in glucose uptake activity in 3T3-L1 cells, with a maximum response at a concentration of 50µg/ml (Figure 1A). TMH fraction also exhibited a similar activation of glucose uptake suggesting that acid hydrolysis has not affected the activity of the compounds (data not shown). Subsequent fractionation led to the identification of EA as an active principle in the TMH fraction. Treatment with EA for 30 min induced basal glucose uptake in 3T3-L1 adipocytes in a concentration dependent manner. A maximumum of 1.9 fold increase

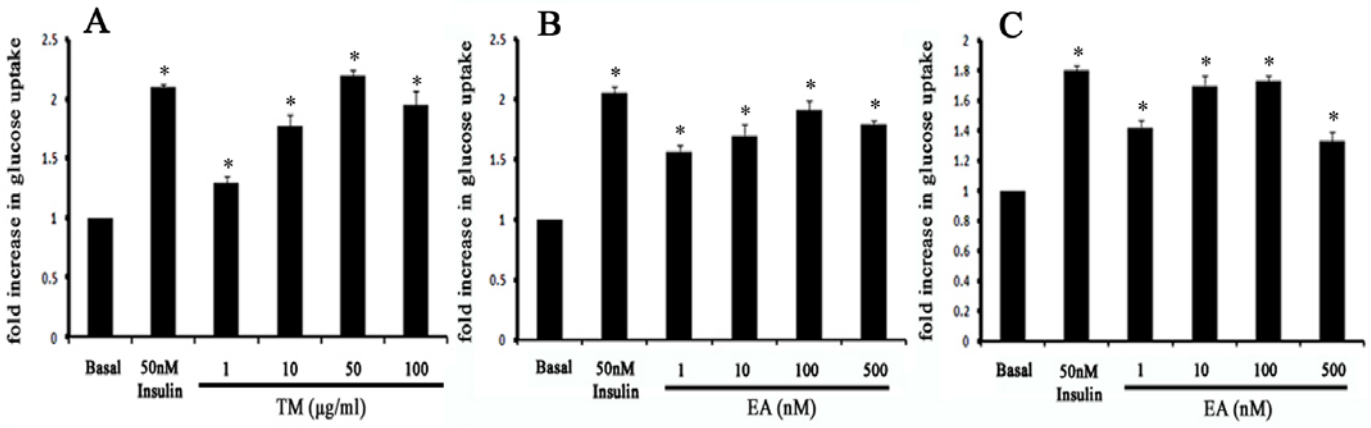

Figure 1: Effect of TM and EA on glucose uptake. Differentiated cells were serum starved for 2 hours and incubated with varying concentrations TM or EA for 30 minutes. Graph showing increase in glucose uptake upon treatment with different concentrations of TM in 3T3-L1 adipocytes (A). Graph showing concentration dependent increase in glucose uptake with EA in 3T3-L1 cells (B) and C2C12 cells (C). Cells incubated with vehicle (0.1% DMSO) alone were used to measure the basal rate of glucose uptake and 50 nM insulin as positive control in both cell types. Data shown are fold increase in glucose uptake compared to the basal. Values are shown as the mean ± S.D. of three different experiments carried out in duplicates. *P<0.05 vs. unstimulated cells.

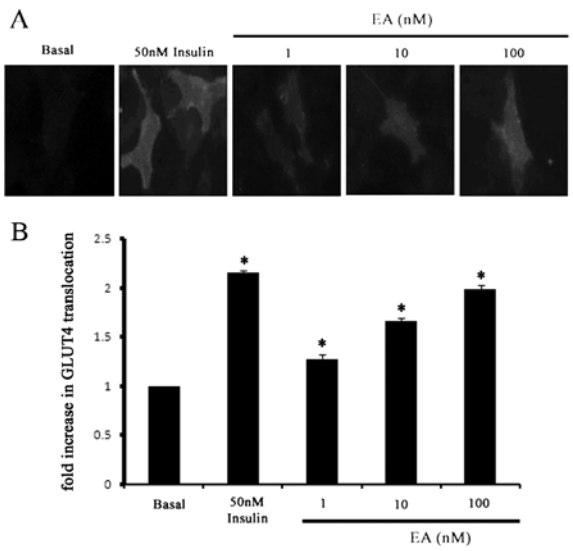

Figure 2: Effect of EA on GLUT4 translocation. 3T3-L1 preadipocytes stably expressing myc-GLUT4-GFP chimera were serum starved for 2 h and incubated with indicated concentrations of EA or insulin for 30 min. Myc epitope externalization was measured by indirect immunofluorescence. (A) Images showing cell surface GLUT4 and (B) showing fold increase in fluorescence intensity of secondary antibody upon treatment with insulin or EA compared to the basal. Cells treated with vehicle (0.1% DMSO) were used to measure non-specific fluorescence. Values are shown as the mean ± S.D. of three different focal planes of the same experiment. *P<0.05 vs. unstimulated cells.

in glucose uptake was observed with 100nM EA and further increase in concentration reduced the rate of glucose transport (Figure 1B). EA (1-500nM) was also found to stimulate glucose uptake in C2C12 myotubes. There was a 1.7 fold increase in glucose uptake activity at 100 nM concentration beyond which the activity was reduced (Figure 1C). 50 nM insulin was used as the positive control in these assays.

Ellagic acid stimulated GLUT4 translocation in transfected 3T3-L1 preadipocytes

GLUT4 translocation from an intracellular pool to the cell surface is the primary factor responsible for insulin induced glucose uptake in adipocytes and skeletal muscles. This process can be studied *in vitro* by employing a chimeric GLUT4 construct with an epitope at an extracellular domain so that the extent of the epitope externalization directly matches with the amount of GLUT4 present on the plasma membrane [18]. To determine whether the increased glucose uptake by EA was a result of enhanced GLUT4 translocation, cell surface expression of GLUT4 was monitored. Upon treatment with different concentrations of EA, the rate of GLUT4 translocation was increased in a dose dependent manner and 100nM EA showed a 2 fold increase in 3T3-L1 cells (Figure 2A and 2B). This increase in GLUT4 translocation correlates with the increase in glucose uptake observed at the same concentration. EA treatment was also found to induce GLUT4 translocation in differentiated myotubes as assessed from plasma membrane sheet assay (data not shown).

EA stimulates the activation of AMPK but not of Akt

Since EA was stimulating GLUT4 translocation our next attempt

was to delineate the signaling mechanism involved in this process. To evaluate the role of protein kinase B (PKB/Akt), classical insulin signaling pathway intermediate, in EA stimulated glucose transport, differentiated adipocytes were treated with 100 nM EA for 30 minutes and the phosphorylation status of Akt was examined. Unlike insulin, EA had no effect on Ser473 phosphorylation and activation of Akt in 3T3-L1 adipocytes and C2C12 mytubes (Figure 3A and 3B). This suggests that EA induced glucose transport is not mediated through Akt pathway.

AMPK activation is known to stimulate glucose transport in adipocytes and muscles in an insulin independent manner [19]. We have analyzed the phosphorylation status of AMPK in response to 100 nM EA treatment in 3T3-L1 adipocytes and C2C12 myotubes. A well known AMPK activator, berberine was used as the positive control [15]. EA induced AMPK activation in both 3T3-L1 adipocytes (Figure 3C) and C2C12 myotubes (Figure 3D). There was a 1.7 fold increase in AMPK phosphorylation in response to EA in 3T3-L1 adipocytes and a 1.8 fold increase in C2C12 myotubes. These results suggest that AMPK activation is involved in mediating the metabolic effect of EA.

EA induced activation of ERK and atypical PKC

Since EA was activating AMPK our next attempt was to identify the downstream targets. Both AMPK and Akt are known to mediate its effect on GLUT4 through phosphorylating and inactivating Akt substrate of 160 kDa (AS160), a rab GTPase activating protein (GAP) normally bound to GLUT4 vesicles restricting its translocation [20]. The phosporylation status of AS160 was analyzed after treatment with 100 nM EA for 30 minutes. An increase in AS160 phosphorylation was not observed with EA in either adipocytes or muscles, where as 50 nM insulin enhanced AS160 phosphorylation in both cell lines (Figure 4A and 4B). This means that EA effect on GLUT4 translocation is independent of AS160.

Another pathway that operates downstream to AMPK is ERK/Phospholipase D (PLD)/aPKC pathway [21]. The effect of EA on ERK1/2 activation in adipocytes and myotubes was investigated. As seen in (Figure 4C and 4D), phosphorylated ERK1/2 was significantly raised in adipocytes and muscles respectively. Next the phosphorylation status of atypical PKC ζ/λ was analyzed. Treatment with EA resulted in a 1.4 fold activation of PKC ζ/λ in both 3T3-L1 adipocytes (Figure 4E) and C2C12 myotubes (Figure 4F). Therefore protein phosphorylation studies with EA suggest that its stimulatory effect on GLUT4 translocation is achieved through stimulating AMPK-ERK-aPKC pathway.

Effects of inhibitors on EA signaling

Inhibitors of AMPK, MEK and PI3K were employed to analyze their effects on EA stimulated glucose transport in C2C12 myotubes. It was found that glucose transport stimulated by EA was inhibited by treatment with AMPK inhibitor Compound C and MEK inhibitor PD98059 (Figure 5). However, PI3K inhibitor wortmannin was not found to have a significant effect on EA stimulated glucose uptake (Figure 5). Compound C effectively blocked EA induced AMPK phosphorylation (Figure 6A). Interestingly it was observed that application of compound C also inhibits ERK1/2 phosphorylation to a similar extent, suggesting that AMPK lies upstream to ERK in the EA signaling pathway (Figure 6B).

Discussion

In the present study, we have shown for the first time glucose uptake stimulatory property of EA in both adipocyte and muscle cell lines. EA is a polyphenol naturally occurring in berries and nuts mainly as ellagitannins [22]. Many studies have reported beneficial effects of EA in preventing various kinds of cancer [23-25]. EA has also been shown to act as antioxidant [26], antimicrobial [27] and antimutagenic agent [28]. However, so far no study has reported any antidiabetic potential for the compound. *Terminalia arjuna* (Combretaceae) is an ayurvedic plant used for the treatment of various ailments [29] and its leaf extract has recently been shown to possess antihyperglycemic activity in streptozotocin-induced diabetic rats [30]. The study reported here is a bioassay directed fractionation and identification of EA as an active principle from *Terminalia arjuna* leaves that induces GLUT4 translocation and glucose uptake in both adipocyte and muscle cell lines.

EA stimulated GLUT4 translocation and glucose uptake was mediated through AMPK activation while there was no effect on Akt activation. AMPK is a serine threonine protein kinase, considered as

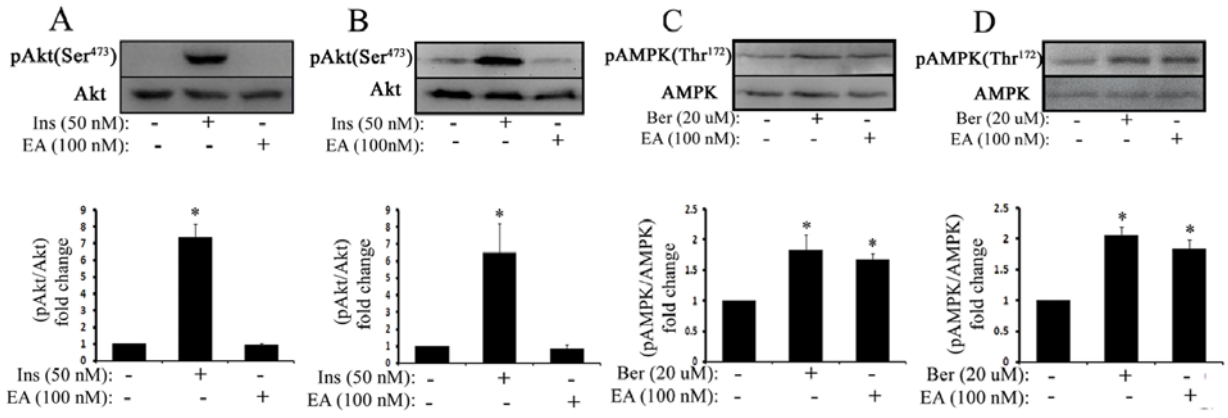

Figure 3: Effect of EA on phosphorylation status of Akt and AMPK. Differentiated adipocytes and myotubes were serum starved for 2h followed by treatment with insulin or berberine (Ber) and EA as indicated for 30 min and phosphorylation status of Akt and AMPK was determined by western blot analysis. Treatment with EA did not stimulate Akt phosphorylation in adipocytes (A) or muscles (B) but stimulated AMPK phosphorylation in adipocytes (C) and muscle cells (D). The protein bands were scanned and the intensities were determined using Bio-rad's quantity one software. Data are average of at least three independent experiments. *P<0.05 compared with basal.

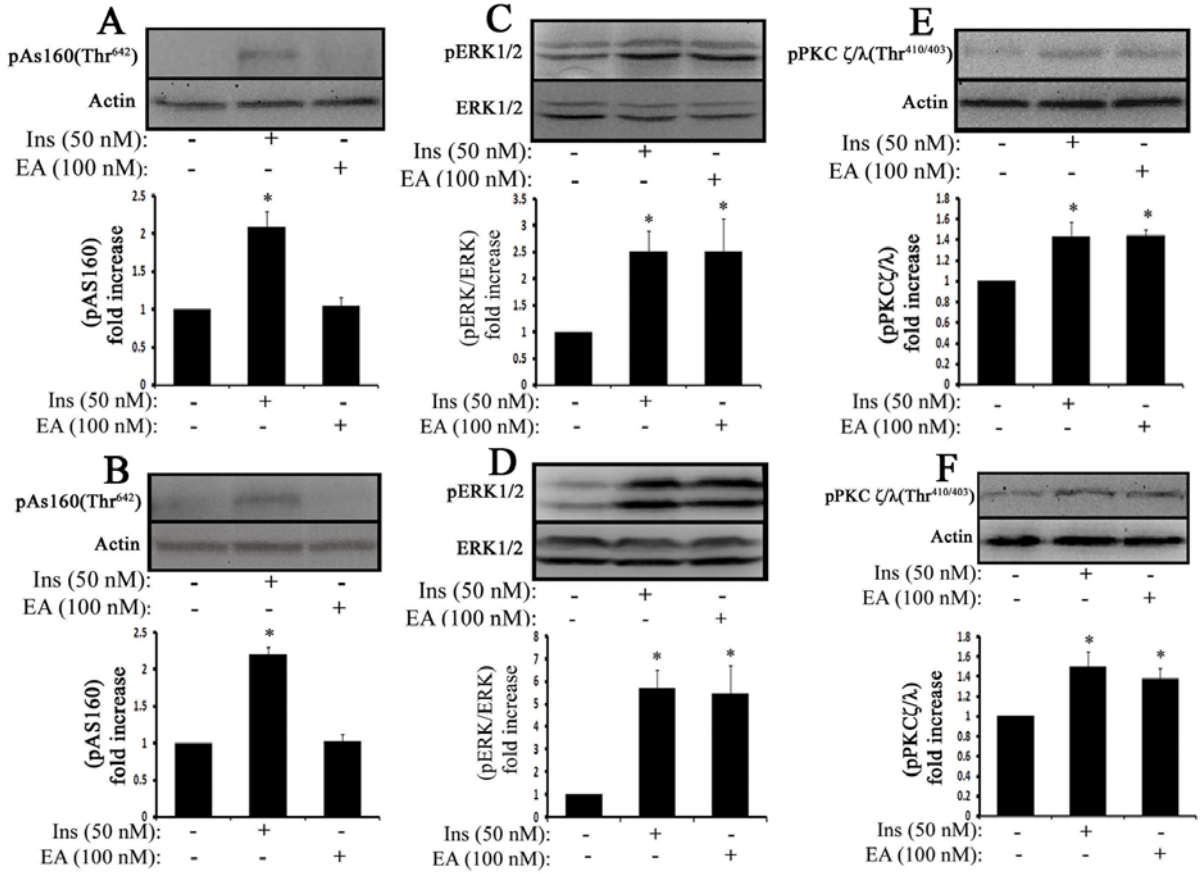

Figure 4: Effect of EA on phosphorylation status of AS160, ERK 1/2 and aPKCζ/λ. Differentiated adipocytes and myotubes were treated with 50 nM insulin or 100 nM EA for 30 min. EA neither stimulated AS160 phosphorylation in adipocytes (A) nor in muscles (B). EA induced ERK and aPKC activation in both adipocytes (C & E) and muscles (D & F). Data shown are representative immunoblots of 3-5 independent experiments. Bars represent mean ± S.D. *P<0.05 compared with basal.

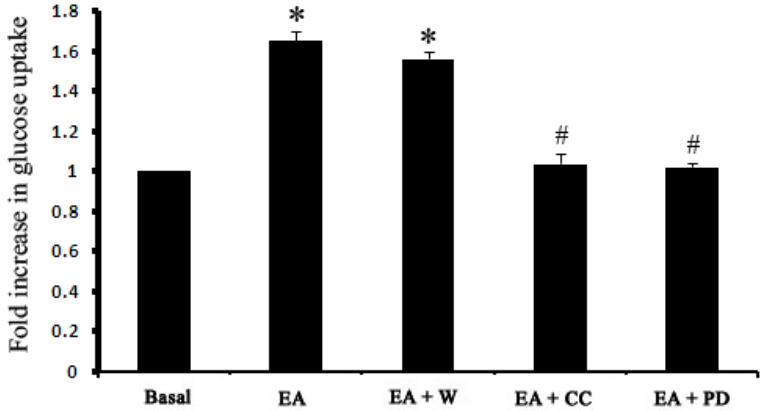

Figure 5: Effect of inhibitors on EA stimulated glucose transport. C2C12 myotubes were serum starved for 2 h, treated with or without 50 μM Compound C (CC) for 1 hour or 50 μM PD98059 (PD) for 30 min or 100 nM wortmannin (W) for 20 minutes. After inhibitor treatment, cells were stimulated with 100 nM EA in the presence of either PD or CC. Cells were also stimulated with EA in the absence of inhibitors. Cells incubated with vehicle (0.1% DMSO) were used to measure the basal rate of glucose uptake. Values are shown as the mean ± S.D. of three different experiments carried out in duplicates. *P<0.05 vs. unstimulated cells. #P<0.05 vs. EA stimulated cells.

the metabolic sensor of the mammalian cells, and is activated by a rise in the cellular AMP:ATP ratio [5]. AMPK is activated in response to different metabolic stress' to facilitate a PI3K independent mode of glucose transport [19,31]. Therefore, it is a crucial player in regulating blood glucose homeostasis. The antidiabetic drug, metformin is a potent stimulator of AMPK and is widely used in diabetes therapy [32]. Several plant derived compounds have been shown to activate glucose transport through AMPK activation. Berberine is one such compound

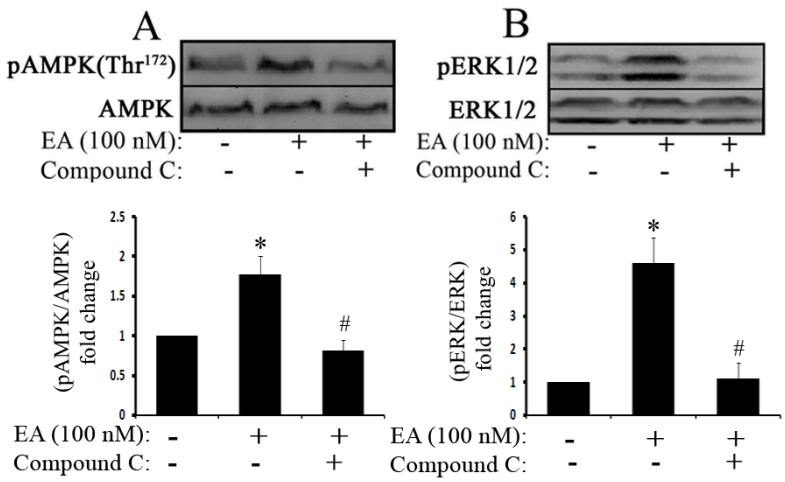

Figure 6: Effect of compound C on AMPK and ERK activation. Differentiated C2C12 myotubes were serum starved for 2 h and treated with 50 μM Compound C (CC) for 1 hour followed by treatment with 100 nM EA in the presence CC for 30 minutes. EA induced phosphorylation of AMPK (A) and ERK 1/2 (B) were reduced by CC treatment. Images shown are representative of three independent experiments and the quantitative data are average of three experiments. *P<0.05 vs. unstimulated cells. #P<0.05 vs. EA stimulated cells.

which mediates hypoglycemic effects through AMPK activation [15]. Curcumin, a principal curcuminoid of turmeric [33], salidroside, a bioactive component from *Rhodiola rosea* [34] and cryptotanshinone, a quinoid diterpene [35] were also reported to have AMPK mediated stimulatory effect on glucose uptake in adipocytes and muscles.

Activated AMPK is known to trigger GLUT4 translocation by phosphorylating and inactivating AS160 which normally acts to tether GLUT4 containing vesicles inside the cell [20]. Treatment with EA did not enhance the level of AS160 phosphorylation in either adipocytes or muscle cells suggesting that AS160 does not have any role in EA induced increase in GLUT4 translocation and glucose uptake. Another pathway that acts downstream of AMPK is aPKC pathway. AMPK activators, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and metformin increase glucose transport in L6 muscle cells through sequential activation of ERK, PLD and aPKC [21]. ERK mediated PLD activation generates phosphatidic acid which in turn activates aPKC. AMPK activator, dinitrophenol has also been found to activate glucose transport through ERK/PLD/aPKC pathway [36]. Sorbitol (osmotic stress) [37] and extracellular glucose [38] mediated GLUT4 translocation also occurs in PI3K independent, aPKC dependent manner. In the present study, EA was found to stimulate phosphorylation of ERK as well as aPKC in both adipocyte and muscle cell lines. Though the proximal signals involved in aPKC activation differ with respect to various stimuli, aPKC has undoubtedly been proven to be a master player in GLUT4 translocation. The mechanism by which aPKC activation contributes to GLUT4 trafficking is not clearly defined. PKC ζ is proposed to mediate its effect on GLUT4 translocation through actin remodeling [39]. PKC ζ has also been shown to interact with munc 18c in an insulin regulated manner suggesting an involvement of this interaction in enhanced GLUT4 translocation to the plasma membrane [40]. PKC λ has been shown to cause association of a small GTPase-binding protein Rab4 with motor protein kinesin that plays a role in translocating GLUT4 vesicles to the cell surface [41].

Studies have shown that AMPK activation is dispensable for some insulin independent glucose transport like contraction and hyperosmolarity [42]. In order to check whether AMPK activation caused by EA is requisite for glucose transport stimulation, a pharmacologic inhibitor of AMPK, compound C was employed. Since AMPK activation is known to induce ERK activity through MEK, an MEK1 inhibitor PD98059 was also employed to test whether MEK/ERK activation is essential for glucose uptake. It was found that glucose transport induced by EA was markedly inhibited by treatment with compound C as well as PD98059. Application of compound C also blocked EA induced ERK activation. These studies indicate that both AMPK and ERK activation is essential for mediating EA effect and that AMPK lies upstream to ERK in the signaling pathway. Application of wortmannin ruled out the possibility of PI3K involvement in the glucose uptake stimulatory effect of EA.

In conclusion our data suggest that EA stimulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes and C2C12 myotubes is mediated through AMPK-ERK-aPKC pathway. Further studies are needed to understand how EA induced aPKC ζ/λ activation mediates GLUT4 translocation and subsequent vesicle docking and fusion with the plasma membrane.

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References

1. James DE, Brown R, Navarro J, Pilch PF (1988) Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. Nature 333: 183-185.
2. Cushman SW, Wardzala LJ (1980) Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. J Biol Chem 255: 4758-4762.
3. Marette A, Richardson JM, Ramlal T, Balon TW, Vranic M, et al. (1992) Abundance, localization, and insulin-induced translocation of glucose transporters in red and white muscle. Am J Physiol 263: C443-452.

4. Hausdorff SF, Fingar DC, Morioka K, Garza LA, Whiteman EL, et al. (1999) Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. DissociationoOf insulin-stimulated glucose uptake and glut4 translocation. J Biol Chem 274: 24677-24684.

5. Fujii N, Jessen N, Goodyear LJ (2006) AMP-activated protein kinase and the regulation of glucose transport. Am J Physiol Endocrinol Metab 291: E867-877.

6. Frojdo S, Vidal H, Pirola L (2009) Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta 1792: 83-92.

7. Vijayakumar MV, Singh S, Chhipa RR, Bhat MK (2005) The hypoglycaemic activity of fenugreek seed extract is mediated through the stimulation of an insulin signalling pathway. Br J Pharmacol 146: 41-48.

8. Punithavathi VR, Prince PS, Kumar R, Selvakumari J (2011) Antihyperglycaemic, antilipid peroxidative and antioxidant effects of gallic acid on streptozotocin induced diabetic Wistar rats. Eur J Pharmacol 650: 465-471.

9. Herrera C, Garcia-Barrantes PM, Binns F, Vargas M, Poveda L, et al. (2011) Hypoglycemic and antihyperglycemic effect of Witheringia solanacea in normal and alloxan-induced hyperglycemic rats. J Ethnopharmacol 133: 907-910.

10. Tan MJ, Ye JM, Turner N, Hohnen-Behrens C, Ke CQ, et al. (2008) Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. Chem Biol 15: 263-273.

11. Liu F, Kim J, Li Y, Liu X, Li J, et al. (2001) An extract of Lagerstroemia speciosa L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. J Nutr 131: 2242-2247.

12. Giron MD, Sevillano N, Salto R, Haidour A, Manzano M, et al. (2009) Salacia oblonga extract increases glucose transporter 4-mediated glucose uptake in L6 rat myotubes: role of mangiferin. Clin Nutr 28: 565-574.

13. Abe D, Saito T, Sekiya K (2006) Sennidin stimulates glucose incorporation in rat adipocytes. Life Sci 79: 1027-1033.

14. Prasad CN, Anjana T, Banerji A, Gopalakrishnapillai A (2010) Gallic acid induces GLUT4 translocation and glucose uptake activity in 3T3-L1 cells. FEBS Lett 584: 531-536.

15. Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, et al. (2006) Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. Diabetes 55: 2256-2264.

16. Zhang Y, Li X, Zou D, Liu W, Yang J, et al. (2008) Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid berberine. J Clin Endocrinol Metab 93: 2559-2565.

17. Witters LA (2001) The blooming of the French lilac. J Clin Invest 108: 1105-1107.

18. Lampson MA, Racz A, Cushman SW, McGraw TE (2000) Demonstration of insulin-responsive trafficking of GLUT4 and vpTR in fibroblasts. J Cell Sci 113: 4065-4076.

19. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ (1998) Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 47: 1369-1373.

20. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, et al. (2006) Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes 55: 2067-2076.

21. Sajan MP, Bandyopadhyay G, Miura A, Standaert ML, Nimal S, et al. (2010) AICAR and metformin, but not exercise, increase muscle glucose transport through AMPK-, ERK-, and PDK1-dependent activation of atypical PKC. Am J Physiol Endocrinol Metab 298: E179-192.

22. Bakkalbasi E, Mentes O, Artik N (2009) Food ellagitannins-occurrence, effects of processing and storage. Crit Rev Food Sci Nutr 49: 283-298.

23. Losso JN, Bansode RR, Trappey A 2nd, Bawadi HA , Truax R (2004) In vitro anti-proliferative activities of ellagic acid. J Nutr Biochem 15: 672-678.

24. Umesalma S, Sudhandiran G (2011) Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. Eur J Pharmacol 660: 249-258.

25. Aiyer HS, Gupta RC (2010) Berries and ellagic acid prevent estrogen-induced mammary tumorigenesis by modulating enzymes of estrogen metabolism. Cancer Prev Res (Phila) 3: 727-737.

26. Han DH, Lee MJ, Kim JH (2006) Antioxidant and apoptosis-inducing activities of ellagic acid. Anticancer Res 26: 3601-3606.

27. Wohlfarth C, Efferth T (2009) Natural products as promising drug candidates for the treatment of hepatitis B and C. Acta Pharmacol Sin 30: 25-30.

28. Stoner GD , Mukhtar H (1995) Polyphenols as cancer chemopreventive agents. J Cell Biochem Suppl 22: 169-180.

29. Terminalia arjuna. (1999) Altern Med Rev 4: 436-437.

30. Biswas M, Kar B, Bhattacharya S, Kumar RB, Ghosh AK, et al. (2011) Antihyperglycemic activity and antioxidant role of Terminalia arjuna leaf in streptozotocin-induced diabetic rats. Pharm Biol 49: 335-340.

31. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, et al. (2000) Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. Diabetes 49: 527-531.

32. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, et al. (2002) Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes 51: 2074-2081.

33. Kim JH, Park JM, Kim EK, Lee JO, Lee SK, et al. (2010) Curcumin stimulates glucose uptake through AMPK-p38 MAPK pathways in L6 myotube cells. J Cell Physiol 223: 771-778.

34. Li HB, Ge YK, Zheng XX , Zhang L (2008) Salidroside stimulated glucose uptake in skeletal muscle cells by activating AMP-activated protein kinase. Eur J Pharmacol 588: 165-169.

35. Kim EJ, Jung SN, Son KH, Kim SR, Ha TY, et al. (2007) Antidiabetes and antiobesity effect of cryptotanshinone via activation of AMP-activated protein kinase. Mol Pharmacol 72: 62-72.

36. Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, et al. (2002) Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport. J Biol Chem 277: 23554-23562.

37. Sajan MP, Bandyopadhyay G, Kanoh Y, Standaert ML, Quon MJ, et al. (2002) Sorbitol activates atypical protein kinase C and GLUT4 glucose transporter translocation/glucose transport through proline-rich tyrosine kinase-2, the extracellular signal-regulated kinase pathway and phospholipase D. Biochem J 362: 665-674.

38. Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert ML, Quon MJ, et al. (2001) Glucose activates protein kinase C-zeta /lambda through proline-rich tyrosine kinase-2, extracellular signal-regulated kinase, and phospholipase D: a novel mechanism for activating glucose transporter translocation. J Biol Chem 276: 35537-35545.

39. Liu XJ, Yang C, Gupta N, Zuo J, Chang YS, et al. (2007) Protein kinase C-zeta regulation of GLUT4 translocation through actin remodeling in CHO cells. J Mol Med 85: 851-861.

40. Hodgkinson CP, Mander A, Sale GJ (2005) Protein kinase-zeta interacts with munc18c: role in GLUT4 trafficking. Diabetologia 48: 1627-1636.

41. Imamura T, Huang J, Usui I, Satoh H, Bever J, et al. (2003) Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. Mol Cell Biol 23: 4892-4900.

42. Fujii N, Hirshman MF, Kane EM, Ho RC, Peter LE, et al. (2005) AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. J Biol Chem 280: 39033-39041.