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Kaempferitrin inhibits GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes

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ABSTRACT

Insulin stimulated GLUT4 (glucose transporter 4) translocation and glucose uptake in muscles and adipocytes is important for the maintenance of blood glucose homeostasis in our body. In this paper, we report the identification of kaempferitrin (kaempferol 3,7-dirhamnoside), a glycosylated flavonoid, as a compound that inhibits insulin stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. In the absence of insulin, we observed that addition of kaempferitrin did not affect GLUT4 translocation or glucose uptake. On the other hand, kaempferitrin acted as an inhibitor of insulin-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes by inhibiting Akt activation. Molecular docking studies using a homology model of GLUT4 showed that kaempferitrin binds directly to GLUT4 at the glucose transportation channel, suggesting the possibility of a competition between kaempferitrin and glucose during the transport. Taken together, our data demonstrates that kaempferitrin inhibits GLUT4 mediated glucose uptake at least by two different mechanisms, one by interfering with the insulin signaling pathway and the other by a possible competition with glucose during the transport.

GLUT4 plays an important role in stimulating glucose uptake into muscles and adipocytes [1]. This is achieved by increasing the membrane translocation and activation of GLUT4 in an insulin dependent or independent manner [2,3]. GLUT4 is a 12 transmembrane facilitative glucose transporter (GLUT) protein, which belongs to Major Facilitator Super (MFS) family [4,5]. As adipocytes and skeletal muscles are the major sites for glucose disposal, defects in GLUT4 translocation and activation result in decreased glucose uptake by these cells. This leads to a concomitant rise in blood glucose level and ends up with diabetes [6].

The structural uniqueness of GLUT4 is characteristic for its membrane trafficking as well as glucose transportation activity. Studies have shown that different motifs in carboxy and amino terminal ends play key roles in GLUT4 translocation [7–10]. But this translocation alone is not sufficient to increase the glucose uptake in muscles and adipocytes. The transporter activity of GLUT4 is also important [11]. It is reported that the seventh *trans*-membrane domain of GLUT4 is important for its substrate selection and determining the Km for 2-deoxy glucose [12]. Further studies on this *trans*-membrane region revealed the presence of a conserved QLS domain in different high affinity glucose transporters like GLUT1, GLUT3 and GLUT4 [13]. Since GLUT4 is responsible for glucose uptake in insulin sensitive cells [14], it is important to search for modulators of GLUT4 translocation and glucose transport, and to identify how these compounds interact with GLUT4.

Extracts from different plants were reported to modulate glucose uptake activity in cultured cells. However, very few studies have been carried out to isolate and characterize the active compound/s from the plant extract and understand the molecular mechanism by which these compounds affect glucose uptake [15–19]. Here we found that the polyphenolic fraction from the leaf extract of *Bauhinia acuminata*, a species native to tropical southeastern Asia, inhibits insulin stimulated glucose uptake in 3T3-L1 adipocytes. Further purification of this polyphenolic fraction identified a flavonol glycoside kaempferitrin (kaempferol 3,7-dirhamnoside) as the active compound that inhibits insulin stimulated GLUT4 translocation and glucose uptake. Molecular docking studies demonstrated that kaempferitrin directly interacts with the substrate binding site present in the glucose transportation channel in GLUT4. Together, our data suggests that kaempferitrin inhibits GLUT4 translocation and glucose uptake in adipocytes by inhibiting insulin stimulated GLUT4 translocation and by directly blocking the glucose transport channel in GLUT4.

Materials and methods

Chemicals and reagents. All cell culture solutions and supplements were obtained from Sigma (St Louis, MO, USA). 3T3-L1 cells were obtained from National Centre for Cell Sciences, Pune, Maharashtra, India. Primary antibody against GLUT4 was a kind gift from

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Dr. Samuel W. Cushman, NIDDK, National Institutes of Health Building, Bethesda, USA and Cy3-conjugated anti-rabbit secondary antibodies were from Cell signaling technology (Beverly, MA, USA). Organic solvents and other chemicals used for extraction and purification were of the highest analytical grade.

Plant extraction and column purification. Fresh leaves of *B. acuminata* were dried in hot air oven for 5 hrs at 50°C and powdered. The leaf powder was extracted with methanol and fractionated by petroleum ether, chloroform, methyl ethyl ketone (MEK) and water. MEK fraction was further purified by column chromatography. Several fractions were collected and pooled based on the similarity in thin layer chromatography (TLC) pattern. Positive flavonoid fraction was further purified by preparative TLC in a bioassay directed manner and the active fraction was eluted out from the plate. This fraction was subjected to various color reactions, TLC, High Performance Liquid Chromatography (HPLC) and was compared with an authentic sample of kaempferitrin.

Cell culture and differentiation. 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. 3T3-L1 fibroblasts were differentiated into adipocytes 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. The medium was subsequently replaced with fresh culture medium after 2 days and then every other day till the cells attained adipocyte morphology.

Plasma membrane sheet assay. Differentiated 3T3-L1 cells grown on cover slips were serum starved for 2 h and were induced either with insulin alone or with various concentration of kaempferitrin in the presence or absence of insulin for 30 min. Plasma membrane sheet was prepared following standard protocol [20] with slight modifications. Briefly, after experimental treatments, cover slips were washed in phosphate buffered saline (PBS) and treated with 0.5 mg/ml poly-L-lysine in PBS. The cells were swollen by three rapid washes in hypotonic buffer (0.33 × Buffer B), transferred to buffer B (70 m MKCl, 30 mM HEPES, 5 mM $MgCl_2$, 3 mM EGTA, pH 7.4), and briefly sonicated to generate a lawn of plasma membrane fragments attached to cover slip. These were fixed with 2% paraformaldehyde and incubated with anti-GLUT4 antibody for 1 h at room temperature. Cover slips were washed and counter stained with Cy3-conjugated anti-rabbit secondary antibody for 30 min, and membrane GLUT4 was visualized under fluorescent microscope. The images were taken with a 40× objective (Olympus-X-71, Olympus America Inc, USA), and the total surface fluorescence of the secondary antibody was taken into consideration. Fluorescent intensity was calculated by image Pro-plus (version 5.1.2) software.

Glucose uptake assay. Differentiated cells were induced with various concentrations of kaempferitrin in the presence or absence of 50 nM insulin 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 with 10 μM 2-deoxy D-glucose and 0.1 μCi 2-deoxy-D-[^{3}H]-glucose. Glucose uptake was terminated after 10 min 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 liquid scintillation counter (Beckman Coulter, LS 6500, Beckman Coulter Inc. USA).

Protein phosphorylation study. Fully differentiated adipocytes were serum starved for 2 h and treated with 50 nM insulin and varying concentrations of kaempferitrin for 30 min. Cells were washed twice with ice-cold PBS and lysed in a buffer containing 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 and 1 μg/ml leupeptin for 5 min. Cells were scraped, briefly sonicated for 5 s, and centrifuged at 14000g for 10 min to remove the cell debris. Total protein was estimated and was resolved on a 10% SDS polyacrylamide gel, transferred to Immun-Blot PVDF membranes (Bio-Rad), blocked using 5% (w/v) non-fat dried milk in a

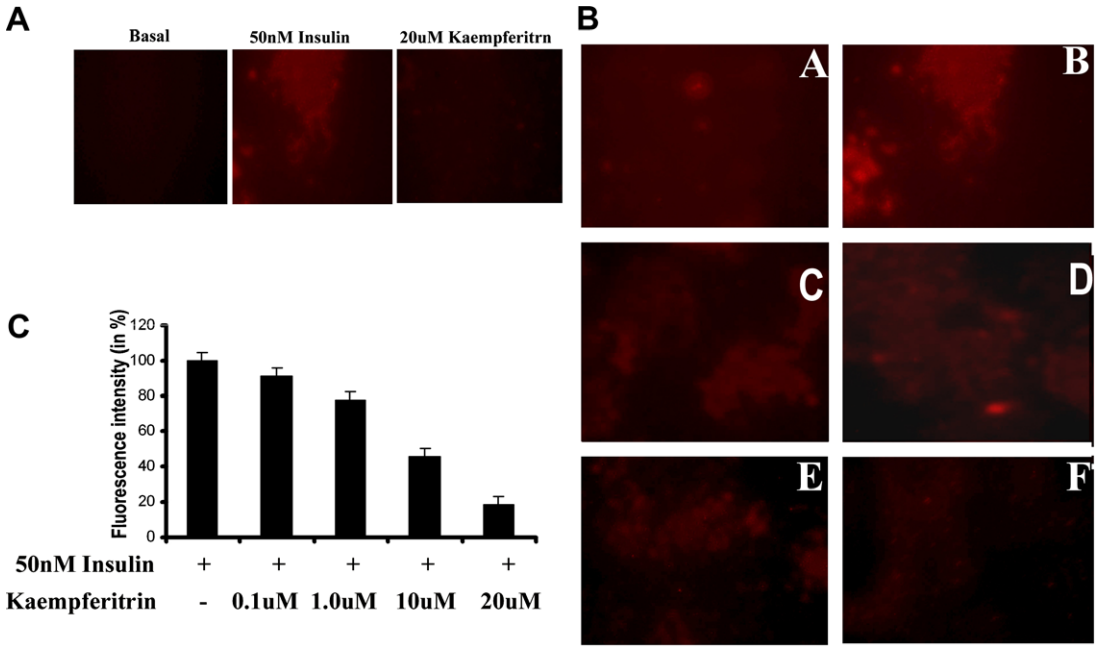

Fig. 1. Effect of kaempferitrin on GLUT4 translocation. Differentiated 3T3-L1 adipocytes were serum starved for 2 h and incubated with DMSO, 50 nM insulin or 20 μM kaempferitrin for 30 min. Plasma membrane lawn was prepared as explained in materials and methods and were fixed and membrane GLUT4 was labeled with Cy3 by indirect immunofluorescence. (**A**) Kaempferitrin does not stimulate membrane translocation of GLUT4 in 3T3-L1 cells. (**B**) Concentration dependent inhibition of insulin stimulated GLUT4 translocation by kaempferitrin. A – DMSO, B – 50 nM insulin, C to F – 0.1, 1.0, 10 and 20 μM kaempferitrin, respectively, added along with insulin and incubated for 30 min. (**C**) Graph showing the percentage inhibition in GLUT4 translocation at various concentrations of kaempferitrin. Values are shown as the mean ± SD of three different focal planes of the same experiment.

buffer containing 10 mM phosphate, 150 mM NaCl (pH 7.4) and 0.05% Tween20 for 1 h at room temperature and then incubated for 2 h with anti-phospho Akt antibody. Immunoreactive bands were visualized by developing the membrane with BCIP/NBT mixture.

Kaempferitrin docking. Kaempferitrin docking studies were performed using AUTODOCK 3 [21]. The structural coordinates of GLUT4 were used from our previous study [22]. The potential active sites used for docking studies were obtained from the PASS program [23]. Center of Grid Map used for docking was the center of active site from the PASS program and the other parameters were set as default. Ligand coordinates are obtained by translating the SMILES, which is obtained from the PubChem compound database using the program SMILES translator [24]. The docked results have been analyzed using the molecular viewer, PyMOL [25].

Results and discussion

Extraction and isolation of kaempferitrin from B. acuminata leaves

Initial extraction enriched phenolic compounds in MEK fraction as was inferred by positive ferric reactions and this fraction showed inhibition of GLUT4 translocation and glucose uptake activity (data not shown). In order to isolate the active component, MEK fraction was subjected to column chromatography as described in materials and methods. All ferric chloride positive fractions were pooled and concentrated. TLC analysis showed three UV positive bands and the major UV and ferric chloride positive band was eluted from the TLC plate. HPLC analysis of this fraction showed the presence of a single major compound with a characteristic UV absorption at 238, 264 and 344. These properties suggest that the compound could be kaempferitrin [26]. This argument was supported by direct comparison of TLC, HPLC, UV and NMR data with an authentic sample of kaempferitrin. For further studies, we used commercially available kaempferitrin.

Kaempferitrin inhibits insulin stimulated GLUT4 translocation in differentiated 3T3-L1 cells

GLUT4 generally resides in specialized compartments and translocates to the membrane based on various stimuli like insulin [3,15,17]. In order to determine the effect of kaempferitrin on GLUT4 translocation, plasma membrane sheet assay was performed in differentiated 3T3-L1 cells. As reported earlier, cells incubated with 50 nM insulin for 30 min showed 2.8 ± 0.2 fold increase in GLUT4 translocation compared to basal level [27,28]. Addition of kaempferitrin in the absence of insulin did not increase GLUT4 translocation even at the maximum concentration (20 μM) used in the assay (Fig. 1A). To identify whether kaempferitrin regulates insulin stimulated GLUT4 translocation, varying concentrations of kaempferitrin were added along with insulin and incubated for 30 min. We found that kaempferitrin showed a dose-dependent reduction in insulin stimulated GLUT4 translocation (Fig. 1B) with a maximum of 80% reduction at 20 μM concentration (Fig. 1C). These data suggest that kaempferitrin inhibits insulin dependent GLUT4 translocation.

Glucose uptake in 3T3-L1 cells is inhibited by kaempferitrin in a concentration dependent manner

Insulin stimulates glucose uptake in differentiated 3T3-L1 adipocytes by increasing GLUT4 translocation and also by increasing the intrinsic transporter activity of GLUT4 on the membrane [29,30]. Kaempferitrin inhibited insulin stimulated GLUT4 translocation in adipocytes. Since GLUT4 translocation is essential for glucose transport, inhibition of insulin stimulated GLUT4 translocation by kaempferitrin should also inhibit glucose uptake in these cells. It is also possible that, kaempferitrin and glucose can compete to interact with plasma membrane bound GLUT4 and thereby inhibit glucose transport. To analyse these different possibilities, glucose uptake assay was performed in differentiated 3T3-L1 adipocytes under various conditions. In the first case, varying concentrations of kaempferitrin were added along with insulin and incubated for 30 min. Kaempferitrin was excluded during glucose uptake and thus exclude the possibility of a competitive binding during glucose uptake. Under this condition, kaempferetrin inhibited the glucose uptake in a concentration dependent manner and 85% reduction in insulin stimulated glucose uptake was observed at a concentration of 20 μM kaempferitrin (Fig 2A). It is interesting to note that the observed extent of inhibition of glucose uptake and GLUT4 translocation were similar at 20 μM concentration. This suggests that kaempferitrin interferes with insulin signaling pathway and inhibits glucose uptake.

In order to verify the possibility of direct interaction of kaempferitrin with GLUT4 and inhibition of glucose uptake, we added

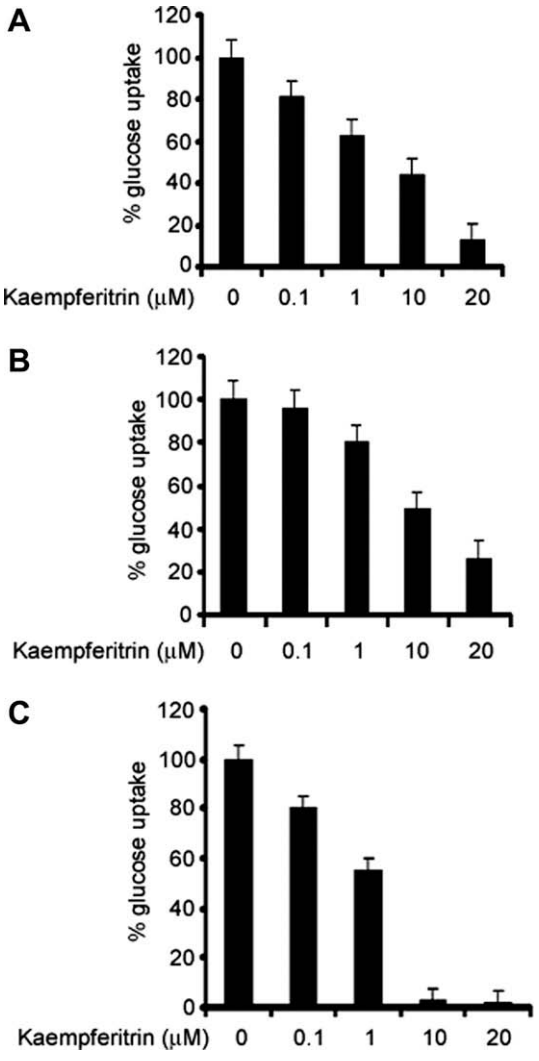

Fig. 2. Effect of kaempferitrin on insulin stimulated glucose uptake. Glucose uptake activity was analyzed by measuring the uptake of radiolabeled glucose in differentiated 3T3-L1 cells. Cells were serum starved for 2 h and stimulated with 50 nM insulin for 30 min, followed by glucose uptake for 10 min. (**A**) Kaempferitrin added during insulin stimulation; (**B**) Kaempferitrin added along with glucose in insulin stimulated cells during glucose uptake; (**C**) Kaempferitrin added during insulin stimulation as well as during glucose uptake. Data shown are percentage inhibition of glucose uptake in insulin stimulated cells. Values are the mean ± SD of three different experiments carried out in duplicates.

varying concentrations of kaempferitrin along with 2-deoxy glucose in cells pre-stimulated with 50 nM insulin. In this case, kaempferitrin was not added along with insulin to prevent kaempferitrin from inhibiting the insulin mediated GLUT4 translocation to the membrane. Under this condition, we found that kaempferitrin inhibited glucose uptake in a concentration dependent manner and 75% inhibition was observed at 20 μM concentration (Fig. 2B). This result suggests the possibility of kaempferitrin competing with glucose for interaction sites in the membrane GLUT4. Since high level of glucose uptake inhibition was observed irrespective of whether kaempferitrin was added along with insulin or with glucose, one would expect a complete inhibition when kaempferitrin is added along with insulin during the stimulation and also added along with 2-deoxy glucose during uptake. When we carried out such an assay, we observed complete inhibition of glucose uptake even at a lower concentration (10 μM) of kaempferitrin (Fig 2C). These observations suggest that kaempferitrin inhibits glucose uptake in adipocytes by at least two mechanisms. One, by interfering with the insulin signaling pathway that results in decreased GLUT4 translocation and glucose uptake and another by directly interacting with GLUT4 and thereby competing with glucose for the transport.

Kaempferitrin inhibits insulin stimulated Akt phosphorylation

Kaempferitrin inhibited insulin stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. Insulin mediated GLUT4 translocation and glucose uptake is regulated by a complex signal transduction cascade involving the phosphorylation and activation of several key molecules. Activation of Akt (Protein kinase B) is one of the key events in this cascade [31]. Down regulation of Akt phosphorylation was shown to reduce the insulin mediated GLUT4 translocation and glucose uptake [32]. We investigated the effect of kaempferitrin on insulin mediated Akt phosphorylation and observed that kaempferitrin reduced the Akt phosphorylation at S473 position in a concentration dependent manner (Fig. 3). Thus, inhibition of insulin stimulated Akt activation by kaempferitrin might play a role in the observed inhibition of GLUT4 translocation and glucose uptake. Bazuine et al., have shown that genistein, an isoflavone-derivative acted as direct inhibitor of insulin-induced glucose uptake without affecting insulin-induced tyrosine kinase activity of insulin receptor or activation of protein kinase B. However, they did not determine the rate of GLUT4 translocation [33]. Although, there are studies using flavonoid derivatives to assess their effect on inhibition of insulin-induced glucose uptake [34], this is the first report showing the effect of a glycosylated flavonoid, kaempferitrin on the inhibition of glucose uptake induced by insulin.

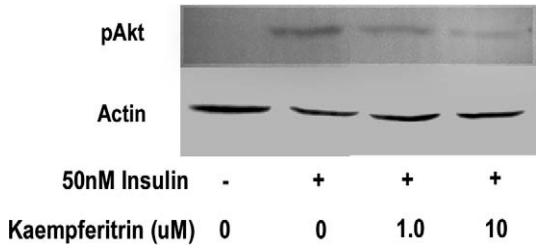

Fig. 3. Kaempferitrin reduces insulin stimulated Akt phosphorylation. Differentiated adipocytes were treated with 50 nM insulin and varying concentrations of kaempferitrin for 30 min. Equal amount of protein and resolved in SDS–PAGE. After transferring to PVDF membrane, Akt phosphorylation was detected by western blot analysis.

Kaempferitrin directly interacts with the glucose transport channel in GLUT4

When kaempferitrin was added along with glucose, our results showed a competitive inhibition in glucose uptake. This suggests the possibility that kaempferitrin interacts with GLUT4. To confirm this, we carried out docking studies of kaempferitrin using the GLUT4 model generated in our laboratory [22]. We obtained different docking poses of kaempferitrin in the glucose transport pore of GLUT4 with favorable energies. Further analysis revealed that kaempferitrin forms hydrogen bonds with amino acid residues such as G43, N176, Q177, Q298, S301, N333, and N431 in the glucose transport channel. Among these residues, Q177, Q298, N333 were reported to be important for glucose transport [13]. Fig. 4A and B shows a favorable mode of interaction with GLUT4 and the important residues involve in the interaction with kaempferitrin. These results suggest that kaempferitrin directly interacts with QLS site, a conserved motif in high affinity glucose transporters, which is critical for the binding and transport of D-glucose through GLUT4 [13].

In the present study, we have shown that kaempferitrin inhibits GLUT4 mediated glucose uptake in differentiated 3T3-L1 cells by interfering with insulin signaling pathway and also by directly interacting with membrane GLUT4. Jorge et al. have reported that kaempferitrin stimulates glucose uptake in rat soleus muscle [35].

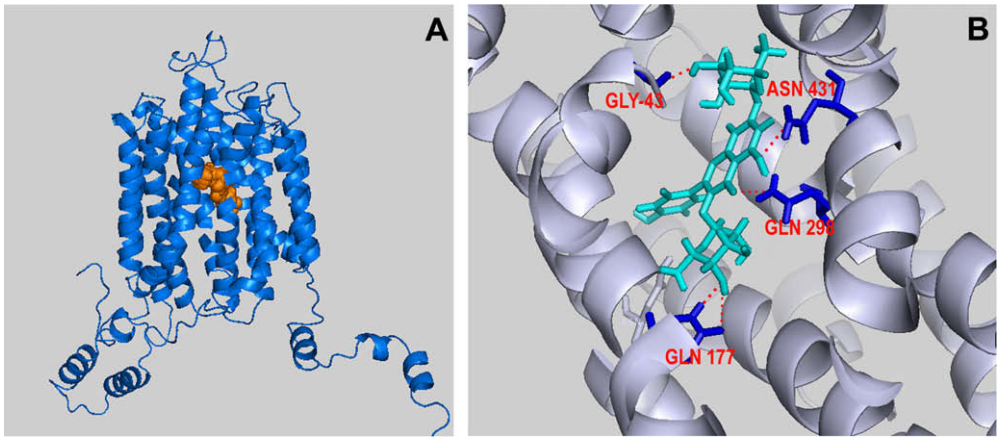

Fig. 4. Interaction of kaempferitrin with GLUT4. (**A**) A view of kaempferitrin docked in the glucose transport channel. (**B**) Enlarged image showing amino acid residues interacting with kaempferitrin docked in the glucose transportation pore.

This suggests that the effect of kaempferitrin on insulin mediated glucose uptake might be a cell type specific function.

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