

MECHANISM OF ACTION OF ANTI-DIABETIC PROPERTY OF CINNAMIC ACID, A PRINCIPAL ACTIVE INGREDIENT FROM THE BARK OF CINNAMOMUM CASSIA.

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ABSTRACT

Glucose lowering potential of Cinnamon extracts is reported in a variety of studies and experimental models. Cinnamic acid is one of the main constituents, isolated from the bark of *Cinnamomum cassia* and is known to possess anti-diabetic properties. In the present manuscript, we are reporting *in vitro* glucose uptake data using cinnamic acid, conducted in skeletal muscle cells, L6 to elicit its mechanism of action. Our studies suggest that cinnamic acid increases glucose uptake in an insulin independent manner and acts through activation of α - and β adrenoceptors in skeletal muscle cells.

Keywords: Cinnamic acid, Glucose uptake, L6 cells, α adrenoceptors, β adrenoceptors, AMPK.

Plant products and derivatives are being intensively researched nowadays for various pharmacological conditions. A number of medicinal herbs have been found to possess hypoglycemic activities. Cinnamon is one of these. There have been several reports available till date suggesting the glucose lowering potential of cinnamon extracts in a variety of studies and experimental models (1-5). Cinnamic acid, being one of the main constituents, isolated from the bark of *Cinnamomum cassia* (6) (Figure 1a), we investigated its glucose lowering potential by conducting a few *in vitro* experiments. Some of these finding have been reported in our earlier manuscript in J of Diabetes (7). Previously, we have reported that cinnamic acid activates glucose uptake in L6 cells in a PI3 kinase independent manner. Since insulin mediated glucose transport is severely impaired in type 2 diabetes patients and skeletal muscles account for ~85 % of the total glucose metabolized,

there is a great degree of interest, in identifying agents which can stimulate glucose transport in skeletal muscle in an insulin independent manner. Since we have already shown that cinnamic acid works in a PI3 Kinase independent manner, a downstream partner of insulin receptor, we further investigated the partners of insulin independent mechanism of action of cinnamic acid in L6 cells and the findings are reported in this manuscript.

Out of several mechanisms proposed so far, involvement of adrenoceptors has been suggested to be an important contributor of insulin independent glucose uptake in skeletal muscle using L6 cells (8,9). Several *in vitro* and *in vivo* studies have shown that activation of α - and β - adrenoceptors increases glucose uptake in skeletal muscle cells in an insulin independent manner (8,10). Adrenoceptors are G coupled receptors and are classified into 3 main subtypes: α 1-, α 2- and β - adrenoceptors. In our previous study since we have already shown that cinnamic acid increases glucose uptake in a PI3 kinase independent manner, we further wanted to explore whether it acts through α and β adrenoceptors. Though some preliminary data of cinnamic acid and its analogs is reported in mouse myoblast, C2C12 cells suggesting the stimulatory effect of these molecules on α 1A adrenoceptors in *in vitro* binding assay (12), detailed characterization of this phenomenon through glucose uptake in L6 cells however is not available. In comparison to C2C12, L6 cells represent a more extensively studied model. C₂C₁₂ cells are also reported to have a defective post insulin signaling pathway while L6 cells not only have an intact insulin pathway, they also express both α and β adrenoceptors (8,9). In the present study we conducted experiments in L6 cells to test the effect of α and β adrenoceptor antagonists on cinnamic acid mediated glucose uptake. We hereby suggest that insulin independent mechanism of glucose uptake by

cinnamic acid occurs through activation of adrenoceptors in L6 cells.

There is also some evidence suggesting that activation of adrenoceptors leads to activation of AMP related kinase (AMPK) (11) Activation of α adrenoceptors agonist phenylephrine has been found to increase the AMPK activity in L6 cells. We therefore also confirmed if there is any participation of AMPK in cinnamic acid stimulated increase in glucose uptake through activation of adrenoceptors.

In the present manuscript we are reporting data which suggests that cinnamic acid mediated glucose uptake in L6 cells is through activation of α and β adrenoceptors and is independent of AMPK.

MATERIALS AND METHODS

1.1 Chemicals and reagents

All cell culture solutions and supplements were purchased from Life Technologies Inc., 2-DeoxyD-[$^1\text{-}^3\text{H}$] glucose and hybond C membrane

were obtained from Amersham Pharmacia Biotech, UK. Insulin, cinnamic acid, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) and all other fine chemicals were obtained from Sigma–Aldrich, St. Louis. Compound C was procured from Calbiochem.

1.2 Cell culture

Rat skeletal muscle cell line L6 from ATCC, was maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% (v/v) penicillin/streptomycin, in a 5% CO₂ environment. Cells were transferred to DMEM with 2% FCS for 4 days post-confluence to allow their differentiation. The extent of differentiation was established by observing multi-nucleation of cells. In the present experiment, 90% of the myoblasts were fused into myotubes. Mouse 3T3-L1 cells were also obtained from ATCC and maintained in high glucose DMEM with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin, and were

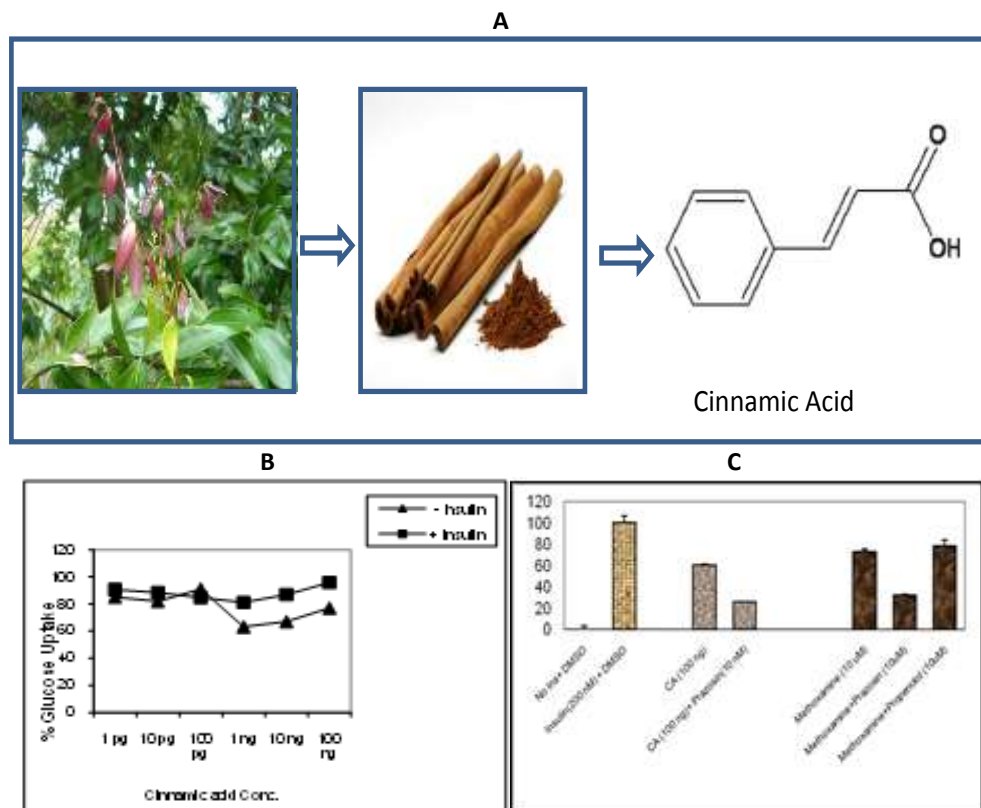


Fig.1 Glucose uptake experiment in L6 cells (A)Cinnamic acid is a component of bark of Cinnomum cassia (B)combination of cinnamic acid and insulin on glucose uptake. (C)Effect of prazosin on cinnamic acid mediated glucose uptake.

differentiated 2 days post-confluence by addition of 500 μM isobutylxanthine, 25 μM dexamethasone, and 4 $\mu\text{g/ml}$ insulin (13) and after 3 days, cultured with the addition of insulin only. This medium was replaced every 3 days. Cells were completely differentiated into adipocytes after 10 days and used in experiments.

1.3 Measurement of 2-deoxy-d-[1-³H] glucose

Differentiated L6 myoblast cells, at a density of 5×10^5 or 3T3-L1 adipocytes at the density of 1.5×10^5 per well, grown in 24-well plate (BD Falcon), were subjected to glucose uptake as reported (14,15). In brief, the differentiated cells were serum starved for 5 h and were incubated with cinnamic acid for 24 h. For synergy experiments, cells were further stimulated with insulin (100nM) for 10 min. After incubation, cells were rinsed once with HEPES-buffered krebs ringer phosphate solution (118mM NaCl, 5mM KCl, 1.3mM CaCl_2 , 1.2mM MgSO_4 , 1.2mM KH_2PO_4 and 30mM HEPES, pH 7.4) and were subsequently incubated for 15 min in HEPES-buffered solution containing 0.5 $\mu\text{Ci/ml}$ 2-deoxy-d-[1-³H] glucose. The uptake was terminated by aspiration of media. Cells were washed thrice with ice cold HEPES buffer solution and lysed in 0.1% SDS. The lysates were transferred to 96 well plate (Packard) with glass fibre paper and air dried overnight. This plate was used to measure the cell-associated radioactivity by liquid scintillation counting. Glucose uptake values were corrected for non-specific uptake in the presence of 10 μM cytochalasin B. All the assays were performed in duplicates and repeated thrice for concordancy. Results were expressed as % glucose uptake with respect to solvent control. Rosiglitazone (50 μM) was used as the positive control.

RESULTS

2.1 Cinnamic acid increases glucose uptake in L6 cells through activation of α and β adrenoceptors.

Glucose uptake experiments were carried out using 2-deoxy-d-[1-³H] glucose in L6 cells. Our results suggest that glucose uptake was significantly increased in presence of insulin and cinnamic acid (100ng/ml) in L6 cells but combination of insulin did not have any significant additive effect (Figure 1b).

Methoxamine (a nonselective α adrenoceptor agonist) also increased glucose uptake in L6 cells (Figure 1c). Cinnamic acid mediated increase in glucose uptake was decreased when cells were pretreated with prazosin (α_1 adrenoceptor inhibitor) and propranolol (β adrenoceptor inhibitor) when tested at 10 nM single concentration. To confirm the specificity of action, we also kept conditions where cells were pretreated with prazosin and propranolol prior to addition of methoxamine. Though methoxamine mediated increase in glucose was decreased by prazosin, propranolol did not decrease methoxamine mediated glucose uptake, suggesting the specificity of the inhibitors in elucidating the role of adrenoceptor mediated glucose uptake (Figure 1c). We further generated the dose response curves using different concentrations

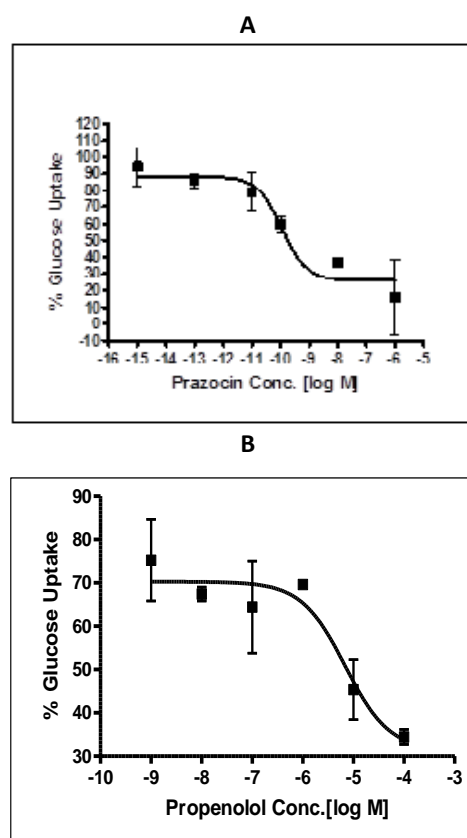


Fig.2. Effect of prazosin and propranolol on cinnamic acid mediated glucose uptake in L6 cells (A)Dose response curve of cinnamic acid mediated glucose uptake in presence of prazosin. (B)Dose response curve of cinnamic acid mediated glucose uptake in presence of propranolol.

of $\alpha 1$ and β AR antagonists. We found that cinnamic acid mediated increase in glucose uptake was blocked by pretreatment with prazosin, $\alpha 1$ AR antagonist in a dose dependent manner (Figure 2a). Increase in cinnamic acid mediated glucose was further carried out in presence of β adrenoceptor blocker propranolol and was found to be inhibited in presence of propranolol in a dose dependent manner (Figure 2b).

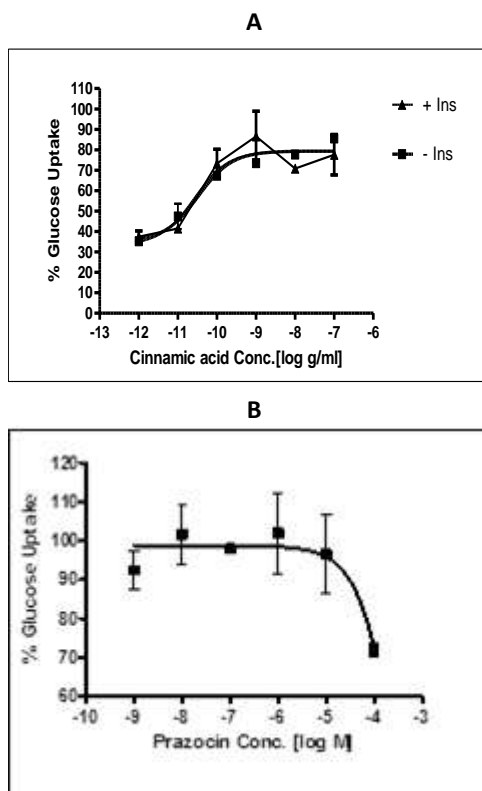


Fig 3 Cinnamic acid mediated glucose uptake in 3T3L1 cells (A) Cinnamic acid mediated glucose uptake in presence and absence of 30 nM insulin. (B) Effect of Prazosin on Cinnamic acid mediated glucose uptake.

We also tested the cinnamic mediated glucose uptake in adipocyte cells, 3T3L1. Cinnamic acid increased glucose uptake in 3T3L1 cells in a dose dependent manner but combination with insulin did not have any synergistic effect (Figure 3a). Pre-treatment with prazosin, however, did not inhibit the cinnamic acid mediated glucose uptake in these cells (Figure 3b) ruling out the possibility of participation of mechanism other than β adrenoceptors in cinnamic acid mediated glucose uptake in 3T3L1 cells.

2.2 AMPK agonist AICAR synergistically increases glucose uptake in L6 cells when used in combination with cinnamic acid.

In next set of studies to check the participation of AMPK in cinnamic acid mediated glucose uptake, we carried out experiments using AMPK agonist AICAR, cinnamic acid and a combination of the two. We found that AICAR increases the glucose uptake and has an additive effect on cinnamic acid mediated glucose uptake. We also tested AMPK inhibitor, compound C in our set up (Figure 4). Though compound C is already known as an AMPK inhibitor in β cells but it was found ineffective in

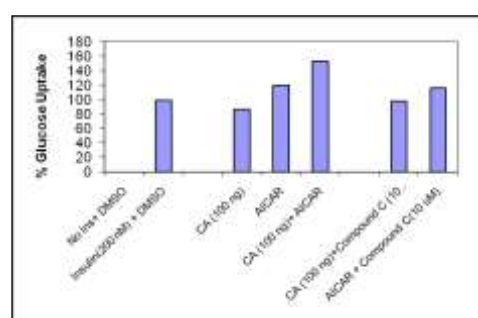


Fig 4.Effect of AMPK activator, AICAR on cinnamic acid mediated glucose uptake in L6 cells

inhibiting the AMPK mediated response in L6 cells, as reported earlier. We tested this in our set up to confirm the same for insulin and also for cinnamic acid mediated glucose uptake in L6 cells. We observed that compound C could not decrease either cinnamic acid mediated or AICAR mediated glucose uptake in L6 cells.

DISCUSSION

Cinnamic acid and its derivatives have been reported to show antidiabetic property in a variety of *in vitro* and *in vivo* models. In addition to the glucose lowering properties, it has been reported that m-hydroxy cinnamic acid and p-methoxy cinnamic acid and frulic acid significantly increased insulin secretion in INS-1 cells(16). In an earlier study, it has also been shown that the daily administration of a cinnamic acid derivative, p-methoxycinnamic acid in diabetic rats suppresses the activity of hepatic gluconeogenic enzyme G6pase and increase the activities of 3 glycolytic enzymes HK, GK and phosphofructokinase in liver(17). With

regard to anti-diabetic property of cinnamic acid, we have previously shown that cinnamic acid mediated glucose uptake in L6 cells is PI3 Kinase independent(7). In the present manuscript, we have further elucidated the mechanism of action of cinnamic acid which is independent of insulin signaling pathway. Our data suggests that cinnamic acid increases glucose uptake by >80 % at the highest tested concentration of 100ng/ml in absence of insulin. However, no synergistic increase in glucose uptake was observed when cinnamic acid was used in the presence of insulin. We have shown that cinnamic acid increases glucose uptake in L6 cells through α and β adrenoceptors. More experiments however are required to identify the adrenoceptor subtypes and this remains the subject matter of future studies.

Our current finding further strengthens our belief that cinnamic acid is an important bioactive component of *Cinnamomum cassia*. Since cinnamic acid mediated anti-diabetic activity is not dependent on the components of insulin signaling pathway, it may have a good chance to show efficacy in insulin resistant states and the states of disrupted insulin signaling. *In vitro* toxicity of cinnamic acid was also tested in L6 cells by MTT assay and it was not found to be toxic up to 1 μ g/ml concentration. (Data not shown).

REFERENCES

1. Qin B, Nagasaki M, Ren M, Bajotto G, Oshida Y, Sato Y, Cinnamon extract (traditional herb) potentiates *in vivo* insulin-regulated glucose utilization via enhancing insulin signaling in rats, *Diabetes Res Clin Pract*, 62 (2003) 139.
2. Khan A, Safdar M, Ali K M, Khattak K N, Andersan R A, Cinnamon improves glucose and lipids of people with type 2 diabetes, *Diabetes Care*, 26 (2003) 3215.
3. Qin B, Nagasaki M, Ren M, Bajotto G Oshida Y, Sato Y, Cinnamon extract prevents the insulin resistance induced by a high-fructose diet, *Horm Metab Res*, 36 (2004) 119.
4. Verspohl E J, Bauer K, Neddermann E, Antidiabetic effect of *Cinnamomum cassia* and *Cinnamomum zeylanicum in vivo* and *in vitro*, *Phytother Res*, 19 (2005) 203.
5. Radosevich J I, Shenequa D, Polansky M M, Baedke D A, Ingebristen TS, Anderson RA. Regulation of PTP-1 and Insulin Receptor kinase by fractions from Cinnamon: Implications for Cinnamon Regulation of insulin signaling. *Hormone Res*50 (1998) 177.
6. Ross, F M, Analysis of cinnamon oils by high pressure liquid chromatography, *J Chromatogr*,118 (1976) 273.
7. Lakshmi B S, Sujatha S, Anand S, Sangeetha KN, Narayanan R B, Katiyar C, Kanaujia A, Duggar R, Singh Y, Srinivas K, Bansal V, Sarin S, Tandon R, Sharma S, Singh S, Cinnamic acid, from the bark of *Cinnamomum cassia*, regulates glucose transport via activation of GLUT4 on L6 myotubes in a phosphatidylinositol 3-kinase-independent manner, *J of Diabetes*, 1 (2009) 99.
8. Hutchinson D S & Bengtsson T, α 1A-Adrenoceptors Activate Glucose Uptake in L6 Muscle Cells through a Phospholipase C-, Phosphatidylinositol-3 Kinase-, and Atypical Protein Kinase C-Dependent Pathway, *Endocrin*, 146 (2005) 901.
9. Nevzorova J, Bengtsson T, Evans B A, Summers R J, Characterization of the β -adrenoceptor subtype involved in mediation of glucose transport in L6 cells, *Br J Pharma*, 2137(2002) 9.
10. Tanishita T, Shimizu Y, Minokoshi Y, Shimazu T, The β 3-adrenergic agonist BRL37344 increases glucose transport into L6 myotubes through a mechanism different from that of insulin, *J Biochem (Tokyo)*, 122 (1997) 90.
11. Hutchinson D S & Bengtsson T, AMP-activated protein kinase activation by adrenoceptors in L6 skeletal muscle cells, *Diabetes*, 55(2006) 682.
12. Liu I M, Tsai C C, Lai T Y, Cheng J T, Stimulatory effect of isoferulic acid on 1A-adrenoceptor to increase glucose uptake into cultured myoblast C₂C₁₂ cell of mice. *Autonomic Neurosci: Basic and Clin*, 88 (2001) 175.

13. Kayali A G, Eichhorn J, Haruta T, Morris A J, Nelson J G, Vollenweider P, Olefsky J M & Webster N J, Association of the insulin receptor with phospholipase C-gamma (PLCgamma) in 3T3-L1 adipocytes suggests a role for PLC gamma in metabolic signaling by insulin, *J Biol Chem*, 273(1998) 13808.
14. Yonemitsu S, Nishimura H, Shintani M, Inoue R, Yamamoto Y, Masuzaki H, Ogawa Y, Hosoda K, Inoue G, Hayashi T, Nakao K, Troglitazone induces GLUT4 translocation in L6 myotubes, *Diabetes*, 50 (2001)1093.
15. Wiese R J, Mastick CC, Lazar D F, Saltiel A R, Activation of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes, *J Biol Chem* 270 (1995) 3442.
16. Adisakwattana S, Moonsan P, Yibchok-Anun S, Insulin-releasing properties of a series of cinnamic acid derivatives in vitro and in vivo, *J Agric Food Chem* 56 (2008) 7838.
17. Adisakwattana S, Roengsamran S, Hsu WH, Yibchok-anun S Mechanisms of antihyperglycemic effect of p-methoxycinnamic acid in normal and streptozotocin-induced diabetic rats, *Life Sci*. 78 (2005) 406.