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Elucidation of hypoglycemic action and toxicity studies of insulin-like protein from *Costus igneus*

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ABSTRACT

We have reported earlier, an orally active insulin-like protein (ILP) from *Costus igneus* having potent hypoglycemic property in STZ-induced diabetic Swiss mice. The blood glucose level was reduced significantly within two hours after feeding ILP orally in an oral glucose tolerance test. The present study elucidates the mechanism underlying the hypoglycemic action of ILP. Mechanism of action of ILP was studied in differentiated L6 myotubes. 2-NBDG uptake stimulated by ILP was studied in differentiated L6 myotubes under normoglycemic, hyperglycemic and induced insulin resistant conditions. ILP treatment significantly increased 2-NBDG uptake in differentiated L6 myotubes. The levels of insulin signaling molecules IRS-1 and GLUT-4 were assessed in ILP treated L6 myotubes by immunoblot analysis of cytoplasmic and plasma membrane fractions respectively. Immunoblot analysis revealed an increase in cytoplasmic IRS-1 with a concomitant increase in GLUT-4 translocation to the plasma membrane in a time dependent manner. Toxicity studies of ILP were performed on normal as well as diabetic Swiss albino mice. ILP did not show any toxicity in the acute and sub-chronic toxicity studies in normal as well as diabetic Swiss albino mice. Mass spectrometry was carried out to identify ILP. MALDI TOF/TOF MS analysis of ILP revealed sequence homology with the predicted protein from *Physcomitrella patens*. Our study reveals that ILP acts via insulin signaling pathway and can be used as oral insulin mimetic.

1. Introduction

Diabetes mellitus is a chronic illness that requires continuing medical care to prevent acute and long term complications. The increasing pace of research is reflected in growing number of antidiabetic agents. There seems to be a renewed interest in naturally occurring antidiabetic compounds across the world today. One of the important areas of research is to identify orally active insulin mimetic molecules.

After the discovery of insulin, Banting and Best reported the presence of insulin-like materials in germinating potatoes and rice (Banting et al., 1922; Best and Scott, 1923). Best and Scott suggested that the hormone analogous to insulin must be present wherever glucose is metabolized (Best and Scott, 1923). Since then the plants have been explored for the presence of insulin-like antigens. Khanna et al. (1974) first reported the presence of insulin in plants and patented a process of isolation of insulin from the fruits of bitter gourd (*Momordica charantia*) (Khanna et al., 1976). Insulin-like antigens are identified from leaves and aerial parts of green plants, red algae, cyanobacteria and fungi (Silva et al., 2002). A protein showing hypoglycemic effects and amino acid sequence homology to animal insulin as well as cross reactivity with anti-insulin antibodies was isolated from *Canavalia ensiformis* seed coat, the fruits of *Vigna unguiculata* and characterized (Oliveira et al., 1999; Venancio et al., 2003). Detection of insulin-like peptides has also been reported in a range of plants like *Spirulina*, *Bauhinia variegata* and a number of other species across the entire plant kingdom (Anwer et al., 2012; Azevedo et al., 2006; Virdi et al., 2003).

Costus igneus N. E. Br. popularly known as 'insulin plant' in India belongs to the family Costaceae. It grows in tropical Africa, Asia, Australia, as well as in North, Central and South America. It is reported that diabetic people in India eat one leaf of Insulin plant

Abbreviations: BCA, bicinchoninic acid; ILP, insulin-like protein from *Costus igneus*; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GLUT-4, glucose transporter-4; IR, insulin receptor; IRS-1, insulin receptor substrate-1; 2-NBDG, [2-{N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino}-2-deoxyglucose]; PBS, phosphate buffered saline; STZ, streptozotocin; TNF-α, tumor necrosis factor-α.

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daily to keep their blood glucose low (Devi and Urooj, 2008). The aqueous and ethanolic extracts of *C. igneus* have shown to possess potent anti-diabetic effect in alloxan-induced and STZ-induced diabetic rats (Bhat et al., 2010; Kumudhavalli and Jaykar, 2012). Shetty and coworkers reported that regular consumption of the leaves of *C. igneus* in conjunction with other treatments provided effective glycemic control in diabetic patients (Shetty et al., 2010).

In our previous study, we reported the presence of a novel, orally active insulin-like protein (ILP) (5.6118 kDa) from the leaves of *C. igneus* (Joshi et al., 2013). We isolated and purified ILP, which was found to be similar to insulin in its hypoglycemic action. The hypoglycemic effect exerted by ILP on insulin responsive cells was comparable to that of insulin. The ILP also retained its bioactivity when administered orally in STZ-induced diabetic mice (Joshi et al., 2013). The present work is, therefore aimed at the investigation of the mechanism underlying the hypoglycemic action of orally active ILP. We have also attempted to identify the ILP using mass spectrometric analysis.

2. Results

We have previously reported the presence of a novel insulin-like protein (ILP) from *C. igneus* that was found to be orally active and revealed its functional similarity with that of human insulin (Joshi et al., 2013). In the present study, we further demonstrated the mechanism underlying the hypoglycemic action of ILP.

2.1. ILP stimulates glucose uptake in L6 myotubes

Skeletal muscles form the major sites of action for insulin mediated glucose uptake. The effect of ILP treatment on glucose uptake in L6 myotubes was investigated using 2-NBDG, a fluorescent glucose analogue. NBDG probe provide a convenient and rapid measurement of spatiotemporal glucose flux (Lee et al., 2013).

The effect of ILP on 2-NBDG uptake was studied in differentiated L6 myotubes under both normoglycemic as well as hyperglycemic conditions. Cells treated with 100 nM insulin and 4.45 μM ILP significantly increased 2-NBDG uptake in both normoglycemic and hyperglycemic conditions compared to control cells (Fig. 1A). The fluorescence intensity of 2-NBDG internalization into myotubes was calculated using ImageJ software. The fluorescence intensity of 2-NBDG uptake in cells treated with insulin under normoglycemic conditions was considered as 100%. ILP stimulated 2-NBDG uptake was found to be 96.5% and was comparable to that of insulin stimulated uptake. Under Normoglycemic condition, both insulin and ILP significantly stimulated 2-NBDG uptake compared to control cells (44.5%). Under hyperglycemic conditions, 2-NBDG uptake was increased up to 93% due to treatment with insulin and 91% due to treatment with ILP which was found to be significantly higher than control (40%) (Fig. 1B).

TNF-α is known to induce insulin resistance in skeletal muscles by inhibiting signaling from the insulin receptor and is used to analyze the mechanism of insulin mimetic activity (Lee et al., 2013; Steinberg et al., 2006; Hotamisligil et al., 1994). To analyze whether ILP acts via insulin receptor signaling, 2-NBDG uptake was carried out in L6 myotubes treated with TNF-α. It was found that the ILP stimulated 2-NBDG uptake was sensitive to TNF-α treatment which was reduced by 50% compared with uptake in normoglycemic condition (Fig. 1A and B). Inhibition of ILP stimulated glucose uptake by TNF-α in myotubes suggested that ILP perhaps exerts its hypoglycemic action via insulin receptor signaling pathway.

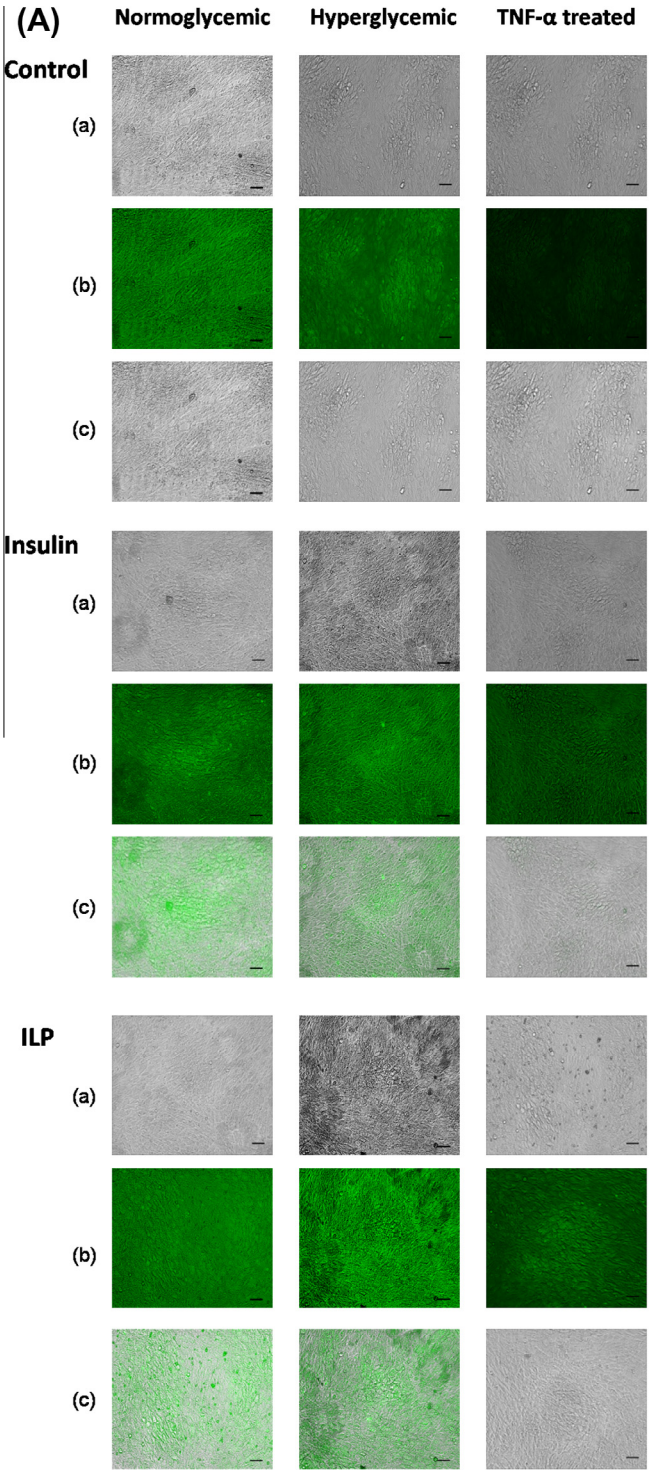

Fig. 1. 2-NBDG uptake in L6 myotubes. (A) 2-NBDG uptake in representative micrographs of differentiated L6 myotubes after treatment with 4.45 μM ILP and 100 nM insulin (positive control) respectively. (a) bright field, (b) fluorescent, (c) overlay images. Scale bar = 100 μm. (B) The percentage fluorescence intensity for 2-NBDG uptake in L6 myotubes was calculated using imageJ software. The data represent mean ± SD, $n = 3$, $^{*}P < 0.05$ anti-GLUT-4, anti-insulin, TNF-α treated cells compared to ILP treatment alone (paired student's *t*-test).

2.2. ILP acts via insulin signaling pathway

Insulin stimulated glucose uptake is achieved by the phosphorylation of IRS proteins and translocation of the insulin sensitive

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