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# Insulin-mimetic action of conglutin-γ, a lupin seed protein, in mouse myoblasts

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**KEYWORDS**
*Lupinus albus*;
Conglutin-γ;
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**Abstract** *Background and aims:* Lupin seed is referred to as an antidiabetic product in traditional medicine. Conglutin-γ, a lupin seed glycoprotein, was found to cause a significant plasma glucose reduction when orally administered to rats in glucose overload trials. Conglutin-γ was identified as being responsible for the claimed biological activity, and the aim of this work was to envisage its hypothetical insulin-mimetic cellular mechanism of action. Insulin is responsible for proteosynthesis control through IRS/AKT/P70S6k/PHAS1 pathways modulation, glucose homeostasis through PKC/Flotillin-2/caveolin-3/Cbl activation and muscle differentiation/hypertrophy via muscle-specific MHC gene transcription control.
*Methods and results:* To assess whether conglutin-γ modulates the same insulin-activated kinases, myoblastic C2C12 cells were incubated after 72 h of differentiation with 100 nM insulin or 0.5 mg/mL (~10 μM) conglutin-γ. Metformin-stimulated cells were used as a positive control. The effect on the above mentioned pathways was evaluated after 5, 10, 20 and 30 min. In the control cells medium insulin, conglutin-γ and metformin were not added. We demonstrated that insulin or conglutin-γ cell stimulation resulted in the persistent activation of protein synthetic pathway kinases and increased glucose transport, glut4 translocation and muscle-specific gene transcription regulation.
*Conclusions:* Our results indicate that conglutin-γ may regulate muscle energy metabolism, protein synthesis and MHC gene transcription through the modulation of the same insulin signalling pathway, suggesting the potential therapeutic use of this natural legume protein in the treatment of diabetes and other insulin-resistant conditions, as well as the potential conglutin-γ influence on muscle cells differentiation and regulation of muscle growth.
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## Introduction

White lupin seed conglutin-γ is a mono-glycosylated protein consisting of two disulphide bonded subunits of 30 and 17 kDa (amino acid sequence available at TrEMBL, accession number: Q9FSH9). This 47 kDa monomeric unit undergoes a pH-dependent reversible association to tetramer at neutral to slightly alkaline pH values [1,2]. Conglutin-γ in its native conformation is unusually resistant to proteolysis by trypsin [3]. Conglutin-γ was found to interact with insulin *in vitro* with a Kd around $7 \times 10^{-5}$ M and, most importantly, to significantly reduce plasma glucose in rodents in doses ranging from 30 to 120 mg/kg body weight [4]. Therefore, this protein was identified as the responsible molecule for the claimed anti-diabetic properties of lupin seeds in traditional medicine. Since insulin-binding to its own receptor causes a series of phosphorylation/dephosphorylation reactions, [5] which lead the insulin signal from the receptor to the final metabolic and myogenic pathways (Fig. 1), we firstly hypothesize that the effect of conglutin-γ on blood glucose is due to an insulin-mimetic effect of the protein at the level of the intracellular pathway insulin receptor/IRS-1/PI-3-kinase, eventually leading to the recruitment and translocation of GLUT4 (Fig. 1). Secondly, as insulin promotes muscle anabolism, we hypothesize the activation of a protein synthetic and myogenic process by conglutin-γ.

The aim of this work was to test the effect of conglutin-γ in an *in vitro* model of mouse myoblasts, assessing the modulation of muscle-specific genes and the phosphorylation/activation of intracellular kinases involved in the insulin signalling cascade. Our results indicate that conglutin-γ shares, with insulin, common effects on the intracellular kinases tested in this work, suggesting a possible therapeutic indication as an insulin-mimetic agent.

## Methods

### Materials

Anti-actin (I-19), anti-AKT (C-20), anti—caveolin-3 (A-3), anti-4e (P-2), anti—flotillin-2 (H-90), anti-IRS1 (H-165), anti-p-IRS1 (Tyr 632), anti-myogenin, anti-PI3-Kinase p85α (Z-8), anti-PKC (H-300), anti —α-tubulin (TU-16), anti-phospho-ERK (E-4), anti-MHC, anti-phospho-Cbl (Tyr700) and anti-phosho-p70S6 kinase (Thr421/Ser 424), anti-PHAS1(P-2), monoclonal or polyclonal primary antibodies and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other reagents were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Mouse C2C12 myoblastic cells were purchased from the European Collection of Animal Cell Cultures (ECACC).

### Purification of lupin conglutin-γ

Conglutin-γ was purified using a combination of anion and cation exchange chromatography [1]. For the estimation of purified conglutin-γ concentrations, 4 optical measurements at 280 nm were made. The extinction coefficient of 1 for a solution of 1 mg/ml was used, according to ref. [2].

### Experimental protocol

C2C12 myoblasts were cultured at 37 °C (5% CO2) in a growth medium (GM) containing DMEM, 20% (v/v) fetal bovine serum, 1% penicillin—streptomycin and 1% L-glutamine. Seventy per cent confluent myoblasts (Ctu) were differentiated in DMEM supplemented with 1% of horse serum and antibiotics (DM) for 72 h. Cells used as control (Ct) were maintained in DM for the duration of the experiment. Other three groups of cells were placed in DM with the selective addition respectively of insulin (I; 100 nM), conglutin-γ (Cg; 10 μM) and metformin (Mf; 400 μM) and lysed at 0, 5, 10, 20 and 30 min after the stimuli addition. For immunofluorescence analysis differentiated C2C12 myoblasts were stimulated with insulin, conglutin-γ and metformin for 30 min. In differentiated cells immunostained with anti-MHC, the DAPI stained nuclei per myotube, myotubes length and diameter were determined and expressed as average.

### Electrophoretic techniques and immunoblotting analysis

Conglutin-γ SDS-PAGE was performed in NuPAGE Novex Bis-Tris 10% gels by using a XCell SureLock Mini-Cell (Invitrogen, Milan, Italy). SeeBlue Plus2 Prestained Standard and SimplyBlue SafeStain (Invitrogen, Milan, Italy) were used. For protein blot analysis, the gel was transferred to nitrocellulose transfer membrane (Protran®, Whatman ® Schleicher & Schuell) by blotting according to Towbin et al. [6] on a Transblot Electrophoretic Transfer Cell (Bio-Rad, Milan, Italy). The membrane was blocked with 3% fish gelatin for 2 h and washed three times with 0.25% fish gelatin solution both in PBS buffer (10 mM Na phosphate, pH 7.4, containing 150 mM NaCl), then soaked for 2 h in PBS buffer containing rabbit anti-conglutin-γ in the ratio 1,500/1 (v/v). The antiserum was prepared and immuno-affinity purified as previously described [7]. The bands were revealed by using horseradish peroxidase conjugate with goat-antirabbit antiserum 2,000/1 (v/v) (Bio-Rad, Milan, Italy) and hydrogen peroxide with 4-chloronaphtol as substrate. C2C12 myofibers were homogenized as described [8]. Aliquots of 30 μg supernatant proteins from the different samples were resolved by 8% (p-IRS1 Tyr 632, IRS1, MHC, myogenin and p-CBL), 10% (PI3K p85, AKT1, p-p70 S6K and PKC), 12% (eIF-4E, p-ERK1, p-ERK2, caveolin-3 and flottilin-2) and 15% (PHAS1) SDS-PAGE. Electrophoresed proteins were transferred as described [8] and the membranes incubated with species-specific secondary antibodies. Immunoreactive bands were visualized by an enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Total cell lysates of 1—2 mg of protein were immunoprecipitated with 10 μl antibodies against p-IRS (Tyr 632). The immunoprecipitated samples were subjected to SDS-PAGE and Western Blot analysis, which was conducted as described previously.

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