Narrow-leafed lupin (*Lupinus angustifolius* L.) seed β-conglutins reverse back the induced insulin resistance in pancreatic cells

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Abstract

Insulin resistance (IR) is the main contributing problem leading to the development of the type 2 diabetes. In this study, we have purified recombinant β-conglutin proteins (rβ1 to rβ4, and rβ6) from narrow-leafed lupin (NLL) by using affinity-chromatography.

The objective of this study was to evaluate the capacity of these β-conglutins to improve IR state using *ex vivo* and in vitro systems. rβ1, rβ3, and rβ6 produced lower levels of pro-inflammatory mediator nitric oxide (~ -7-fold in all cases); up-regulated mRNA expression levels of IRS-1 (+201, +173, +192%) and Glut-4 (+286, +121, +147%); increased levels of p85-PI3K (+188, +187, +137-fold) and Glut-4 (+503, +548, +515-fold) proteins; higher levels of phosphorylation of insulin signalling pathway activator p-IRS-1, and downstream mediators as p-Akt, p-Cbl, and p-Caveolin; as well as improved glucose uptake in insulin resistant (IR-C) culture cells.

β-conglutin proteins were able to suppressing the oxidative stress produced by insulin induced resistance on PANC-1 control (C) cells by strongly reducing proteins oxidative carbonylation induced by ROS, and balancing the metabolic homeostasis in IR-C cells through regulation of mRNA expression. At the same time, β-conglutins are able to reduce the levels of pro-inflammatory mediator nitric oxide; and promoting anti-oxidative capacity of cells by increasing the levels of reduced glutathione.

These results suggest NLL β-conglutins might play a fundamental role as functional food components, since β-conglutins nutraceutical properties could enhance the effectiveness of dietary improvement of type 2 diabetes complications.

Keywords: vicilins, β-conglutins, *Lupinus angustifolius*, nitric oxide, proteins carbonylation, oxidative stress, insulin signalling pathway, diabetes.

Introduction

Diabetes mellitus is the most important disease worldwide after cancer and cardiovascular diseases[1]. Lifestyle and diet changes with an excessive caloric food intake are the main factors for the growing incidence of diabetes[2]. The International Diabetes Federation estimated that in 2015, more than 400 million people diagnosed with diabetes worldwide. This number will increase over 600 million by 2040, and people with type 2 diabetes (T2DM) will account for more than 90% of this number (https://www.idf.org/).

Development of T2DM is associated with the failure of pancreatic β cells that display the main functions of insulin synthesis and secretion. T2DM courses with insulin resistance (IR), a fundamental aspect associated with severe complications, including cardiovascular disease, retinopathy, nephropathy, and neuropathy. Thus, it is important to prevent and manage T2DM; therefore, an improvement on insulin sensitivity of sensible tissues is an important approach in diabetes prevention[3].

Various factors such as glucotoxicity, lipotoxicity, chronic inflammation, endoplasmic reticulum stress, dysregulation of adipokine secretion, and oxidative stress are implicated in the pathogenesis of IR[4]. In this regard, IR induces hyperinsulinemia, and defects in glycogen synthesis that plays an important role in glucose homeostasis, and a significant decrease in glycogenesis[5].

Therefore, at molecular level, phosphorylation of key proteins in the insulin signalling pathway affecting negatively or positively downstream effectors, e.g. serine or tyrosine phosphorylation of insulin receptor substrate isoform 1 (IRS-1) is a crucial mediating mechanism for IR development[6]. IRS-1 tyrosine and Akt phosphorylation represent an insulin sensitization mechanism in insulin-resistant cells[7]. Furthermore, enhancing the phosphorylation of Akt substrate of 160 kDa (AS160), increase the migration of glucose transporter-4 (GLUT-4) and the glucose uptake of insulin-resistant cells in the absence of

insulin[8], suggesting that they can be substitute for insulin to promote glucose clearance.

Pancreatic islets β cells may easily suffer from oxidative stress leading to IR. Elevated plasma free fatty acid (FFA) and glucose levels play a key role in the development of IR, since these metabolites are accompanied with excessive generation of reactive oxygen species (ROS) and increased lipid accumulation[9]. In this regard, high levels of ROS are an important trigger of IR, and contribute to the onset and development of T2DM, and ROS levels are negatively correlated with insulin sensitivity[10]. Thus, reduction of ROS level would be an important contributor for the improvement of IR, as another effective approach in the management of IR and T2DM[11].

Thus, interest in searching for effective therapeutic strategies to tackle this problem has increased worldwide. Food bioactive compounds that can treat IR or provoke insulin-independent glucose disposal should be helpful in preventing β-cell damage, and managing T2DM and related disorders. Sweet lupin seed bioactive components as fibre, polyphenols, digestible carbohydrates, and storage proteins as globulins, have displayed different health benefits, particularly in the area of dyslipidaemia, hyperglycaemia and T2DM, and hypertension prevention[12,13,14]. However, the mechanism/s at how these lupin seed compounds, and particularly specific proteins, improve inflammatory-related diseases remains to be elucidated.

In the present study, we used human pancreatic PANC-1 cells as a model of induced IR aimed to validate the ability of IR improvement by narrow-leafed lupin (NLL) β-conglutins by activation of insulin signalling pathway genes expression up-regulation and key proteins phosphorylation, anti-oxidative enzymes improvement and ROS production reduction, as well as T2DM metabolic makers improvement.

Material and Methods

Expression of genetic constructs

Bacterial β-conglutins expression were developed using the pET28a(+) vector containing each individual β-conglutin isoform β1, β2, β3, β4, and β6 (GenBank accession number HQ670409, HQ670410, HQ670411, HQ670412, and HQ670414, respectively), linked to 6xHis-Tag.

Proteins expression and purification

Proteins were expressed in Rosetta™ 2(DE3) pLysS Singles™ Competent Cells (Novagen) following the method developed by Jimenez-Lopez *et al.* (2016)[15].

Bacterial pellets were used for recombinant conglutin isoforms β1, β2, β3, β4, and β6 purification. The purity of the protein samples was >95% and typical yields were ~5–25 mg/mL. Purified β-conglutin proteins concentration was estimated using Pierce Coomassie (Bradford, Bio-Rad, USA) Protein Assay Kit (Thermo Fisher, USA).

β-conglutin antibody production

The polyclonal antiserum production was performed in rabbit by using a synthetic peptide as an immunization epitope that is commonly shared in the five NLL β-conglutin protein isoforms (Agrisera, Sweden). The antibody was affinity-purified being total IgG quantified by ELISA.

Cell culture

Human pancreatic PANC-1 cells were supplied by the Cell Bank of the University of Granada (CIC-UGR, Granada, Spain). PANC-1 cells were grown in poly-L-lysine-coated flasks (~2.0–2.5 × 10^6 cells/mL) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum at 37 °C under 5% CO_2 in humidified air. Cells were grown as monolayers and detached from cultures by trypsinization, and frequently sub-cultured to be used in the exponential growth phase for all experiments. Cells were washed twice with phosphate-buffered solution (PBS, Sigma) and treated with 0.25% tryp-EDTA (Lonza, Switzerland) for 10 min under 5% CO_2 in humidified air at 37 °C.

The trypsinization effect was neutralized with culture medium. The cells were collected after centrifugation at 1000 ×g for 5 min and washed with PBS for viability assays. Cell counting and viability were checked using a Countess II FL Automated Cell Counter (Thermo Fisher, USA) at the beginning and the end of each experiment using representative wells. Cell viability was monitored as > 95%.

MTT assay for cell viability

Assays of cell viability were performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to Riss *et al.* (2016)[16]. Briefly, PANC-1 cells were seeded in 96-well plates in the normal culture medium: 1×10^3 cells in 100 µL of medium per well in 200 µL of DMEM with FBS and antibiotics. After overnight incubation, cells were treated with LPS alone, or each of the β1, β2, β3 β4, and β6 purified protein alone or as a mix at indicated doses in two replicates. After the treatments, the cells were tree time washed with PBS avoiding any interference of the phenolic compounds with the MTT. 200 µL of free red-phenol DMEM containing 1 mg/mL of MTT was added, and the cells incubated for 3 h. Viable cells with active metabolism convert MTT into formazone crystals (purple colour), which was solubilized with 200 µL of DMSO, and the absorbance measured at test (570 nm) and reference (690 nm) wavelengths using a microplate reader (iMark microplate reader, Bio-Rad, USA).

Insulin resistance PANC-1 cell model and Glucose uptake

PANC-1 control cells (C) were seeded in 96-well microtiter plates in DMEM supplemented with 10% (v/v) FBS, under standard cell culture conditions (humidified atmosphere, 5% CO2 and 37°C), at a density of 2×10^4 cells/mL in 200 mL of growth media. The following protocol was used to determine the optimal dose of insulin and treatment duration required to establish insulin-resistant IR_PANC-1 (IR-C) cells.

The cells were divided into two groups with six replicates per group: 1) the cells were

cultured in 200 µL complete medium (group named C); 2) insulin-treated cells between $3x10^{-5}$ and $3x10^{-9}$ nmol/L when the cells become adherent (IR-C). The cells were then cultured for 24, 48 and 72 h. Following the defined culture periods, the glucose concentration in the culture media was detected using the glucose oxidase method, according to the manufacturer's guideline (Abcam, UK). Insulin resistant PANC-1 cell culture (IR-C) were stablished for insulin concentration $3x10^{-7}$ nmol/L cultured during 24h, and with the lower amount of glucose uptake[17]. At this state, we consider that cells are insulin sensitive or insensitive to determine whether β-conglutin proteins can improve insulin-dependent glucose uptake capacity of the cells.

Using IR-C epithelial cells, these cells were divided into seven groups, each with six replicates: The control group (C), 200 µl normal medium; the model group, IR-C; and the IR-C β-conglutin groups (β1, β3, β6, β2, β4 treated with 5µg of protein, respectively). After 24h of culture, 2 µl of supernatant was collected from each sample. Glucose concentration was detected as above.

IR-C cells were cultured to 80% confluence and then treated with β1, β2, β3 β4, or β6 purified proteins for 24 h. 5 µg of each purified conglutin was added to cultures. After the treatments, cells were harvested for further analyses.

Quantitative real-time PCR

Real-time quantitative PCR technology was used to assay IRS-1, GLUT-4 mRNAs expression from each experimental group. Total RNA was isolated from C cells using the RNeasy Tissue RNA isolation kit (Qiagen, Germany). First strand cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, USA). For gene expression assays, cDNA was prepared, diluted and subjected to real-time polymerase chain reaction (PCR), amplified using TaqMan technology (LightCycler 480 quantitative PCR System, Roche, Switzerland). Primers and probes were used from the commercially available TaqMan Gene Expression

Assays (GenBank accession no: NM_005544.2, Assay ID: Hs00178563_m1; GenBank accession n°: NM_001042.2, Assay ID: Hs00168966_m1, respectively). Relative changes in gene expression levels were determined using the 2−ΔΔCt method. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of β-Actin (GenBank accession n°: NM_001101.3, Assay ID: Hs99999903_m1, Applied Biosystems, used as the housekeeping gene) detection, referred to as ΔCT, where the relative mRNA levels were presented as unit values of 2∧ [CT (β-Actin)–CT (gene of interest)], having CT as the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline. PCR efficiency was determined by TaqMan analysis on a standard curve for targets and endogenous control amplifications, which were highly similar.

Immunoblotting phosphorylation analysis of IRS-1 and downstream effectors in IR-C cells

After treatments, IR-C cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer for 1 h, supplemented with a 1/100 dilution of protease and phosphatase inhibitor cocktails (Sigma, USA). After centrifugation at 12,000 × g at 4 °C for 30 min, the supernatant was stored at –80 °C until use. Proteins levels, including Glut-4 transporter, IRS-1, phospho-IRS-1 (Tyr612), p85-PI3K, Akt, phospho-Akt (Ser473), phospho-caveolin-1 (Tyr14), and phospho-CBL (Tyr700) (Santa Cruz, USA), were detected and evaluated by Western blot with primary antibodies. Briefly, equal amounts of protein samples were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) on a wet transfer apparatus (Bio-Rad, USA). The PVDF membranes were blocked with 5% non-fat dry milk or bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 2 h, and then incubated with primary antibodies overnight at 4 °C. Immunoblots were washed three

200 times (each for 5 min with TBS-T), followed by 1 h incubation in peroxidase conjugated
201 secondary antibody at room temperature. After washing the membrane four times (10 min)
202 with 15 mL of TBS-T buffer, the membranes were developed by Western Lightning™ Plus-
203 ECL (Perkin Elmer Life Sciences, USA). The blots were monitored by a luminescent image
204 analyser (LAS-3000, Fujifilm, Minato, Tokyo, Japan) to acquire the data for further analysis
205 and comparison.

206 **Antioxidant Enzymatic Activities Assays**

207 After 24h-incubation, the treated culture media was removed and cells washed with 4°C
208 phosphate buffered solution (PBS, Sigma, USA). C, and IR-C cells, and IR-C cells challenged
209 with different β-conglutin isoforms cells were collected for the GSH, SOD and Catalase
210 activity (Canvax, Spain), following manufacturer's instructions. Data were statistically
211 analysed by the t-test.

212 **Participant information**

213 Fourteen type 2 diabetic patients and fourteen healthy control subjects were involved for this
214 study. The subjects were unrelated and diagnosed from the coverage area of the 'Pedro
215 Martínez' hospital (A.G.S. North East Granada, Spain). All study procedures were performed
216 using samples and protocols previously approved by the ethics committee of 'Pedro Martínez'
217 hospital (A.G.S. North East Granada, Spain). Medical staff of the 'Pedro Martínez' hospital
218 (A.G.S. North East Granada, Spain) involved in this study obtained the full written and
219 informed consent for the procedures from all participants of this study.

220 The whole study was developed according to the international declarations in research ethics:
221 (1) The Declaration of Helsinki (DoH, Edinburgh 2000), ethical guidelines for physicians and
222 other participants in medical research of the World Medicine Association (WMA,
223 www.wma.net).

224 (2) Council of Europe Convention for the Protection of Human Rights and Dignity of the

225 Human Being with regard to the Application of Biology and Medicine (Oviedo 1997,
226 www.coe.int).
227 (3) Universal Declaration on Bioethics and Human Rights adopted by UNESCO's General
228 Conference on 19 October 2005 (www.unesco.org).
229 (4) CIOMS/WHO International Ethical Guidelines for Biomedical Research Involving
230 Human Subjects (1993, reviewed in 2001, www.cioms.ch).
231 Moreover, this study was developed according to the Spanish research and ethics laws:
232 1) LAW 14/2007, of 3rd July on Biomedical Research.
233 2) ROYAL DECREE 65/2006, of 30 January on setting requirements to import and export
234 biological samples (when applicable).
235 3) ROYAL DECREE 1301/2006, of 10 November, on setting quality and security standards
236 to donate, obtain, evaluate, process, preserve, store and distribute human cells and tissues, and
237 approving the managing regulation for using them in humans.
238 4) ORDER of 14 April 2000, adapting the annexes of the Royal Decree 2043/1994 of 14th
239 October as regards inspection and verification of good laboratory practices, to the technical
240 progress.
241 T2DM group includes patients that were screened and diagnosed according to the American
242 Diabetes Association guidelines[18]. Furthermore, anthropometric data were collected; i) Body
243 mass index (BMI) was calculated as weight in kilograms divided by the square of height in
244 meters; ii) Blood pressure and heart rate were measured in a standardized manner. After
245 subjects remained resting for a period of time at least of 5 min, blood pressure and heart rate
246 were measured twice using a standard mercury sphygmomanometer and a heart rate monitor
247 with a wrist receiver, respectively. In both cases, the mean of the two values was used for
248 analysis; iii) We obtained samples of venous blood collected from the cubital vein in 4 mL
249 lithium–heparin tubes; iv) Biochemical parameters were obtained. Fasting plasma glucose

was measured by standard biochemical methods, where fasting can be defined as no caloric intake for at least 12 h; v) Glycated haemoglobin (HbA1c) was measured using standard automated laboratory techniques, by high-performance liquid chromatography.

When calling patients diagnosed with T2DM for blood samples extraction, these receiving treatment for T2DM, e.g. metformin, sulfonylureas, thiazolidinedione or other medications that might affect inflammation process were excluded from this study.

Whole blood Culture

Venous blood was drawn into lithium–heparin tubes (BD Vacutainer System, Germany) in the morning. Participants were fasted for 12 hours before blood collection (Fasting consisted in no food or drink intake but water). Within 3 h, whole unseparated blood was diluted 1:3 with Dulbecco's modified Eagle's medium (DMEM) and HEPES 2.4%; (Invitrogen, Germany), and agitated gently in 50mL tubes (Greiner Bio-one, Germany); aliquots (1mL) were seeded in 24-well plates (Nunc, VWR International GmbH, Germany) and cultured for 24 h at 37°C under 5% CO_2 in humidified air. Assays were performed for each blood included positive and negative controls, and separate cultures challenged with 15 μg of each purified β-conglutin (β1, β2, β3, β4, β6, respectively). After treatments, blood samples were centrifuged at 700 g for 5 min at 20°C; obtained supernatants were aliquoted and stored at -20°C until further analysis.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density-gradient centrifugation on Histopaque 1077 (Sigma, USA), washed three times in Hanks' balanced salt solution (Life Technologies, USA), and re-suspended in complete medium consisting of RPMI 1640 (Sigma, USA) supplemented with penicillin, streptomycin and L-glutamine (100 U/mL, 100 μg/mL, and 2 mM, respectively) (Sigma, USA).

Polymerase chain reaction (PCR) array for diabetes-inflammatory response

Total RNA was extracted from isolated PBMC of type 2 diabetic patients using an RNeasy

Mini Kit (Qiagen, Germany). 2 μg total RNA was converted to complementary DNA (cDNA) using an RT2 First-Strand kit (Qiagen, Germany). The resulting cDNA was mixed with RT2 SYBR Green qPCR (Qiagen, Germany) and aliquoted into a commercially available diabetes-obesity and inflammation pathway-specific real-time RT2 Profiler PCR Array (PAHS-023Z, Qiagen, Germany). Quantitative real-time PCR (qPCR) was performed using a Light Cycler 480 (Roche, Switzerland), where samples were run as duplicates for 40 cycles: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 40 cycles of 60 °C for 1 min. Cycle thresholds were measured, and the relative expression of genes calculated by comparing Ct values. The difference in gene expression was evaluated by calculating the fold-change in expression levels based on at least a 2-fold up or down change in comparison or the gene expression levels of the control group. All gene expression levels were normalized to the housekeeping genes – β-Actin, hypoxanthine phosphoribosyl transferase 1, β-2-microglobulin, and glyceraldehyde-3-phosphate dehydrogenase - which did not vary significantly between the study groups.

Determination of intracellular ROS (Oxyblot assay) and nitric oxide

Proteins were extracted from control C and IR-C cell cultures that were/were not treated with individual β-conglutin isoforms, following either control or treatments company instructions (EMD Millipore, USA). 25 μg of total proteins were loaded onto 12% polyacrylamide gels for protein separation. Proteins separated by SDS-PAGE were electrotransferred to PVDF membranes. The OxyBlot^{TM} Protein Oxidation Detection Kit (EMD Millipore, USA) was used according to the manufacturer's instructions for immunoblot detection of carbonyl groups introduced into proteins by reaction with ROS. Measurements were made at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

The total amount of NO production including nitrite/nitrate contents from cultured C cell samples and IR-C cells before and after β-conglutin proteins challenges were measured using

a commercial assay kit ab65328 (Abcam, UK). Briefly, samples from all experimental groups were deproteinized according to the manufacturer's instructions. An equal amount (30 μL) of samples and standards were loaded into 96-well microtiter plates. Nitrate reductase, enzyme cofactor, and assay buffer was added, followed by 1 h incubation at room temperature by Enhancer, Griess Reagent R1 and Greiss Reagent R2. Immediately after incubation, measurements at 540 nm were carried out in a microplate reader (iMark Bio-Rad, USA), and the value of the blank control (medium without cells) was subtracted. Total nitrite/nitrate concentrations were calculated by using a standard curve.

Statistics

Values from all assays were carried out at in triplicate and data were shown as means ± standard deviations (SEM). Statistical significant differences ($p < 0.05$) of the data were assessed using SPSS software by analysis of variance followed by Dunnett analysis.

Results and Discussion

Purification of β-conglutin isoforms

Purification of β-conglutins was accomplished according to the methodology of Jimenez-Lopez *et al.* (2016)[15]. Protein elution was performed by using an imidazole linear gradient (0–300 mM). SDS–PAGE analyses of the eluted fractions displayed a single protein band of ~ 69 kDa (Suppl. Fig. S1). Purified recombinant β-conglutins exhibited a level of purity > 95%, and a concentration of 5–25 mg/mL. Analysis by immunoblotting using the anti-β-conglutin protein antibody confirmed the identity of the recombinant proteins (Suppl. Fig. S1).

β-conglutins do not affect the cellular viability of PANC-1 pancreatic cells

Before assessing the IR improvement activity of β-conglutin proteins, we evaluated their effect on cell viability and potential cytotoxicity. To investigate whether β-conglutin isoforms could have a cytotoxic effect, an MTT assay was performed on insulin induced-resistance IR-C cells after separately adding each β-conglutin isoform protein, as well as a mix of the five

β-conglutins for 24 h. Neither individual β-conglutins nor the mix of β-conglutins had significant effect on cell viability (Suppl. Table S1), when compared to the unchallenged IR-C group. The addition of insulin in the absence of β-conglutin was used as a positive control.

To complete the feasibility studies of the β-conglutins, cell viability was assayed using trypan blue exclusion in IR-C pancreatic cells treated either with increasing concentrations of each β-conglutin, or the mix of conglutins, for a 24 h period. As expected, there were no differences in cell viability after 24 h of incubation in the presence of higher concentrations of each β-conglutin, or in the mix of β-conglutins containing 5 or 10 μg when compared to the control (Suppl. Table S1).

These results suggest that either individual β-conglutin proteins or the mix of isoforms do not compromise the integrity of IR-C pancreatic cells. Moreover, cell viability did not differ significantly from the control assays upon exposure to higher concentrations of β-conglutins.

Participant information

The clinical parameters of the population studied are summarized in Suppl. Table S2. As expected, the T2DM patients showed classical parameters for BMI, and in values of fasting glycaemia ($p < 0.002$), blood pressure ($p < 0.0025$), heart rate ($p < 0.001$), and HbA1c ($p < 0.001$), when compared to the control study group of healthy volunteers.

Insulin induced resistance IR-C cell model

IR plays a key role in the development of inflammatory-related pathologies such as T2DM and obesity[19]. Furthermore, IR derives in several metabolic disorders including hyperglycaemia, hyperinsulinemia and hypertriglyceridemia[20]. Thus, the alleviation of IR by NLL seed proteins as β-conglutins may contribute to the inhibition of these disorders.

In order to establish an *in vitro* insulin resistant model of pancreatic cells and evaluate the effects of different insulin concentrations on glucose metabolism in the cell model, C cells were incubated with insulin in a concentration range between 5×10^{-5} and 5×10^{-9} nmol/L to

build an insulin-resistant cell (IR-C) model (Suppl. Fig. S2). The cells were stimulated with fresh insulin for 24 h, and the glucose uptake of these cells was analysed. As shown in Suppl. Fig. S2A, following 3×10^{-7} nmol/L insulin incubation of C cells, there was the most significant decrease in the consumption of extracellular glucose ($P < 0.05$) compared with control without insulin pre-treatment. In Suppl. Fig. S2B, the addition of 3×10^{-7} nmol/L insulin to C cells induced a time-dependent decrease ($P < 0.05$) in the glucose consumption in the period 24-48 h compared to C cells without insulin pre-treatment. These results showed that IR-C cells have maintained the state of insulin-resistant in a period of 48 h after insulin incubation. After that, cells are acquiring a normal condition as C cells, which is consistent with the increasing glucose uptake showed after 72h in Suppl. Fig. S2B and where non-significant differences in glucose consumption were displayed compared to C cells without insulin pre-treatment.

β-conglutins increase glucose uptake and insulin signalling pathway genes expression in IR-C cells

IR is observed in a wide variety of pathophysiological states and it is a key cause of diabetes and pre-diabetes status[21]. Improving IR is the main approach to prevent and treat diabetes. Hyperglycaemia and hyperinsulinemia result from IR, which may affect different insulin target organs, e.g. liver and skeletal muscle[22]. The alleviation of IR by NLL seed proteins as β-conglutins may contribute to the inhibition of insulin dependent organs to improve the T2DM state. However, the molecular mechanisms underlying the effects leading glucose homeostasis and IR are still unclear.

The different NLL β-conglutins might play an important role on glucose homeostasis. The treatment of pancreatic insulin induced resistance IR-C cells with β-conglutins were performed to determine their effects on GLUT-4 mRNA level and increasing total GLUT4 protein for potential improvement of glucose uptake in IR_PANC-1. We have demonstrated

that treatment with conglutin proteins β1, β3 and β6, induced glucose uptake by IR-C cells showing comparable results since increasing β-conglutin quantity treatment resulted in an incensement of the glucose uptake in IR-C cells, particularly until 5 μg of proteins, which induced a glucose uptake of 60% in comparison to IR-C cells ($p < 0.05$) (Fig. 1). A small decrease was displayed when β-conglutin proteins (10 μg) was added to the cell culture.

A similar effect was showed between members of the other β-conglutins group. On the contrary to what happened to β1, β3, and β6, treatment with conglutins β2 and β4 maintained glucose uptake at comparable levels than IR-C cells (without β-conglutin proteins challenge).

Therefore, the analysis of key genes in the insulin signalling pathway as IRS-1 and GLUT-4 has showed the up-regulation of the expression for these genes. The increased expression of IRS-1 and GLUT-4 was achieved when conglutin β1, β3 and β6 were incubated with IR-C cells (IRS-1: +201%, +173%, and +192%; GLUT-4: +268%, +121%, and +147%, respectively) ($p < 0.05$) (Fig. 2A). Furthermore, IRS-1 and GLUT-4 mRNA expression did not increase in IR_PANC-1, being at similar levels to control when cell culture was challenged with conglutin β2 and β4 (Fig. 2B). In this regard, protein synthesis of p85-PI3K and GLUT-4 increased when β1, β3 and β6 challenged the IR-C cell culture (p85-PI3K: +188%, +187%, and +137%; GLUT-4: +503%, +548%, and +515%, respectively) ($p < 0.05$), in comparison to C cells. No significant differences in total GLUT4 protein expression were observed between IR-C cell culture when challenged with β2, and β4.

These findings suggest that β-conglutin proteins would be able to significantly reduce blood glucose level by promoting glucose uptake by cells and alleviating hyperglycaemia via increasing GLUT-1 concentration. In this regard, improving IR is also achieved through up-regulation of IRS-1 may mediates the activation of the insulin signalling pathway leading to decreasing blood glucose by cells glucose uptake[23]. Furthermore, glucose uptake is mediated by the recruitment of glucose receptor GLUT4 to the plasma membrane. At the cellular level,

400 the ability to uptake glucose is determined by controlling the amount of the GLUT4 glucose
401 transporter present in the plasma membrane. At molecular level, increased expression of
402 GLUT-4 may increase glucose uptake, together with an increasing trend of insulin signalling
403 key proteins phosphorylation[24].

404 **Conglutin β1, β3 and β6 increased the phosphorylation of IRS-1 and downstream**
405 **effectors**

406 Chronic insulin treatment or IR usually results in the reduction of insulin-stimulated tyrosine
407 phosphorylation of insulin receptor[7]. Insulin-resistant target cells exhibit impaired insulin-
408 stimulated glucose uptake and defective insulin signal transduction[25]. The Ser/Thr
409 phosphorylation of insulin receptor and substrates IRS proteins are considered to be a
410 molecular basis for IR. The insulin receptor is a tyrosine kinase that undergoes ligand-
411 stimulated auto-phosphorylation and activation of its intrinsic substrate kinase activity. Once
412 activated, the receptor phosphorylates intracellular substrates on tyrosine, including members
413 of the insulin receptor substrate family (IRS1/2/3/4), and downstream effectors as Cbl or
414 APS[26]. The insulin receptor-dependent tyrosine phosphorylation of both IRS1 and IRS2 are
415 critical in maintaining proper glucose homeostasis through its interaction with the PI3K[27].
416 This interaction appears to serve a dual function by stimulating PI3K activity and targeting
417 the enzyme to a critical intracellular site[28].

418 We treated the IR-C cells with β-conglutin proteins to investigate the molecular mechanism
419 by which these proteins improve insulin sensitivity. Thus, we studied the effects of β-
420 conglutins on insulin signalling pathway in C cells at basal level or after insulin induced
421 resistance IR-C cells. As shown in Fig. 3A, basal phosphorylation of Akt at serine 473 and
422 IRS-1 at tyrosine 612 in C and IR-C cells were analysed after β-conglutin treatments.
423 Furthermore, after conglutin β1, β3 and β6 challenges elevated Akt and IRS-1
424 phosphorylation in IR-C (Fig. 3A and Fig. 3B), were the p-IRS1/IRS1 and pAkt1/Akt1 were

425 increased (+250, +283, +275-fold; and +315, +396, +303-fold, respectively). In addition to
426 increasing levels of IRS-1 phosphorylation, we also found that mRNA expression level of
427 IRS-1, and IRS-2 were up-regulated when T2DM blood culture were challenged with β1, β3,
428 and β6-conglutins in comparison to control (T2DM blood untreated) (Table 1), as well as
429 down-regulation of the mRNA expression level of protein tyrosine phosphatase, non-receptor
430 type 1 (PTPN1), a protein that can dephosphorylate the phospho-tyrosine residues of the
431 activated insulin receptor kinase, thus inhibition of the PTPN1 increase insulin sensitivity by
432 preventing inhibition of IRS-1 dephosphorization (Table 1). These results show that using
433 NLL β-conglutins improves the insulin signalling in insulin induced resistant cells, which also
434 could improve IR state.
435 The translocation of GLUT4 is predominantly mediated through the insulin-dependent p85-
436 PI3K/AKT/GLUT4 signal pathway to form glycogen storages[29], which involves several
437 pivotal genes, such as PKB, p85-PI3K, IRS-1, and insulin receptor (IR). When insulin binds
438 to IR induces tyrosine phosphorylation of IR and IRS-1, which subsequently activate and
439 phosphorylate IRS-1, p85-PI3K, AKT and PKB/AKT2, and induce GLUT4 translocation[30].
440 Thus, the activation of IRS-1, p85-PI3K and AKT could be direct evidence to demonstrate the
441 specific effects of NLL β-conglutin proteins only on the insulin-dependent pathway. Our
442 results showed increased tyrosine phosphorylation of IRS-1, which could reduce glucose level
443 (increase glucose uptake) and IR in hyperglycaemic and T2DM state[7], in agreement with the
444 IRS-1 and GUT-4 mRNA increased expression (Fig. 2A), and p85-PI3K and GLUT-4
445 proteins synthesis (Fig. 2B). In addition, we also found increased levels (up-regulation) of
446 PKB after T2BM blood culture treated with β1, β3, and β6 (Suppl. Table S3). It has been
447 proven that hyperglycaemia and IR is caused via inhibition of p85-PI3K phosphorylation[30].
448 Consequently, phosphorylation of IR, IRS-1, p85-PI3K, and PKB is essential for GLUT4
449 translocation and anti-hyperglycaemia. In the current study, we have shown increasing

phosphorylation of proteins IRS-1, and Akt [IRS-1(Tyr612), and Akt (Ser473)] up-regulated by NLL conglutins β1, β3, and β6 treatment in insulin induced resistant IR-C cells (Fig. 3). Moreover, NLL β-conglutins also increased IRS-1, and GLUT-4 mRNA and p85 and GLUT4 proteins expression levels (Fig. 2A). These findings demonstrated that NLL β-conglutins may enhance GLUT4 translocation via increasing the phosphorylated proteins in the PKB/GLUT4 pathway, as well as the up-regulation of IRS-1 and GLUT-4 genes' expression at the transcriptional level and corresponding proteins synthesis in insulin resistant IR-C.

In addition to the p85-PI3K activation through IRS-1 phosphorylation (a p85-PI3K-independent pathway), recent data suggest that a second requisite pathway might involve tyrosine phosphorylation events that are restricted to subdomains of the plasma membrane[30]. We have investigated the role of Cbl and caveolin proteins phosphorylation in facilitating GLUT4 translocation to the membrane and glucose uptake. The tyrosine phosphorylation of Cbl results in caveolin-enriched, lipid raft subdomains of the plasma membrane[31], and is required to promote the GLUT4 translocation to the plasma membrane. In this regard, lipid raft subdomains have been implicated as sites for signal initiation through helping in formation of caveolae, a subset of lipid raft microdomains. These small invaginations of the plasma membrane are often enriched in signalling molecules, glycolipids, and cholesterol and have been proposed to act as signalling organelles[32].

In the present study, our data presented in Fig. 3C and 3D show that conglutin β1, β3, and β6 maximally phosphorylate Cbl (Tyr700) and caveolin-1 (Tyr14). Differences among control and these isoforms of β-conglutin treated cells in Cbl and caveolin-1 phosphorylation level were significant ($p < 0.03$). Although the precise function of lipid raft subdomains in assembling this signalling complex remains uncertain, resident structural proteins may play an important role. One such protein is caveolin-1 that is known to undergo tyrosine phosphorylation[33], and when Cbl is phosphorylated, a Cbl-CAP complex is recruited to lipid

rafts through the interaction of CAP with flotillin[34]. In addition, the caveolin family of proteins appears to play a particularly important role in the formation of caveolae and possibly in signalling activities that occur in these microdomains. Caveolin interacts with a number of signalling molecules that are thought to be enriched in caveolae, including a subset of tyrosine kinase receptors as the insulin receptor[34]. Caveolin is also known to undergo tyrosine phosphorylation although this phosphorylation correlates with metabolic responsiveness to insulin. In addition, previous studies strongly support a direct insulin receptor-mediated tyrosine phosphorylation of caveolin on Tyr14 of the protein, which does not require the activation of PI3K or MAPK and is independent of the phosphorylation and translocation of Cbl to the lipid raft microdomains[33]. A possibility remains that the tyrosine phosphorylation of caveolin by the insulin receptor is merely a result of the interaction of these two molecules and, further, that the binding of caveolin to the receptor helps to recruit the latter protein to caveolae, where it initiates the CAP/Cbl signalling pathway[35]. The investigation of this possibility will be the subject of a further work.

This data collectively suggested that NLL β-conglutins i) promote the phosphorylation of p-85-PI3K through activation of IRS-1 by phosphorylated IRS-1, beside to an increases in their mRNA expression ($p < 0.05$); ii) activated the downstream proteins of SOCS3 such as Akt, and could increase the insulin sensitivity by regulating Akt; GLUT4 translocation to the plasma membrane was regulated by iii) its major upstream regulators, via promoting the phosphorylation of IRS-1, Akt, and p85-PI3K; and iv) promoting the phosphorylation of Cbl and caveolin proteins that recruit main activators protein complexes to lipid rafts and caveolae formation. β-conglutin proteins are a potential biochemical composition from the legume NLL to promote glucose uptake and glycaemic control.

Effects of β-conglutin proteins on the metabolism and signalling pathways

A main thing for the IR state is the uncontrolled balance of the glucose metabolism. In this

state, glucose uptake to be used in target tissues is deeply decreased because down-regulation of GLUT-4 transporter in T2DM subjects[36]. In addition, glucose metabolism key enzymes expressions are also dysregulated, e.g. hexokinase or glucose kinase, glucose 1,6 phosphatase and glucose 6-phosphatase, controlling the direction of glycolysis and gluconeogenesis direction[37].

In the current study, we have found that particular β-conglutin isoforms are able to regulate the expression of key enzymes of the glucose metabolism (Fig.1) that may favour increase glucose utilization and degradation instead of gluconeogenic pathway and to activate the metabolism of isolated PBMC from type 2 diabetic patients´ blood. Hexokinase (GK) mRNA level was only affected by β1 treatment (lowered the value) in PBMC samples compared to control group (without β-conglutin proteins challenges), which may help to use glucose in the degradative catabolic pathway (glycolysis instead to the pentose phosphate pathway). The same effect is favoured by the decreasing levels of glucose 6-phosphate dehydrogenase when T2DM blood samples were challenged with β1, β3, and β6-conglutins. Glucose catabolism may be favoured also by decreasing levels of glucose 6-phosphatase (G6Pase), G6Pase catalytic subunit, and mRNA up-regulated levels of fructose 1,6- biphosphatase (F1,6BPase) in T2DM blood treated with β1, β3, and β6 (Table 1). G6Pase catalyse enzymatic reaction that is included in the gluconeogenesis reactions, and confers the capacity to release glucose into the blood[38]. In the present study, simultaneous lowering expression of enzymes that decreased glucose generation (GP and G6Pase) and increase in those involved in glucose utilization (HK) increased glucose utilization, causing improvements in hyperglycaemia (reduction of blood glucose) and IR (insulin sensitivity) in T2DM culture cells.

At the same time, glucose homeostasis is controlled not only by glucose catabolism but also by glycogen synthesis through balancing insulin/glucagon levels. Increasing mRNA levels of glucagon-like peptide 1 receptor (GLP-1R) by β2 and β4 treatments of T2DM blood samples

525 (Suppl. Table S3) is able to activate the insulin secretion of pancreatic beta cells[39] that
526 increasing the insulin/glucagon balance favouring the glucose catabolism since increasing this
527 balance activates key glycolysis metabolism enzymes as phosphofructokinase, and inhibiting
528 gluconeogenesis[40]. In addition, in order to increase this balance insulin/glucagon, β1 and β3
529 down-regulated mRNA synthesis of glucagon and glucagon receptor, as well as increasing
530 mRNA synthesis of insulin by β1, β3, and β6 (Suppl. Table S3).
531 An increased glucokinase (GK) activity enhanced glucose utilization of blood for glucose
532 storage, and glucose uptake in insulin targeted tissues[41]. Glycogen synthesis from different
533 precursors and its degradation are known to be dependent on nutritional and hormonal factors,
534 principally glucose and insulin[40]. Insulin decreased the glucose output by activating glycogen
535 synthesis and glycolysis, and by inhibiting gluconeogenesis. However, in diabetes, the
536 gluconeogenic pathway is aberrantly activated and supplies a relatively larger amount of
537 glucose into the circulation[42]. To control glucose catabolism levels, GSK3β (inhibit the
538 glycogen synthesis) is down-regulated by β1, β3, and β6 treatment of T2DM blood culture
539 (Suppl. Table S3), which allow part of the glucose entering to glycogen biosynthesis.
540 Furthermore, we have also found that mRNA insulin is up-regulated by β1, β3, β6 (Suppl.
541 Table S3), helping to balance glucose catabolism and gluconeogenesis. Thus, β-conglutins
542 can promote the influx of glucose into the cells by GLUT-4 (increased expression levels),
543 which in turn balance glucose oxidation and glucose production in T2DM, improving IR
544 through normalization of glucose metabolism-related enzyme expression levels.
545 Pro-inflammatory cytokines regulate the pancreatic β-cell function. In addition to plasma
546 circulating cytokines, pancreatic β-cells produce various cytokines in response to different
547 physiological and pathological stimuli, playing key roles in regulating β-cells function, and in
548 IR[43]. IR increases the production of pro-inflammatory cytokines in β-cells and plasma. Thus,
549 the T2DM progression, this abnormal situation is characterized by an imbalance in the profile

of pro-inflammatory nitric oxide, cytokines (i.e., interleukins), TNF-α, and INF-γ, and other mediators that play an important role in triggering β-cell dysfunction, IR and T2DM since crosstalk through cytokines in pancreatic β-cells and other tissues may be strongly involved in regulation of β-cell function[44]. Thus, restoring the balance toward to the protective cytokines in β-cells and plasma could prevent and treat β-cell dysfunction, IR and T2DM progression. In the current study, we have demonstrated that NLL β1-, β3-, and β6-conglutin proteins decreased the mRNA production levels of pro-inflammatory mediators NO (Fig. 5), TNF-α, INF-γ, and NFkB1 in T2DM blood culture (Table 1, and Suppl. Table S3). The nuclear factor NF-κB is a key player in inflammatory diseases as it controls the global inflammatory response. NF-κB regulates the expression of a plethora of pro-inflammatory genes in most metabolic tissues, including enzymes as cyclooxygenase (COX)-2, 5-lipoxygenase (LOX), iNOS; cytokines as TNF-α, cell cycle regulatory molecules, and angiogenic factors[45]. In the current study, the mRNA expression level of NF-κB1 (p50) was strongly inhibited by β1, β3, and β6 (Table 1), which may contribute to a reduction in the formation and activation of the NF-κB complex. This reduction in NF-κB mRNA expression level may also be indirectly facilitated by the reduction in INF-γ and TNF–α levels promoted by the same β-conglutin isoforms.

This wide anti-inflammatory capacity may be able to cope with multiple situations that promote a feed-forward process in the establishment of a chronic inflammatory state that is advocated by IR in T2DM. Thus, β-conglutins may be able to reverse the harmful effects of an inflammatory course of various cellular states as different levels as described in Lima-Cabello *et al.* (2018)[14].

Protein kinase, AMP-activated (AMPK) is a conserved serine/threonine kinase, which plays a vital role in cellular energy homeostasis maintenance. AMPK inhibits fatty acid synthesis through restraining phosphorylation of two targets, acetyl-coA carboxylase 1 (ACC1) and

sterol regulatory element-binding protein 1c (SREBP1c)[46]. In addition, AMPK stimulates glucose uptake by the translocation of GLUT4-containing intracellular vesicles across the plasma, as well as inhibits gluconeogenesis for keeping blood glucose levels[47]. In addition, the small GTPase Rab4 participates in the molecular mechanism involved in the subcellular distribution of the glucose transporters GLUT-4 molecules both in basal and in insulin-stimulated conditions, which are highly responsive to insulin for glucose transport. It has been determined that when Rab4 was moderately present, the number of GLUT-4 molecules recruited to the cell surface in response to insulin increased, as well as the insulin efficiency. However, when Rab4 is present in higher level, the amount of GLUT-4 present at the cell surface in response to insulin decreased[48]. In this regard, β1, β3, and β6-conglutins treatment helped to decrease Rab4 mRNA levels in T2DM (Suppl. Table S3) to increase the recruitment to the cell surface of GLUT-4 transporter in order to increase the influx of glucose and improve the IR.

Therefore, in the current work, we have also found that AMPKα1 catalytic subunit, and AMPKγ2 non-catalytic subunit mRNA expression level are up-regulated by β4 and β1, respectively, in T2DM cultures (Suppl. Table S3). These increased levels of this kinase may help to control glucose homeostasis and improve IR, which could be achieved through affecting the mRNA levels of FOXO1, GLP-1R, PDX-1, and PI3K in hepatic and pancreatic tissues. It has been previously found that AMPK decreased for FOXO1[49]. In addition, these findings indicated that AMPK significantly affects mRNA levels (up-regulation) of GLP-1R (activate the insulin secretion of pancreatic beta cells), PI3K (activation of insulin signalling pathway and glucose uptake by GLUT-4), at the same time that down-regulated (lowering) mRNA levels of downstream signalling pathway genes FOXO1 (transcription factor) and PDX-1 (insulin promoter factor 1). AMK activates PI3K/AKT signalling to decrease the cellular expression of FOXO1 and increase that of PDX-1. Brunet et al.[50] found that insulin

represses liver gluconeogenesis through PI3K/AKT phosphorylation and FOXO1 inactivation. Thus, due to these major roles, the anti-diabetic effects promoted by AMPK includes a strong potential of preventing IR, oxidative stress or diabetic disorder, particularly AMPK affecting the decreasing blood glucose, lipid profiles, and serum levels of inflammatory biomarkers, as well regulate the PI3K/FOXO1/PDX-1 signalling pathway. Furthermore, the beneficial biological functions of GLP-1 are mediated by its interaction with its specific receptor, GLP-1R, a member of the B-subclass of the G protein-coupled receptor (GPCR) family. GLP-1 binds to GLP-1R in β-cells and increases cAMP levels. In turn, cAMP molecules individually act on protein kinase A and exchange protein directly activated by cAMP to stimulate insulin secretion and promote β-cell growth[39]. In addition, GLP-1R is a GPCR family member that can activate PI3K that participate in several critical steps in insulin signal transduction and in glucose and lipid metabolism. We suggest that mRNA increasing levels of AMPK by β1 and β4-conglutins could help effectively activate GLP-1R, also up-regulated by β4 and β6-conglutins, which acts on GLP-1 to strengthen insulin/IGF-1 signal, potentiated by the fact that β1, β3, and β6-conglutins up-regulated the mRNA expression levels of both insulin and IGF-1 (Table 1, and Suppl. Table S3). This effect, in turn, may lead to the phosphorylation of downstream PI3K/AKT pathway and inhibits the expression of downstream FOXO1 to promote the nuclear localization of PDX-1 and pancreatic β-cell mass and function.

Transcriptional Activation of metabolism through insulin pathway require a cascade of proteins phosphorylation where implicated multiple kinases, e.g. extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. All these kinases are activated by insulin in sensitive cells. It has been found that activation of p38 MAPK is impaired in insulin resistant cells, where as ERK and JNK were activated by insulin[51]. We have also found that p38 mRNA expression levels are down-regulated in induced insulin

625 resistant IR-C cells. However, treatment of IR-C cell culture with β1, β3, and β6-conglutins
626 are able to highly increase mRNA expression levels of p38 MAPK (Table 1), which would be
627 also a mechanism to increase p38 activity if more available protein is produced in the cell,
628 recovering p38 levels of insulin non-resistant state.

629 **Determination of the effects of β-conglutin proteins in oxidative stress.**

630 Oxidative stress (excess of ROS generation) is the main factor to the onset and the
631 progression of diabetic complications (IR) under obesity or hyperglycaemic conditions[11].
632 Furthermore, oxidative stress may be an important factor for the development of IR and
633 T2DM. Hyperglycaemia causes injury of antioxidant defence system followed by excessive
634 reactive oxygen species (ROS) production, contributing to oxidative stress and mitochondrial
635 dysfunction that in turn aggravate IR[52]. The amount of ROS within the cell is finely
636 modulated by enzymatic and non-enzymatic antioxidant defences such as superoxide
637 dismutases (SODs), catalase (CAT), and glutathione[53]. Therefore, it is necessary to
638 understand the relationship among oxidative stress, and IR for diabetes prevention, and
639 decrease free radical levels may serve to reduce the risk of diabetes and its associated
640 complications[54].

641 Thus, the effect of β-conglutin protein on the capacity of suppressing the oxidative stress
642 produced by insulin induced resistance on C cells was examined. IR-C cells were challenged
643 with individual conglutin isoforms β1 to β4 and β6. We estimated production of ROS
644 capacity by examining the level of protein carbonylation in insulin induced resistant cells and
645 compared to C and IR-C without any challenge using an OxyBlot Protein Oxidation Detection
646 and immunoassay[55]. Protein oxidation is one of the covalent modifications of proteins
647 induced by ROS such as H_2O_2 or other products of oxidative stress, and carbonylation is one
648 of the most commonly occurring oxidative modifications of proteins, which may be
649 responsible for the alteration in protein activity, for example, signalling. Carbonylated

proteins have been identified in many plant species at different stage of growth and development as indication of oxidative modification in proteins[56].

Very low levels of proteins oxidation, as generated through normal metabolic activity were observed in IR-C cells after challenging with conglutin β1, β3, and β6, as well as in non-challenged C cells (Fig. 4). Following challenges with conglutin β2, and β4, protein oxidation remained at similar levels than insulin induced resistant IR-C cells (Fig. 4). These results show that conglutin β1, β3, and β6 effectively reverse back the ROS production (oxidative stress) in pancreatic IR-C cells treated with these β-conglutins. The analyses indicated that NLL conglutins β1, β3 and β6 have the strongest anti-oxidant effect, and these proteins ameliorated the oxidative stress induced by IR in IR-C cell model.

Therefore, IR has been found to be intricately linked with a rapid rise in free radicals and weakening of antioxidants. The Superoxide dismutase (Cu/Zn-SOD) enzymatic activity, a cytoplasmic isoform as a major antioxidant enzyme for removal of free radicals, glutathione (GSH) values and nitric oxide production represent key indicators of the level of oxidative stress[11]. In the current work, it was evaluate the effects of the different isoforms of β-conglutins over antioxidant variables in the *in vitro* model (IR-C). In order to achieve this, we measured the SOD and Catalase activities, GSH levels and NO production in induced resistant IR-C cells before and after individual β-conglutin isoforms challenges (Fig. 5). We found significant increased levels of GSH in IR-C cells after treatment with β1-, β3-, and β6-conglutins (+1197, +1515, +1565-fold, respectively) (Fig. 5A). Levels of GSH remained not altered when challenges were made with β2-, and β4-conglutins compared with IR-C cells. On the other hand, the levels of SOD and Catalase activities were strongly reduced in IR-C after the same treatments with β1, β3, and β6 (SOD: -286, -291, -290-fold; Catalase: -28, -26, -26-fold, respectively) (Fig. 5B and Fig. 5C). These data indicated that increasing levels of GSH and reduction on the SOD and Catalase activities may be regulated by β-conglutins as

consequence of direct or indirect marked effects of these β-conglutin isoforms in preventing lipid peroxidation and proteins oxidative modifications because drastic reduction on oxidative carbonylation (Figure 4) and, in this regard, overall oxidative stress balancing (amelioration) by β-conglutins.

Additionally, nitric oxide is a signalling molecule that plays a key role in the pathogenesis of inflammation, since NO is considered a pro-inflammatory mediator that induces inflammation when over-produced in abnormal situations in an oxidative stress situation (IR), e.g. as result of peroxynitrite production, a potent oxidizing agent from the reaction between superoxide with nitric oxide (NO)[57]. In the current work, the effect on the NO production was determined in IR-C cells challenged with individual β1, β2, β3, β4, β6 isoforms for 24 h. IR-C cultures treated with β1-, β3-, and β6-conglutins significantly reduced the levels of NO compared to IR-C untreated samples (Fig. 5C) [−7.08, −7.03, −7.03-fold, respectively], recovering the NO levels at the C cell (non-insulin resistant) state. Interestingly, adding β2 or β4-conglutins had no effect on NO reduction, which was at comparative level of IR-C cells.

Conclusions

IR contributes to the loss of pancreatic β-cell function with T2DM progression. NLL β-conglutin proteins could improve T2DM by acting on multiple targets and pathways in order to reverse back IR.

In this study, treatments with particular NLL β-conglutin protein isoforms (β1, β3, and β6) to *in vitro* induced IR-C pancreatic culture cells assays promoted i) glucose uptake, which may be mediated by other β-conglutin effects as ii) the up-regulation of mRNA expression in key insulin signalling pathway activation mediators including IRS-1, GLUT-4 transporter, and protein increased synthesis of p85-PI3K and GLUT-4, in addition to iii) the activation of the intracellular IRS-1/PI-3-kinase pathway eventually involved in glucose homeostasis and protein synthesis stimulation, by increasing the level of phosphorylation (active forms) of

700 insulin pathway activator p-IRS-1, and downstream mediators as p-Akt, p-Cbl, and p-
701 Caveolin proteins, which playing an important role in the vesicular transport of GLUT4 to
702 cell surface improving glucose uptake.
703 In addition, β-conglutins improve IR by iv) strongly lowering the oxidative stress of IR-C
704 culture cells as measured by decreasing the level of carbonylation of proteins, and increasing
705 glutathione (GSH) levels and lowering antioxidant enzymatic activities such as SOD and
706 Catalase as immediate effects of β-conglutins in oxidative stress reversion, and the reduction
707 of the mRNA expression levels of pro-inflammatory cytokines as TNF-α, INF-γ; v) reducing
708 the NO production in IR-C, a main source of oxidative stress; and vi) changing the mRNA
709 expression levels (up-/down-regulation) of multiple key mediators of metabolic and signalling
710 transduction pathways, and transcription factors.
711 This study is the first to describe the molecular basis underlying the IR improvement effect of
712 a legume protein family (β-conglutin), particularly NLL β1-, β3-, and β6- conglutin proteins,
713 with strong evidences suggesting that NLL β-conglutins might play a key role as functional
714 food components with multi-pathway intervention with novel efficacious therapeutic options
715 for the prevention and treatment of complications from prediabetes and T2DM.

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722 ## Conflict of Interest Statement

723 The authors have declared that no competing interests exist.

724 ## Authors' Contribution

Conceived and designed the experiments: JCJ-L, and EL-C. Performed the experiments: JCJ-L, and EL-C. Analysed the data: JCJ-L, EL-C, JDA, AC, SM-S, and JL. Contributed reagents/materials/analysis tools: JCJ-L, JDA. Wrote the paper: all authors.

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Figure Legends

Figure 1. The effect of different β-conglutin isoforms in glucose consumption by insulin-resistant IR-C cells. (A) β1-, (B) β3-, (C) β6-, (D) β2- and (E) β4-conglutin at 0, 3, 5 and 10 μg. Values are shown as the mean ± standard deviation. $p<0.05$ represent statistically significant differences associated with each figure.

p*<0.05 β-conglutins treated IR-C cells (μg) *vs* IR-C.

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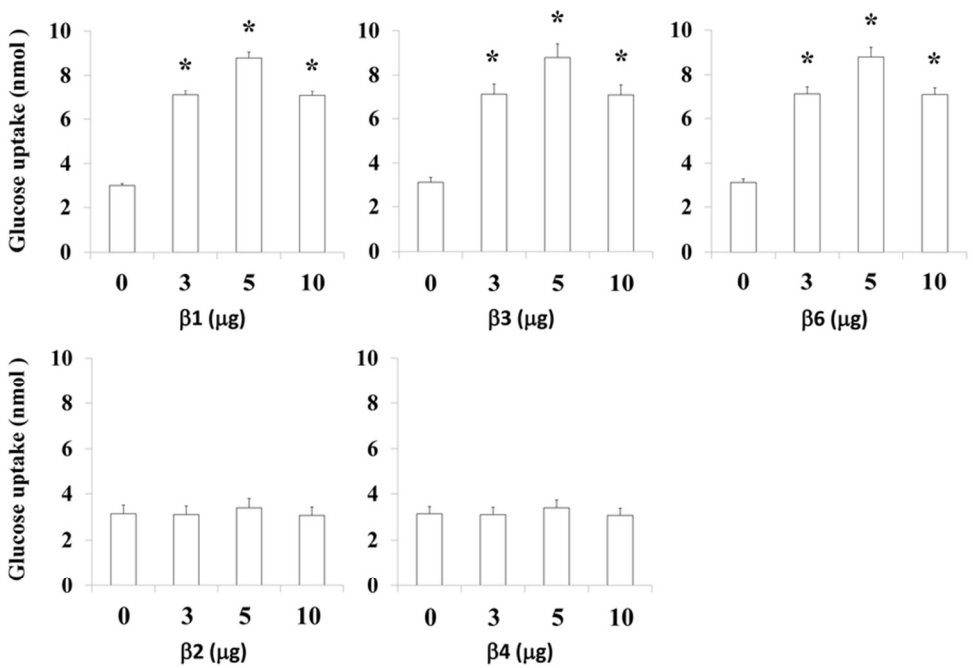
Table 1. Effect of NLL conglutin proteins β1, β3 β6, β2, and β4 on inflammation diabetes-related genes in isolated PBMC from type 2 diabetic patients' blood.

Numbers represent fold-change obtained from qPCR array data analysis of inflammation diabetes-related genes. Treatments with β1, β3, β6, β2, and β4, resulted in a significant up- or down-regulation of these genes compared to control patients. Data represent three

872 independent experiments. Values presenting statistical significant (P<0.05) differences with
873 T2DM are depicted in bold.

Gene name	Gene acronym	Up-/Down-regulation*	T2DM	β1	β3	β6	β2	β4
Metabolic enzymes								
Fructose-1.6-bisphosphatase 1	FBP1	β1, β3, β6↑	–7.76	**3.94**	**3.96**	**3.80**	–6.20	–6.91
Glucose-6-phosphatase	G6PC	β1, β3, β6↓	32.64	**1.87**	**6.96**	**6.88**	33.97	22.88
Glucose-6-phosphatase catalytic subunit	G6PC	β1, β3, β6↓	29.87	**1.26**	**2.93**	**2.90**	30.43	24.72
Glucose-6-phosphate dehydrogenase	G6PD	β1, β3, β6↓	32.49	**7.37**	**2.88**	**2.53**	31.71	23.97
Glucokinase (hexokinase 4)	GCK-HK	β1↓	25.87	**3.23**	24.97	27.96	26.70	23.55
Cytokines and growth factors								
Glucagon	GCG	β1, β3↓	27.37	**7.90**	**4.72**	29.97	29.97	24.97
Glucagon Receptor	GCGR	β1, β3↓	27.37	**7.07**	**3.96**	32.95	25.92	27.67
Cell Signalling								
Insulin-like growth factor binding protein 5	IGFBP5	β1, β3, β6↑	1.35	**29.62**	**27.25**	**31.96**	1.23	0.31
Insulin receptor substrate 1	IRS-1	β1, β3, β6↑	0.97	**17.28**	**16.73**	**16.80**	0.69	0.80
Insulin receptor substrate 2	IRS-2	β1, β3, β6↑	2.00	**22.78**	**24.50**	**30.75**	0.81	2.96
Mitogen-activated protein kinase 14	MAPK14/P38	β1, β3, β6↑	–1.00	**13.83**	**14.80**	**34.89**	–2.55	–3.86
Transcription factors								
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFkB1 (p50)	β1, β3, β6↓	27.93	**7.37**	**2.95**	**1.600**	23.19	21.94

874 * indicate particular β-conglutin isoforms that up- or down-regulated the mRNA expression
875 level of each gene in comparison with the control (T2DM).
876



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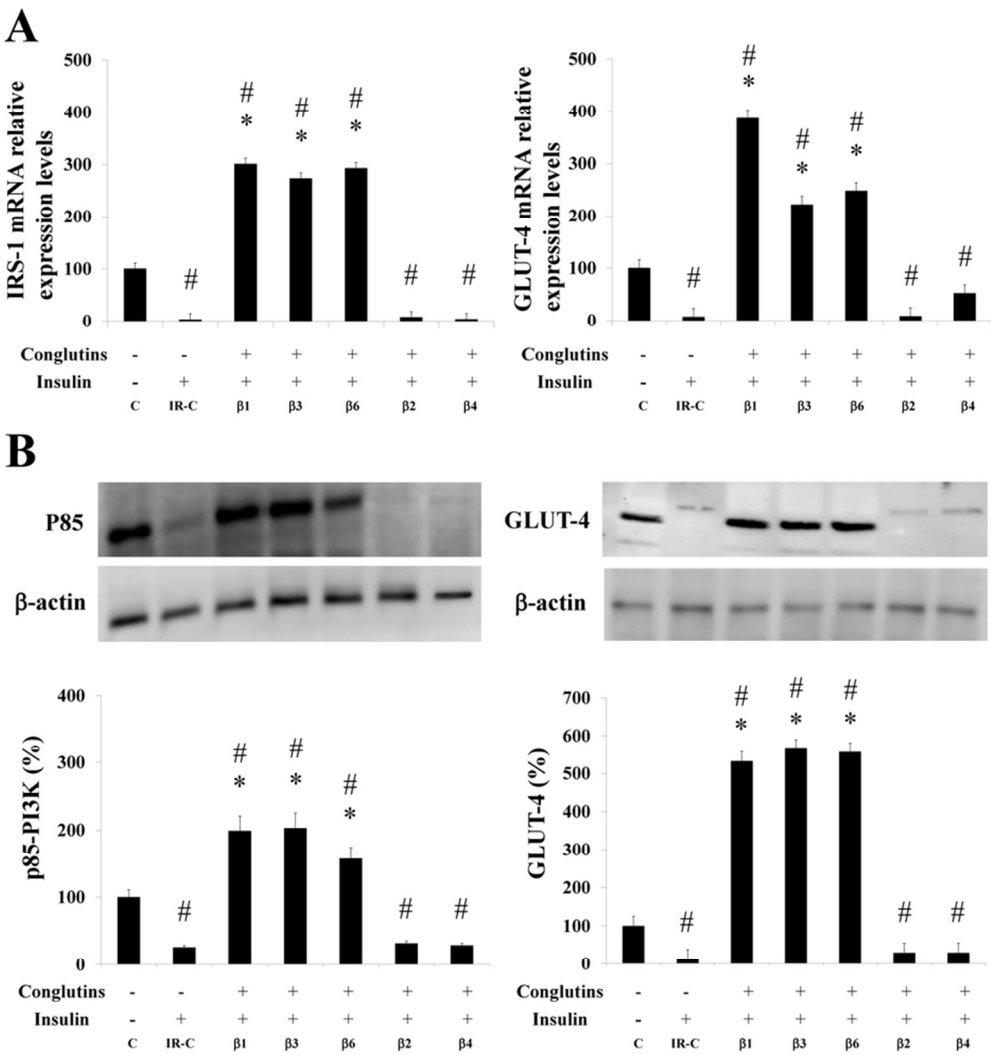

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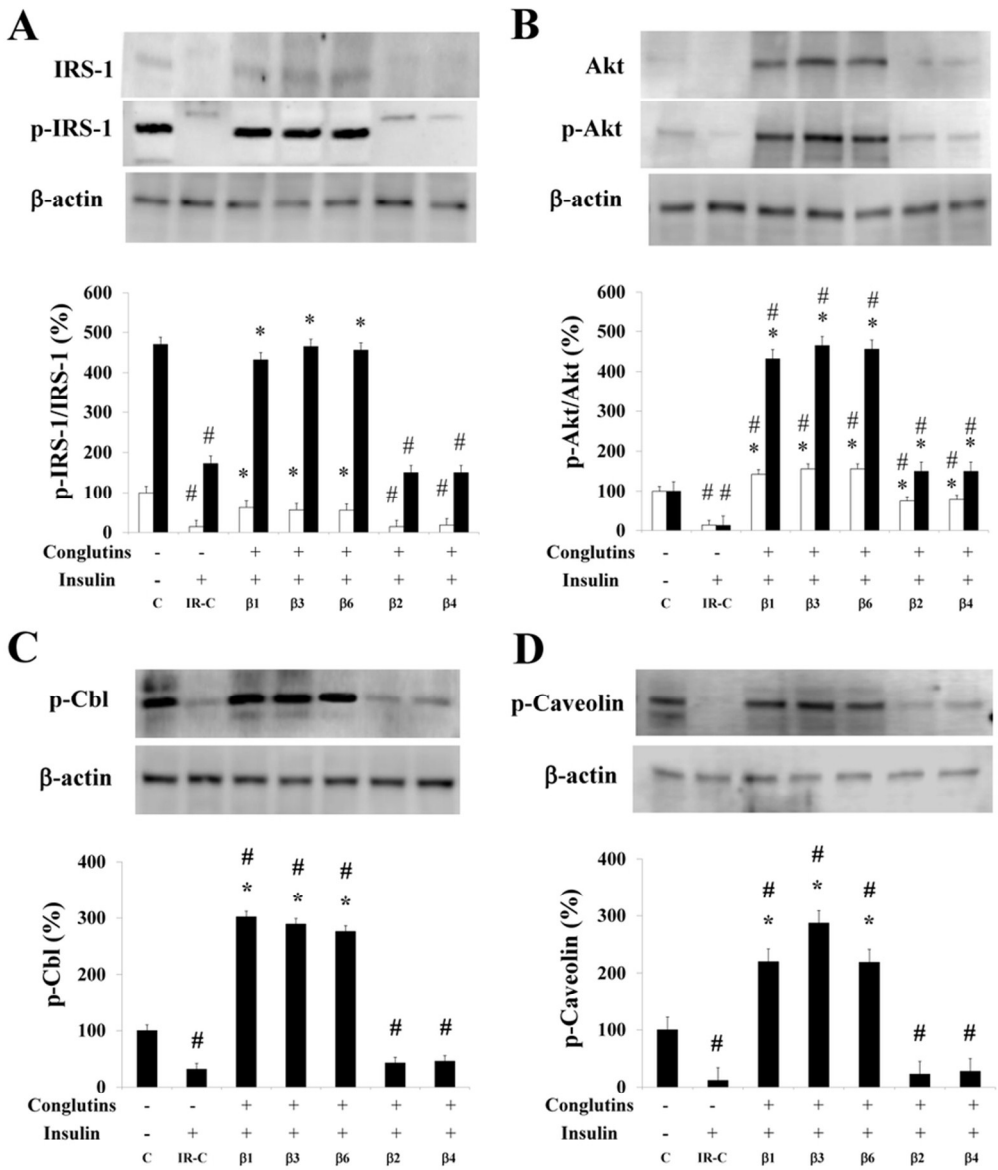

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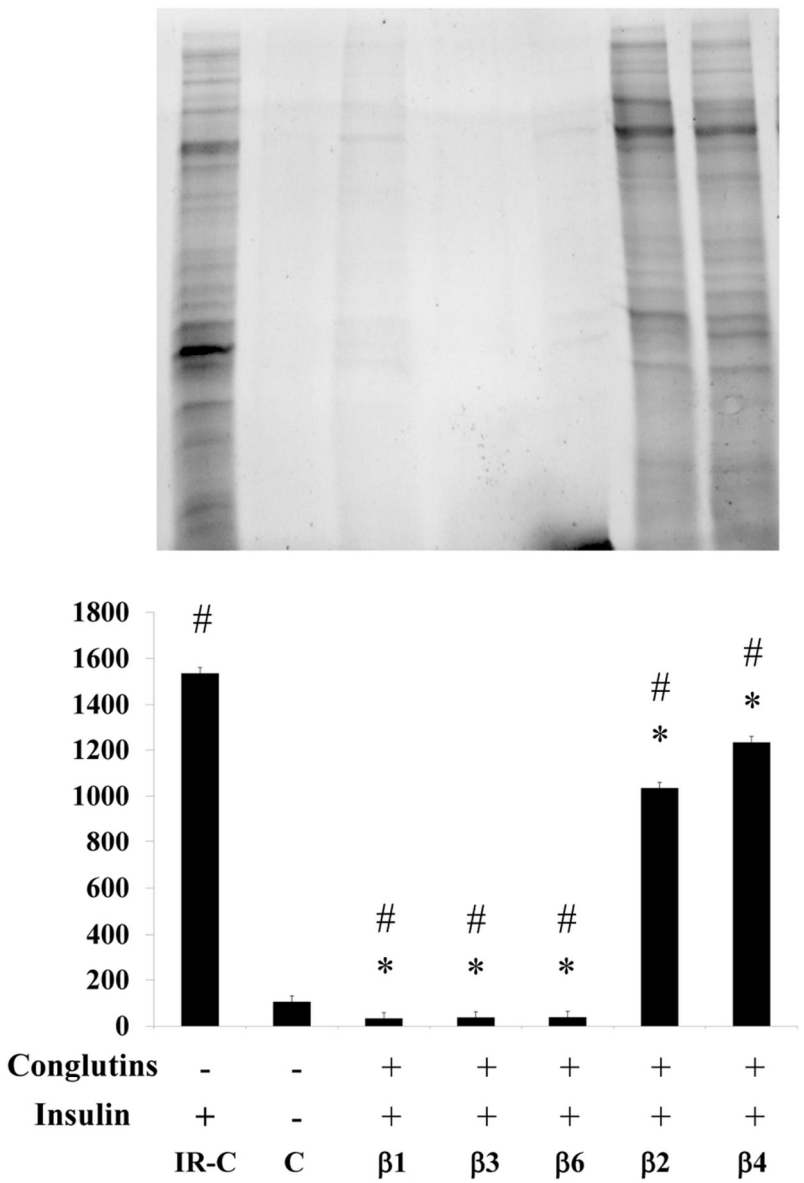

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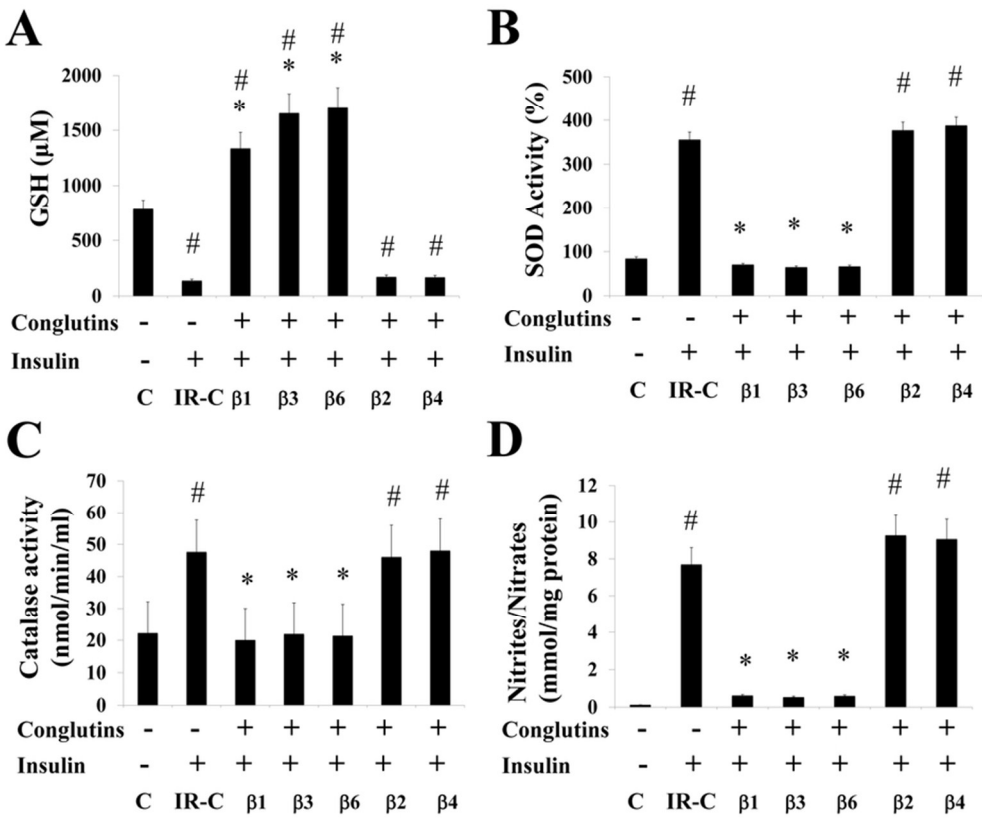

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80x65mm (300 x 300 DPI)