Lupin seed γ-conglutin lowers blood glucose in hyperglycaemic rats and increases glucose consumption of HepG2 cells

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Abstract

The aim of the present study was to evaluate the effect of a chronic oral γ-conglutin treatment in male Sprague–Dawley rats in which hyperglycaemia had been induced by supplying 10% d-glucose in drinking-water. A γ-conglutin dosage of 28 mg/kg body weight was daily administered to animals for 21 d. Plasma glucose, insulin and glucose overloading were monitored. Chronic administration of glucose resulted in a statistically significant (P<0.05) increase in fasting blood glucose (2.5-fold) and insulin (2.7-fold) v. the values recorded in control rats. Simultaneous treatment with γ-conglutin attenuated the rise in plasma glucose (1.9-fold) and insulin (1.8-fold) levels in the glucose-fed rats (P<0.05). Fasting insulin and homeostasis model of insulin resistance were decreased by 34 and 48% (P<0.05), respectively, in the γ-conglutin-treated rats v. the values found in pair-fed animals. To confirm these results with a different approach, HepG2 cells, grown for 24 and 48 h in Dulbecco’s minimum essential medium containing different glucose concentrations (5.5, 11.1 and 16.5 mmol/l), were exposed to 10 μmol/l γ-conglutin with or without 10 mmol/l metformin or 100 nmol/l insulin. γ-Conglutin increased glucose consumption (from 1.5- to 2.5-fold) in HepG2 cells, under all experimental conditions; this effect was more evident after 48 h incubation. Moreover, in this in vitro model, the addition of γ-conglutin potentiated the activity of insulin and metformin in cell glucose consumption. These findings extend the previous ones and suggest the potential use of lupin γ-conglutin in the control of glycaemia.

Key words: Hyperglycaemic rats: Lupinus albus seed protein: γ-Conglutin: HepG2 cells: Glucose consumption

A number of studies on the beneficial effects of plant-derived food proteins have appeared in recent years. Most of these studies evidence the biological activities of some legume seed storage proteins or fragments thereof and those of other cotyledony protein on the markers of chronic diseases, such as lipid disorders, diabetes, hypertension and cancer, which are typical of industrialised societies(1–4). Although the beneficial effects of legume seed dietary intake are the basis for various health claims, some questions still remain to be solved, including the identification of the biologically active peptides, their metabolic fate and mechanism of action, in order to identify the potential of these molecules both in the nutraceutical industry and in drug companies.

Many papers from our groups pointed out the positive effects of soya and white lupin proteins on lipid and glucose metabolism. Investigations on soya polypeptides, namely the purified α′ subunit from 7S globulin(5), a recombinant polypeptide including α′ subunit extension region(6) and peptides from the β subunit(7), have been reported. On the other hand, new data on the potential of γ-conglutin from lupin seed on glucose metabolism control are emerging(8).

γ-Conglutin is a cotyledony white lupin (Lupinus albus, L.) seed glycoprotein with molecular weight of approximately 47 kDa, composed of two disulphide bound heterogeneous subunits of 29 and 17 kDa(9). Its amount in the lupin seeds ranges from 3 to 5%(10). Proteins having sequence homology with γ-conglutin, for which the biological function has not been assessed yet, have also been found in other plant species, including soya(11) and carrot(12).

In a previous paper, the in vitro interaction of γ-conglutin with mammalian insulin(13), as the homologous soya basic 7S protein also does(14), was described, and the kinetic and thermodynamic parameters of binding were also measured. In parallel, the glucose-lowering effect of this protein in normal rats, upon glucose overload trial, was described for the first time. In that study(15), the protein was administered by gavage half an hour before glucose administration, and glycaemia was measured at 30 min intervals. In γ-conglutin-treated rats, a dose-dependent statistically significant reduction in glycaemia

Abbreviations: BW, body weight; DMEM, Dulbecco’s minimum essential medium; HOMA, homeostasis model assessment; IR, insulin resistance.

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was detected, comparable with that of about half a dose of metformin.

Nevertheless, these previous findings need to be confirmed in in vitro models of pre-diabetic or diabetic state in order to assess γ-conglutin actual potential. On this basis, the aim of the present study was to evaluate the effect of a chronic oral administration of γ-conglutin in male Sprague-Dawley rats in which hyperglycaemia was induced by supplying 10% D-glucose in drinking-water \[\text{15}\] in addition to a normal chow diet during 3 weeks. In this animal model, chronic glucose feeding resulted in non-insulin-dependent diabetes as reflected by the increase in both blood glucose and insulin levels. In addition to these trials, further experiments were carried out on HepG2 cells, a human hepatoma cell line, to assess γ-conglutin effects on glucose consumption and to get hints on the possible mechanism.

Materials and methods

Materials

Dry mature seeds of white lupin (L. albus L, var. Multitalia) were kindly provided by Dr Massimo Fagnano, University of Naples, Naples, Italy. Type F, a γ-conglutin-enriched lupin protein isolate, prepared as described in Bez et al. \[\text{16}\], was kindly supplied by Fraunhofer Institute (Munich, Germany). This preparation, the composition of which is detailed in the cited reference, contained about 80% protein and was used as such in the in vitro experiments.

White lupin γ-conglutin purification

A laboratory-scale purified γ-conglutin was utilised in the in vitro assay. The procedure for γ-conglutin purification, as described by Duranti et al. \[\text{17}\], was slightly modified to improve the homogeneity of the preparation. In particular, after conventional chromatographic steps, which included gel permeation chromatography, ion exchange chromatography on both Whatman DE52 diethylaminoethyl-cellulose and carboxymethylcellulose, a further step of metal affinity chromatography was added. For this purpose, the protein solution was loaded onto a nickel column (NiNTA-Agarose; Qiagen, Milan, Italy) equilibrated in 50 mM-Tris–HCl, pH 7.4, containing 0.5 M-NaCl. The protein bound to the matrix was subsequently eluted with 50 mM-sodium acetate, pH 4.5, containing 0.5 M-NaCl. The purified protein was desalted by dialysis against MilliQ water and freeze dried.

For the estimation of purified γ-conglutin concentrations, optical measurements at 280 nm were made. The extinction coefficient of 0.733 for a solution of 1 mg/ml was used \[\text{18}\].

SDS-PAGE was carried out on 12% polyacrylamide gels, according to Laemmli \[\text{19}\] under reducing conditions using a mini-Protein II cell (Bio-Rad, Milan, Italy). The sample buffer contained 0.25 M-Tris–HCl, pH 6.8, 7.5% glycerol, 2% SDS and 5% mercaptoethanol. Samples were heated at 100°C for 5 min before loading. The gels were stained with Coomassie Blue. Densitometric scanning of the gel was carried out by ImageMaster 1D software (Amersham Pharmacia Biotech, Milan, Italy).

Animals, treatments and homeostasis model assessment index

Male Sprague-Dawley CD rats (Charles River Laboratories Italia Srl, Calco-Lecco, Italy), body weight (BW) 150–175 g, were housed in a room with controlled lighting (12 h/d), constant temperature (18°C) and relative humidity (55–65%). Following a 2d adaptation period, during which they were fed a standard diet (pelleted commercial non-purified diet - Mucedola 4RF21; Settimo Milanese, Milan, Italy), the animals were given 10% D-glucose in drinking-water for the entire experimental period. The rats had free access to water. The mean daily water intake was between 20 and 30 ml/rat, estimating an average daily intake of D-glucose about 2–3 g. After 1 week, the animals were divided in two groups of twelve rats according to their BW and plasma glucose concentrations, so that the distribution between the groups was similar. The animals were daily treated (at 09.00 hours) by gavage for 3 weeks as follows: one group (D-glucose-treated) received only the vehicle (1% carboxymethylcellulose), whereas the other group (D-glucose + γ-conglutin) received 100 mg/kg BW type F, corresponding to 28 mg/kg BW γ-conglutin in carboxymethylcellulose. A third group of rats received only the vehicle without D-glucose in drinking-water (controls). Food intake and BW were monitored weekly. At the end of treatment, glucose was determined by an enzymatic method (Sigma-Aldrich, Milan, Italy), and fasting insulin was evaluated using a LINCO Rat Insulin RIA kit (LINCO, Millipore, Bedford, MA, USA) on plasma of overnight fasted animals.

At the end of the experimental period, glucose loading was carried out on 10 h fasted animals. At time 0, each rat was given 2 g/kg BW D-glucose, administered orally. Blood samples were withdrawn from the tail vein of each rat under light diethyl ether anaesthesia at 30, 60, 120 and 180 min following the carbohydrate load. Aliquots of serum were stored at −20°C until assayed. Glucose was determined as described earlier.

To estimate the degree of insulin resistance (IR), the homeostasis model assessment (HOMA) was used as an index of IR according to Madaoui & De Champlain \[\text{20}\]. The HOMA-IR index was calculated using the following equation:

\[
\text{fasting insulin (mU/ml) × fasting glucose (mmol/l) / 22.5.}
\]


Cell cultures and treatments

All culture reagents, glucose-Trinder kit, 1,1-dimethylbiguanide hydrochloride (metformin) and biosynthetic human insulin were obtained from Sigma-Aldrich. The established human...
hepatoma cell line (HepG2) was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in monolayer in 75cm² flasks and maintained at 37°C in a humidified atmosphere of 95% air, 5% CO₂ in Dulbecco’s minimum essential medium (DMEM; 5.5mM glucose) containing 10% fetal bovine serum, non-essential amino acids (1%, v/v), 200mM-L-glutamine, penicillin (10⁵U/l; 0.06g/l) and streptomycin (0.1g/l), and of sodium pyruvate (0.11g/l). To evaluate glucose consumption, 2d before the experiments, cells were seeded in twenty-four-well plates (to give 1.5 x 10⁵ cells/well) with some left blank. After the cells reached confluence, the medium was replaced by DMEM supplemented with 0.2% bovine serum albumin and glucose at various concentrations (5.5, 11.1 and 16.5mM/l). After 12h, the medium was removed and the cells grown in bovine serum albumin–DMEM were exposed, under sterile conditions, to 10μM/L-α-conglutin with or without metformin (10μM/l) and/or insulin (100μM/l), including the blank wells, for 24 or 48h.

At the end of incubation periods, the medium was removed, and glucose concentrations were determined as described previously (Sigma-Aldrich). The glucose concentration of the wells with cells was subtracted from the glucose of the blank wells to obtain the amount of glucose consumption(21). To assess cell viability, culture media from cells exposed to the different compounds were tested by methyltetrazolium salts assay, essentially as described by Lovati et al.(1).

Statistical analyses
Results are reported as means with their standard errors of the mean. Statistical analyses of the individual differences in plasma glucose, insulin, HOMA-IR and in cell glucose concentrations were carried out by one-way ANOVA with Bonferroni’s post hoc test. Results of all analyses were considered to be statistically significantly different at P < 0.05.

Results
Analysis of laboratory-purified and pilot plant-enriched L-α-conglutin preparations for the in vitro and in vivo assays

Fig. 1 shows the SDS-PAGE profile of the two L-α-conglutin preparations used in the present study. The gels were stained by Coomassie brilliant blue. Sample lanes are as follows: M, marker; 1, type F; 2, purified L-α-conglutin.

γ-Conglutin administration reduces plasma glucose in the in vivo model of type 2 diabetes

The dosage of 28 mg/kg BW γ-conglutin was selected according to lowest dosage of the previous trials consisting of acute glucose overloads in normal rats(13). BW were not modified either by glucose feeding or by γ-conglutin treatment in all groups (controls, 244±6 (SE 3.8)g; glucose-treated, 243±7 (SE 5.5)g; glucose-treated + γ-conglutin, 241±00 (SE 8.0)g). As depicted in Fig. 2(a), chronic glucose administration resulted in a statistically significant (P<0.01) increase in fasting blood glucose (2.5-fold); conversely, simultaneous treatment with γ-conglutin attenuated the rise in glucose (1.9-fold) so that the glucose levels in these animals were reduced by 22% in comparison with those recorded in the glucose-treated rats. In glucose-drinking rats, insulin levels increased by 170% (2.7-fold) (Fig. 2(b); P<0.05); the treatment with γ-conglutin reduced this increase to 79% (P<0.05), although the levels remained higher than in control animals. Chronic glucose feeding increased the IR index, as expressed by HOMA-IR, by 582% (Fig. 2(c); P<0.05). γ-Conglutin treatment attenuated this increase by 252% in glucose-fed rats, and the comparison between the HOMA-IR indexes in the two groups of rats (glucose-treated and untreated) showed an improvement in IR by 48% (P<0.05), following daily administration of γ-conglutin.

The oral glucose tolerance test, carried out at the end of experimental period on all animals enrolled in the study, showed (Fig. 3(a)) a statistically significant reduction.
upon 3-week treatment with γ-conglutin, both fasting blood glucose and postprandial blood glucose (2 h) were reduced (−21 and −12%, respectively; *P < 0.05), suggesting improved insulin sensitivity in the treated animals (Fig. 3(b)).

γ-Conglutin improved glucose metabolism in vitro

Glucose consumption was examined in HepG2 cells following incubation with purified γ-conglutin. The dose of 10 μmol/l γ-conglutin was selected from previous experiments, where an up-regulation of LDL receptors was detected in HepG2 cells after pre-incubation with γ-conglutin (4). Cells were grown in DMEM containing different glucose concentrations in order to simulate normal (5.5 mmol/l = 990 mg/l) or moderate high glucose (11.1 mmol/l = 2000 mg/l) and severe hyperglycaemia (16.5 mmol/l = 3000 mg/l) in human subjects. Moreover,
insulin (100 nmol/l) and metformin (10 mmol/l) were used alone or in combination with γ-conglutinin to assess the potential synergism/antagonism in glucose consumption following 24 or 48 h incubation.

As depicted in Fig. 4, the addition of insulin or metformin to HepG2 cells grown in DMEM containing different amounts of glucose induced a statistically significant increase ($P<0.05$) in the glucose consumption after 24 and 48 h incubation. When the glucose in the culture medium was normal (5.5 mmol/l), no statistically significant effect on glucose consumption was detected on the addition of γ-conglutinin in all experimental conditions (control, insulin and metformin). On the other hand, the effect of γ-conglutinin in glucose consumption ($P<0.05$) was evident in cells grown in DMEM with moderate (11.1 mmol/l) or elevated (16.5 mmol/l) glucose content; moreover, the concomitant addition of γ-conglutinin to HepG2 cells exposed to insulin or metformin increased further the glucose consumption, normally stimulated by both compounds. In particular, when the glucose concentration in the culture medium increased from 5.5 to 11.1 mmol/l, the glucose expenditure induced by γ-conglutinin, after 24 h of incubation, was elevated to 109, 60 and 18%, respectively in controls, insulin- and metformin-treated cells (Fig. 4(b)). When the glucose concentration increased from 11.1 to 16.5 mmol/l, the amount of glucose consumed in 24 h was enhanced by 109, 33 and 43%, respectively in controls, insulin- and metformin-treated cells by the addition of γ-conglutinin (Fig. 4(c)). This trend was shared by HepG2 cells incubated for 48 h in the same experimental conditions: the addition of γ-conglutinin induced a statistically significant increase in the amount of glucose consumed as depicted in the Fig. 4((e) and (f)).

The glucose-lowering effect of γ-conglutinin observed in HepG2 cells was not linked to an increment in cell number, due to the glucose level, since we did not observe any change in methyltetrazolium salts optical density (data not shown). In addition, the results obtained following the exposure of HepG2 cells to γ-conglutinin were compared for each time and glucose concentration with the respective controls. Moreover, a previous experiment (data not shown), in which mannitol was added to 5.5 mmol/l glucose DMEM, pointed out that the present results were due to the activity of tested compounds (γ-conglutinin, insulin and metformin) and not to the effect of hyperosmolarity.

**Discussion**

Type 2 diabetes is a chronic metabolic disorder, often characterised by IR, which leads to several secondary complications, including hypertension, atherosclerosis, coronary artery disease and hyperlipidaemia$^{23}$. Approximately 150 million people worldwide are affected by the disease at present, with a projection of 300 million people being affected by 2025. Diabetes has become a serious public health problem, particularly in developed countries$^{24}$. Research in an effective anti-diabetic agent, in addition to those already available, would be of great interest for the treatment of type 2 diabetes. Legume seeds, due to the nutraceutical potentialities of some

![Fig. 4. (a, b, c) Glucose consumption by HepG2 cells after 24 h and (d, e, f) 48 h growth at different glucose concentrations (1, 5.5 mmol/l; 2, 11.1 mmol/l and 3, 16.5 mmol/l). The trials were cells alone (control), treated with insulin (100 nmol/l) or metformin (10 mmol/l) in the absence (white bars) or presence (black bars) of 10 μmol γ-conglutinin. The tests were performed in Dulbecco’s minimum essential medium supplemented with 0.2% bovine serum albumin as detailed under ‘Materials and methods’ section. Values are means, with their standard errors represented by vertical bars of three independent experiments, each performed in quadruplicate. * Mean values were significantly different from those of the γ-conglutinin untreated trials ($P<0.05$).](https://www.cambridge.org/core/da68a28a240d975730e4b9e3f33b16d1)
of its proteins, can provide an alternative to the drug treatment of glucose metabolism disorders. More specifically, lupin flours, such as other pulses, are characterised by a low-glycaemic index, so they can be useful in the prevention of IR in human subjects. Lupin seeds are characterised by a high content of protein, about 35%, and by low levels of isoflavones and anti-nutritional factors. The anti-diabetic activity of toasted lupin seeds was first described, in the middle of the last century, by Ferranini & Pirolli and by Orestano, who proposed lupin as a substitute for the insulin therapy in mild-to-medium diabetes mellitus, but no further studies have been carried out to identify the molecule responsible of this biological effect. A few years ago, the potential of a single oral administration of γ-conglutin, a lupin seed protein, was demonstrated by Magni et al. in rats after glucose overload. In that study, the interaction of the lupin protein with insulin was also shown. However, the role of this interaction has not been elucidated so far.

In the present study, the 10% glucose supplied in drinking-water induced hyperglycaemia and hyperinsulinaemia in rats similar to that observed in human subjects. No side effects have been detected during the experimental period, such as those recorded in animals undergoing streptozotocin or alloxan treatment to mimic diabetes. γ-Conglutin administration has been demonstrated to counteract the plasma glucose increase as well as to improve the insulin sensitivity, normally reduced by the glucose-rich drinking-water. In the γ-conglutin-treated rats, the insulin sensitisation was increased significantly, as indicated by the 48% reduction in the homeostasis model of IR. It is worth noting that the hypoglycaemic effect in vivo was obtained by the use of a preparation, which contained a γ-conglutin amount corresponding to the lowest dose previously used in acute trials of glucose overload. Moreover, lower glucose levels were detected in γ-conglutin-treated rats following oral glucose overload; these results were confirmed by lower glycaemia in fasting and 2h postprandial conditions. The mechanisms underlying the present results, are currently under investigation, but the hypothesis that γ-conglutin could act as an insulin-like agent should not be excluded. Recently, Terruzzi et al. have shown that γ-conglutin may regulate muscle energy metabolism, protein synthesis and major histocompatibility complex gene transcription through the modulation of the same insulin signaling pathway. Moreover, γ-conglutin resistance to proteases at neutral pH values could explain the maintenance of its activity after 48h of incubation, as we have observed in HepG2 cells. The role of γ-conglutin in controlling glucose concentrations has been assessed with the purified protein in cell assays (the present study) and animal models in previous studies; however, the synergic effect of other protein/peptide components in the type F sample cannot be excluded. In addition, the reduced increase in plasma glucose (−24%) and insulin (−33%) levels, recorded in rats following γ-conglutin treatment v. the values found in the pair-fed animals, might be of pharmacological relevance. It is noteworthy, in fact, that these decreases have been obtained by the use of a single daily administration of a purified food protein. Although, these data need to be confirmed by human studies, the potential of lupin seed γ-conglutin to control glycaemia could be considered before developing new therapeutic strategies for the prevention or regression of glucose metabolism modifications.

The linkages between diet and health are no longer a matter of discussion. Moreover, the specific and/or limited effects of current drug treatments for diabetes, combined with dangerous side effects that most of them induce, have fueled the search for alternative medicine. At this point, the anti-diabetic effects of cinnamaldehyde and berberine in rats with type 2 diabetes, involving the up-regulation of tissue retinol-binding protein 4/GLUT4 protein, have been published by Zhang et al.

Furthermore, the specific role of many food components, their synergies and antagonism are still a largely unexplored area. The case of dietary proteins/peptides is particularly intriguing due to the dramatic changes they may undergo from food production to food digestion. On the other hand, the hypothesis that peptides with biological/pharmacological activities can be produced in vivo have been the object of recent papers. Other food proteins, such as cod and soya proteins, improved glucose tolerance and insulin sensitivity in rats, compared with casein; this effect seems due to the different amino acid composition of these proteins, which could be responsible for insulin’s diminished ability to stimulate peripheral glucose transport. At present, we do not know whether γ-conglutin would be able, in vivo, to interfere in the production/secretion of glucagon-like peptide 1 or glucose-dependent insulinotropic peptide, thus acting as incretino-mimetic compound. Further studies, aimed at understanding the protein moiety of γ-conglutin responsible for the glucose-lowering effect and the molecular mechanism thereby, are currently being undertaken.

In conclusion, the present study provides the in vivo and in vitro evidence of the involvement of γ-conglutin on cell glucose homeostasis, thus suggesting the potential use of this food protein in the control of glycaemia in patients with manifest or pre-clinical diabetes as well as for applications as functional foods and dietary supplements.

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