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Original Article

Lupin gamma conglutin protein: effect on *Slc2a2*, *Gck* and *Pdx-1* gene expression and GLUT2 levels in diabetic rats

Roberto de J. Sandoval-Muñíz[a], Belinda Vargas-Guerrero[a], Tereso J. Guzmán[a], Pedro M. García-López[b], Alma L. Martínez-Ayala[c], José A. Domínguez-Rosales[a], Carmen M. Gurrola-Díaz[a,*]

[a] *Instituto Transdisciplinar de Investigación e Innovación en Salud/Instituto de Enfermedades Crónico-Degenerativas, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico*
[b] *Departamento de Botánica y Zoología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Zapopan, Jalisco, Mexico*
[c] *Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, Mexico*

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ABSTRACT

Recently, lupin seed (*Lupinus albus* L., Fabaceae) products have emerged as a functional food due to their nutritional and health benefits. Numerous reports have demonstrated the hypoglycemic effects of lupin's gamma conglutin protein; nonetheless, its mechanism of action remains elusive. To understand the role of this protein on glucose metabolism, we evaluated the effect of administering *L. albus*' gamma conglutin on *Slc2a2*, *Gck*, and *Pdx-1* gene expression as well as GLUT2 protein tissue levels in streptozotocin-induced diabetic rats. While consuming their regular diet, animals received a daily gamma conglutin dose (120 mg/kg per body weight) for seven consecutive days. Serum glucose levels were measured at the beginning and at the end of the experimental period. At the end of the trial, we quantified gene expression in pancreatic and hepatic tissues as well as GLUT2 immunopositivity in Langerhans islets. Gamma conglutin administration lowered serum glucose concentration by 17.7%, slightly increased *Slc2a2* and *Pdx-1* mRNA levels in pancreas, up-regulated *Slc2a2* expression in the liver, but it had no effect on hepatic *Gck* expression. After gamma conglutin administration, GLUT2 immunopositivity in Langerhans islets of diabetic animals resembled that of healthy rats. In conclusion, our results indicate that gamma conglutin up-regulates *Slc2a2* gene expression in liver and normalizes GLUT2 protein content in pancreas of streptozotocin-induced rats.

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Introduction

In recent years, consumers interest in plant-based foods, as well as foods with high protein content, has significantly increased. Although protein intake in some countries exceeds the daily requirements, consumption of specific protein sources may be beneficial for some segments of the population.

In this sense, the genus *Lupinus* is a legume constituted by many species distributed worldwide. Interestingly, its seeds have a high protein content (35–40% dry basis). Lupin seeds are traditionally consumed in some areas for their purported beneficial effects, especially the species *L. albus* L., Fabaceae, whose properties have been widely described (Duranti et al., 2008; Arnoldi et al., 2015; Lucas et al., 2015).

In addition to globulin and albumin proteins, lupin seeds contain other bioactive compounds including alkaloids, tocopherols, carotenoids, and polyphenols (Lampart-Szczapa et al., 2003; Ganzera et al., 2010; Arnoldi et al., 2015). Lupin globulins reduce circulating and tissue lipids as well as glucose levels (Brandsch et al., 2010; Radtke et al., 2015). More specifically, lupin's gamma conglutin (Cγ) protein reduces serum glucose levels in both diabetes- and insulin resistance-induced rats (Vargas-Guerrero et al., 2014; Gonzalez-Santiago et al., 2017). In healthy animals, Cγ attenuates the glycemic peak after a glucose overload similar to the pharmacological agent metformin (Magni et al., 2004).

Alterations in carbohydrate and lipid metabolism are critical in the development of metabolic diseases such as diabetes and its complications (IDF, 2017; ADA, 2017). Type 2 diabetes is characterized by persistent hyperglycemia, resulting from either defective

* Corresponding author.
E-mail: carmen.gurrola@academicos.udg.mx (C.M. Gurrola-Díaz).

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insulin secretion, impaired insulin action, or both (Ozougwu et al., 2013).

The pancreatic and duodenal homeobox 1 (*Pdx-1*) is a major transcription factor that regulates pancreatic β-cells differentiation, development, and function. The targets modulated by *Pdx-1* include the solute carrier family 2 member 2 (*Slc2a2*/*Glut2*) and glucokinase (*Gck*). Both *Slc2a2* and *Gck* play essential roles in glucose transport, insulin secretion, and glucose homeostasis (Watada et al., 1996; Waeber et al., 1996; Ahlgren et al., 1998; McKinnon and Docherty, 2001; Fujimoto and Polonsky, 2009; Pedica et al., 2014). An elevation in circulating glucose levels triggers the insulin secretion by β-cells. This process requires glucose internalization by GLUT2 and its subsequent phosphorylation by GCK (Rorsman and Renstrom, 2003).

Previously, we have shown that the administration of *L. albus*' Cγ moderately increases insulin content and *Ins-1* gene expression in the pancreatic tissue of experimentally-induced diabetic rats (Vargas-Guerrero et al., 2014). However, we were not able to identify reports regarding the effect of Cγ on *Pdx-1*, *Slc2a2*, or *Gck* pancreatic gene expression.

GLUT2 and glucokinase, encoded by the *Slc2a2* and *Gck* genes respectively, play a significant role in hepatic glucose metabolism by promoting glycogen synthesis and lipogenesis. Therefore, diseases like diabetes show altered *Slc2a2* and *Gck* expression (Iynedjian et al., 1988; Yonamine et al., 2016). Interestingly, Cγ promotes higher glucose uptake in HepG2 cells (Lovati et al., 2012; Capraro et al., 2013), indicating that this protein might up-regulate the *Slc2a2* and *Gck* genes.

In previous work, we also investigated the effect of Cγ on the hepatic gluconeogenic genes (glucose-6-phosphatase, *G6pc*; fructose-bisphosphatase 1, *Fbp1*; and phosphoenolpyruvate carboxykinase 1, *Pck1*) and found that Cγ negatively regulates *G6pc* gene expression and lowers hepatic glucose production (Gonzalez-Santiago et al., 2017). However, the effect of Cγ on *Slc2a2* and *Gck* gene expression in the liver of diabetes-induced rats has not been proven.

Although there have been advances in elucidating the Cγ mechanism of action, additional data is needed before it can be fully understood. Consequently, this study aimed to evaluate the effect of Cγ protein from *L. albus* on *Slc2a2* and *Pdx-1* mRNA and GLUT2 protein content in pancreas as well as *Slc2a2* and *Gck* mRNA content in liver of rats with streptozotocin-induced diabetes.

Material and methods

Experimental design and animal group assignment

Male Wistar rats, supplied by the University of Guadalajara Bioterium, were housed in individual cages and placed in an air-conditioned room (24 ± 2 °C) and 55.0 ± 5% relative humidity with a 12 h light–dark cycle. Animals had free access to a standard rodent diet (LabDiet, PMI Nutrition International, St. Louis, MO, USA) and water. All experimental animal procedures were approved by the University of Guadalajara Bioethics Committee (C.I./023/2014) and adhered to the International Guidelines for Care and Use of Laboratory Animals.

Experimental animals were randomly allocated into one of three groups of five rats each as follows: (1) healthy control group (Ctrl), (2) diabetic group without treatment (STZa), and (3) diabetic Cγ-treated group (STZa-Cγ). The Ctrl and STZa groups received 1.5 ml of carrier solution (0.9% w/v NaCl) while the STZa-Cγ group received Cγ (120 mg/kg BW) dissolved in carrier solution. Treatment solutions were administered by oral gavage once a day for seven consecutive days and serum glucose levels measured at the beginning and at the end of the treatment period. After sacrificing the animals, we collected their pancreas and liver to quantify gene expression and GLUT2 protein tissue content.

STZ-induced diabetes

Rats with a fasting glucose levels ≥200 mg/dl 72 h after the streptozotocin (STZ) injection were deemed diabetic and included in the study. Diabetes was chemically-induced in male Wistar rats (200–250 g) by a single intraperitoneal (*i.p.*) injection of STZ (65 mg/kg BW) (Sigma, St. Louis, MO, USA) dissolved in 0.1 M sodium acetate buffer, pH 4.5.

Plant material

Dr. Edzard van Santen from the College of Agriculture, Auburn University, Alabama kindly provided the dry, certified *Lupinus albus* L., Fabaceae, seeds used in this study.

Extraction and validation of Cγ isolation

Cγ was isolated from hexane defatted lupin flour as previously described (Vargas-Guerrero et al., 2014). Briefly, albumins were extracted twice with double distilled water (DDW) at 4 °C under constant stirring for 2 h and a 1:10 (w/v) flour to water ratio. After each extraction, the mixture was centrifuged for 30 min at 10,370 × *g* and 4 °C, and the supernatant discarded. Subsequently, the pellet was suspended in 10% NaCl (pH 7.0), the solution stirred for 12 h at 4 °C, it was centrifuged for 30 min at 4 °C and 10,370 × *g*, and finally the supernatant was saved. The globulins fraction, recovered from the supernatant by precipitation with 85% ammonium sulfate, was dissolved in 0.1 M phosphate buffer and dialyzed against 0.2 M acetate buffer (pH 4.8) for 18 h at 4 °C. The retentate was then centrifuged to separate α-conglutin (in the pellet) from β- and γ-conglutin (supernatant). Finally, the supernatant was dialyzed against DDW for 48 h at 4 °C and the retentate containing Cγ centrifuged. The resulting supernatant solution, containing Cγ, was lyophilized (Freeze Zone, LABCONCO) at −50 °C, 0.036 mbar for 8 h.

The presence of Cγ in the isolate fraction (2 mg per sample) was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% under reducing and non-reducing conditions using a mini Protean® Tetra cell (BioRad, Milan, Italy) equipment (Schagger and von Jagow, 1987). Gels were stained after electrophoresis with Coomassie brilliant blue G-250 (BioRad, Milan, Italy). The relative molecular weight of native and denatured Cγ was determined by comparison with a protein ladder (BenchMark™ Prestained protein ladder, Invitrogen).

Serum collection and glucose quantification

After an overnight fasting period, blood was collected from the retro-orbital plexus of sedated animals and centrifuged for 15 min at 6000 × *g* and 4 °C to separate the serum. Serum glucose concentration (mg/dl) was quantified using the glucose oxidase-peroxidase reagent (BioSystems, Spain) and a spectrophotometer analyzer (BTS 350, BioSystems, Spain). Animals were anesthetized with a single dose of 80 mg/kg BW of tiletamine-zolazepam (Zoletil® 50; Virbac, Carros, France).

GLUT2 immunopositivity in β-cells

Immunohistochemistry established GLUT2 immunopositivity in pancreatic β-cells. The excised pancreas from each animal was washed with saline solution, immediately fixed in 4% *p*-formaldehyde and embedded in paraffin. Four μm thick sections were dewaxed at 62 °C and the tissue rehydrated with graded concentrations of ethanol (10 min) followed by a 10 min incubation in

Table 1
Probes, primers and annealing temperature used in qPCR.

Gene	UPL number	Forward	Reverse	Ta
Slc2a2	122	5′-AAAGCCCCAGATACCTTTACCT-3′	5′-TGCCCCTTAGTCTTTTCAAGC-3′	60 °C
Pdx-1	95	5′-AAAGCCCCAGATACCTTTACCT-3′	5′-TGCCCCTTAGTCTTTTCAAGC-3′	60 °C
Rps18	1	5′-CAGAAGGACGTGAAGGATGG-3′	5′-TCTATGGGCTCGGATTTTCTT-3′	57 °C
Gck	–	5′-CTCTAAGGGGACCAGAAAGGG-3′	5′-GGCATTTGGGAACGAGGGA-3′	63 °C

Abbreviations: Ta, annealing temperature; *UPL*, Universal Probes Library (Roche, Germany).

citrate buffer (pH 6.0) and a 60 min incubation in 5% fetal bovine serum (FBS).

Tissues were later incubated at 4 °C overnight with the GLUT2 primary antibody (Anti-Glucose Transporter GLUT2 antibody Abcam ab 54460, Cambridge, USA) at a 1:100 dilution and rinsed three times for 2 min each with 1 × TBST. Mouse/Rabbit Immunodetector HRP/DAB Detection System (BIO SB, USA) was used to reveal the GLUT2-bounded primary antibody. 3,3′-Diaminobenzidine (DAB) was added to the tissue and incubated at room temperature for 20 min in the dark, and then washed for 5 min with DDW and stained for 5 min with hematoxylin. For negative controls, we replaced the primary antibody with 1× phosphate buffer saline (PBS).

The immunopositive GLUT2 regions were determined by detecting and quantifying the DAB intensity with Motic Images Plus 2.0 software (Motic China Group Co. Ltd., China). The total and GLUT2-positive average areas were calculated for each sample in the experimental groups, and the percent of GLUT2 immunopositivity calculated as follows:

$$\text{GLUT2 immunopositivity } \% = \frac{\text{Average GLUT2 positive area}}{\text{Average total area}} \times 100$$

Slc2a2, Pdx-1 *and* Gck *gene expression*

RNA was isolated from hepatic and pancreatic tissues and reverse-transcribed into cDNA as previously described (Gonzalez-Santiago et al., 2017). Afterwards, we quantified *Slc2a2* (pancreas and liver), *Gck* (liver) and *Pdx-1* (pancreas) gene expression by quantitative real-time PCR (qPCR). The amplification involved an initial denaturation process (95 °C for 10 min), followed by 45 cycles comprised of a denaturation step (95 °C), annealing and elongation (72 °C) steps. Table 1 shows the primer sequences, number of the UPL probes, and annealing temperature for each gene.

For the *Slc2a2*, *Pdx-1*, and *Rps18* gene expression, we used the LightCycler TaqMan Master Mix (Roche, Germany) and Universal Probes Library (UPL, Roche, Germany). To quantify the *Gck* gene expression, we used a LightCycler® FastStart DNA MasterPLUS SYBR Green I Kit (Roche).

Quantification of the gene expression of all reactions was determined in triplicate using a LightCycler 1.5 Instrument® (Roche Applied Science, Mannheim Germany). For negative controls, sterile water replaced the cDNA. The *Rps18* housekeeping gene served as an internal control. We used the $2^{-\Delta\Delta Ct}$ method for the relative quantification of *Slc2a2*, *Pdx-1*, and *Gck* gene expression. The crossing threshold (Ct) values obtained for the target gene were normalized against *Rps18* Ct values.

Glycogen content in hepatic tissue

Hepatic tissues were fixed in 4% *p*-formaldehyde and embedded in paraffin. Four-μm sections were mounted on slides and hydrated in bidistillated water. After, the slides were incubated in 0.5% periodic acid solution for 10 min, stained with Schiff's reagent for 15 min and counterstained with hematoxylin solution for 2 min. The periodic acid-Schiff (PAS) positive sections were interpreted as percentage and cellular distribution of glycogen.

Data analysis

Data analysis was carried out using PASW Statistics v. 18 software (IBM Corp., Chicago, IL, USA). We computed the average serum glucose levels (pre- and post-treatment) and the standard error of the mean (SEM) for each group. We also calculated GLUT2 immunopositivity, as a percentage, for every Langerhans islet analyzed and the *Slc2a2*, *Pdx-1*, and *Gck* gene expression, as relative units (RU) ± SEM. The Wilcoxon test established significant differences between pre- and post-treatment serum glucose levels within each group. The Mann-Whitney U test assessed statistical significance for gene expression, and immunopositivity to GLUT2. p values <0.05 were deemed statistically significant.

Results and discussion

The effect of Cγ on some molecules related to metabolic pathways has been described in previous works (Terruzzi et al., 2011; Lovati et al., 2012; Vargas-Guerrero et al., 2014; Gonzalez-Santiago et al., 2017). However, to further understand the mechanism of action of Cγ, more studies are required. Here, we determined the effect of *L. albus*' Cγ protein on *Slc2a2*, *Gck*, and *Pdx-1* gene expression as well as GLUT2 protein tissue content under diabetic conditions.

We isolated Cγ from defatted lupin flour. After that, we verified its purity by SDS-PAGE under both, reducing and non-reducing conditions. As previously reported, a typical single ∼49 kDa band corresponding to the native protein was found under non-reducing conditions whereas two bands (∼17 and ∼29 kDa) were present under reducing conditions; thus, confirming the presence of Cγ (data not shown) (Vargas-Guerrero et al., 2014).

Subsequently, we validated the STZ-induced diabetes model. As expected, most animals developed marked hyperglycemia after a single STZ *i.p.* dose. Experimental animals with fasting glycemia ≥200 mg/dl were deem diabetic and included in the study.

Cγ treatment diminishes serum glucose levels in diabetic animals

In Fig. 1, the serum glucose levels (pre- and post-treatment) and the SEM for the three experimental groups are shown. After the seven-day treatment period, there was no significant change in glycemia in the Ctrl group ($p > 0.05$).

Conversely, serum glucose level increased by 30.5% in the STZa group, but significantly decreased (−17.7%) with the daily Cγ administration (120 mg/kg BW). These findings are in agreement with results from previous work showing that Cγ intake lowers glucose levels under hyperglycemic conditions (Lovati et al., 2012; Vargas-Guerrero et al., 2014). Glucose uptake by peripheral tissues is relevant to preserve normoglycemia, especially during postprandial state (Alvim et al., 2015; Gannon et al., 2015). In

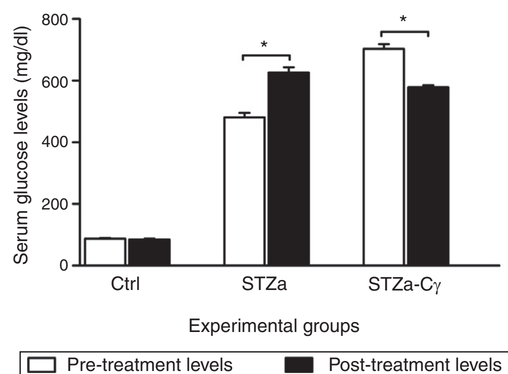


Fig. 1. Effect of the daily intake (120 mg/kg BW) of lupin gamma conglutin (Cγ) on the glycemic status of streptozotocin-induced diabetic rats. Bars and figures represent the average serum glucose levels ± standard error of the mean, after seven days, for the control group without diabetes induction or treatment (Ctrl), the streptozotocin-induced diabetic group without Cγ treatment (STZa), and the streptozotocin-induced diabetic group treated with Cγ (STZa-Cγ). Horizontal lines across bars indicate the statistical significance of the difference between pre- and post-treatment glucose values (* $p < 0.05$).

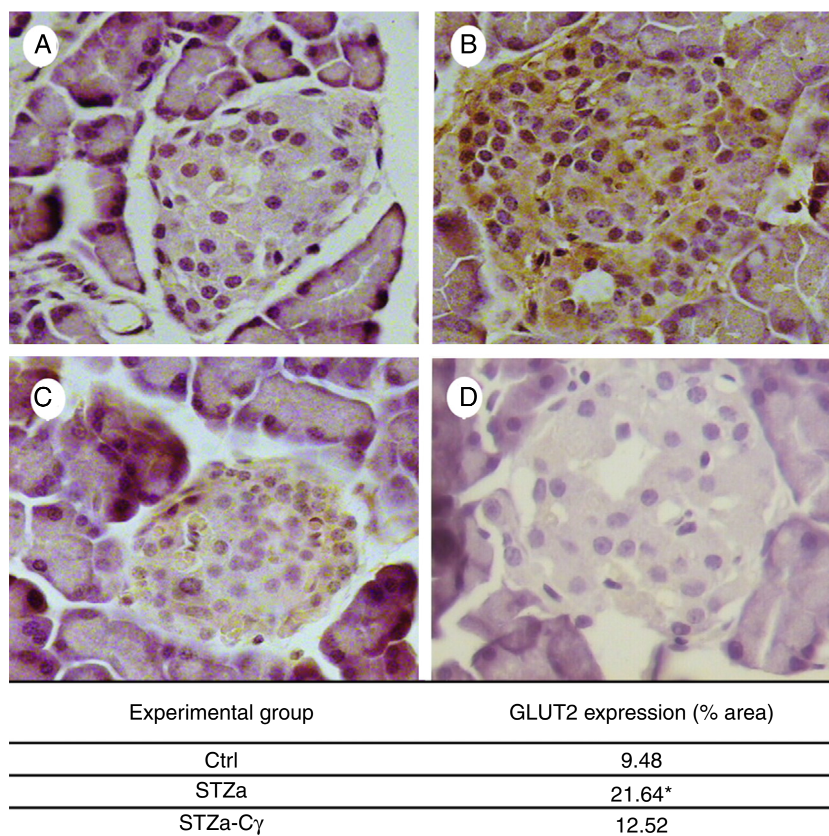
this respect, previous data have shown an ability of Cγ to stimulate glucose uptake and activation of the insulin signaling cascade *in vitro* in C2C12 cells, which suggests a higher GLUT4 membrane translocation (Terruzzi et al., 2011). However, further research is needed since other molecules, or signaling pathways may be involved.

Cγ ameliorates pancreatic GLUT2 protein over-expression to similar levels found in healthy animals and increases Pdx-1 *and* Slc2a2 *gene expression in pancreatic tissue in diabetic rats*

We used immunohistochemistry to evaluate GLUT2 protein content in pancreatic islets. A semi-quantitative analysis was performed to determine the GLUT2 positive area as percentage (%). Immunopositivity to GLUT2 in Langerhans islets of STZa rats was more than two-fold higher (21.64%) than in the Ctrl group (9.48%). Remarkably, Cγ administration to diabetes-induced rats reduced GLUT2 protein content (12.52%) to a level similar to that of the Ctrl group (Fig. 2A–C). The absence of cross-reactivity validated the immunohistochemistry assays (Fig. 2D).

Cγ-induced attenuation of GLUT2 protein expression in diabetic animals may be due to changes in circulating glucose levels. In line with this observation, we found a moderate positive correlation between GLUT2 immunopositivity of Langerhans islets and serum glucose levels (data not shown). Circulating glucose regulates GLUT2 expression in the pancreas (Bae et al., 2010; Chen et al., 1990); thus, glucose uptake by peripheral tissues may influence GLUT2 protein content in β-pancreatic cells.

GLUT2 is crucial for normal β-cell physiology, and abnormal GLUT2 content results in aberrant β-cell function. Also, inhibition of insulin secretion with diazoxide leads to higher GLUT2 expression in rat islets while stimulation of insulin release by glipizide or high extracellular K^+ levels diminishes it (Zhao et al., 2005). Our results suggest that Cγ treatment might improve and regulate β-cells' function through normalization of GLUT2 expression; however, additional research is needed to corroborate this finding.



Experimental group	GLUT2 expression (% area)
Ctrl	9.48
STZa	21.64*
STZa-Cγ	12.52

Fig. 2. GLUT2 protein expression in Langerhans islets (40×) of experimental groups. Streptozotocin-induced diabetes results in a GLUT2 over-expression (B, STZa) as compared to the control group (A, Ctrl). Daily intake of gamma conglutin protein ameliorates GLUT2 over-expression (C, STZa-Cγ) in streptozotocin-induced diabetic animals. The negative control shows absence of cross-reactivity (D). GLUT2 positivity was expressed as immunoreactive area per total islet area (%). * $p < 0.05$, compared to Ctrl group. *Abbreviation*: *GLUT2*, glucose transporter 2.

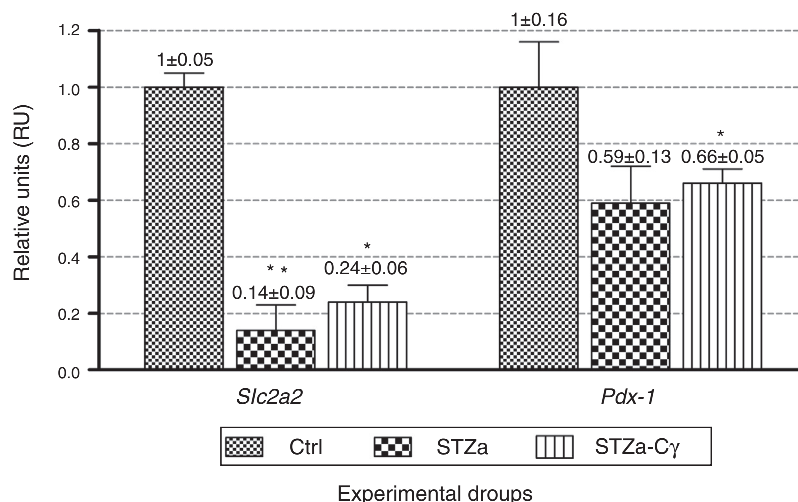


Fig. 3. *Slc2a2* and *Pdx-1* gene expression in pancreatic tissue of streptozotocin-induced diabetic animals with (STZa-Cγ) and without gamma conglutin (STZa) administration. Bars with a star are significantly lower than the control group (Ctrl) at $^{*}p < 0.05$ or $^{**}p < 0.01$. Data represent the mean ± standard error of the mean. *Abbreviations: Ctrl*, group without diabetes induction; *STZa*, streptozotocin control group; *STZa-Cγ*, streptozotocin group treated with Cγ.

Since GLUT2 immunopositivity was found distributed throughout the hepatic tissue, it was not possible to delimit the positive area to quantify the expression of GLUT2 protein, despite several technical modifications in the immunochemistry's assay conditions.

On the other hand, expression of the genes *Pdx-1* and *Slc2a2* was higher in the pancreatic tissue of STZa-Cγ rats as compared to STZa group (Fig. 3). *Pdx-1* is a master transcriptional regulator that modulates β-cell differentiation and function by stimulating the expression of *Ins-1*, *Slc2a2*, and *Gck* genes among others (Ohlsson et al., 1993; Watada et al., 1996; Waeber et al., 1996). A reduced *Pdx-1* gene expression leads to hyperglycemia through loss of β-cell function, which is related to down-regulation of *Slc2a2* and *Gck* (Ahlgren et al., 1998; Gao et al., 2014). *Slc2a2* and *Gck* play a prominent role in glucose-stimulated insulin secretion (GSIS) by pancreatic β-cells. Therefore, a decreased *Slc2a2* expression is associated with β-cells' unresponsiveness to hyperglycemia (Unger, 1991; Thorens et al., 1992; Guillam et al., 1997; Weir and Bonner-Weir, 2004). Our results show a lack of positive correlation between mRNA (*Slc2a2*) and protein (GLUT2) levels. We observed a decreased GLUT2 content and a slightly increased *Slc2a2* mRNA levels in pancreatic tissue after Cγ treatment; however, this effect has been reported for other compounds and explained through protein's half-life modulation (Gremlich et al., 1997).

Unfortunately, it was not possible to quantify expression of the *Gck* gene by qPCR in pancreatic tissue, possibly due to an elevated rate of β-cells death after STZ administration.

Slc2a2 *and* Gck *mRNA levels in hepatic tissue of Cγ-treated rats*

We also measured the expression of the *Gck* and *Slc2a2* genes in hepatic tissue of experimental animals (Fig. 4A and B). As compared to the control group, both STZa and STZa-Cγ showed a significant increase in *Slc2a2* gene expression (Fig. 4A) ($p < 0.05$) and agree with previous reports in diabetes-induced rats (Yonamine et al., 2016). However, the Cγ-treated group showed even higher *Slc2a2* gene expression. The increase in hepatic *Slc2a2* gene expression after Cγ treatment might be indicative of augmented glucose uptake, as observed in hepatic cells *in vitro* (Lovati et al., 2012). This mechanism might explain the reduction of circulating glucose in STZa-Cγ rats (Lovati et al., 2012).

Although *Gck* levels were expected to increase after Cγ treatment, we found decreased *Gck* gene expression in both, STZa and STZa-Cγ groups, as compared to the Ctrl rats ($p < 0.05$) (Fig. 4B). It is possible that Cγ had a transient effect on *Gck* mRNA levels (Arden et al., 2011). Studies with GCK activating molecules have shown this type of response (Agius, 2014).

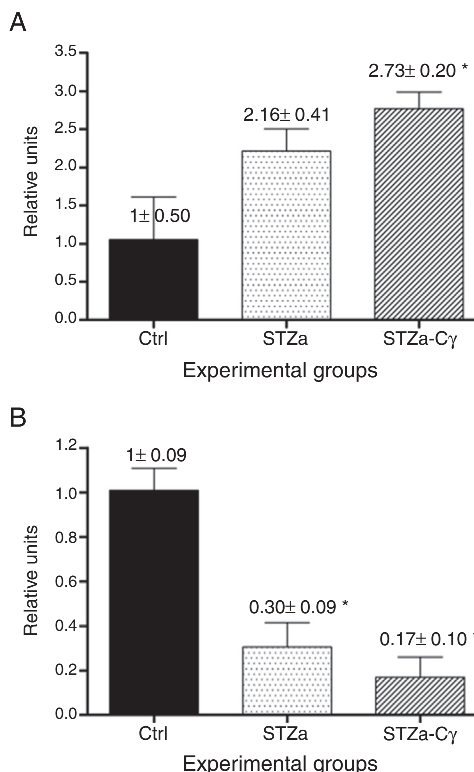
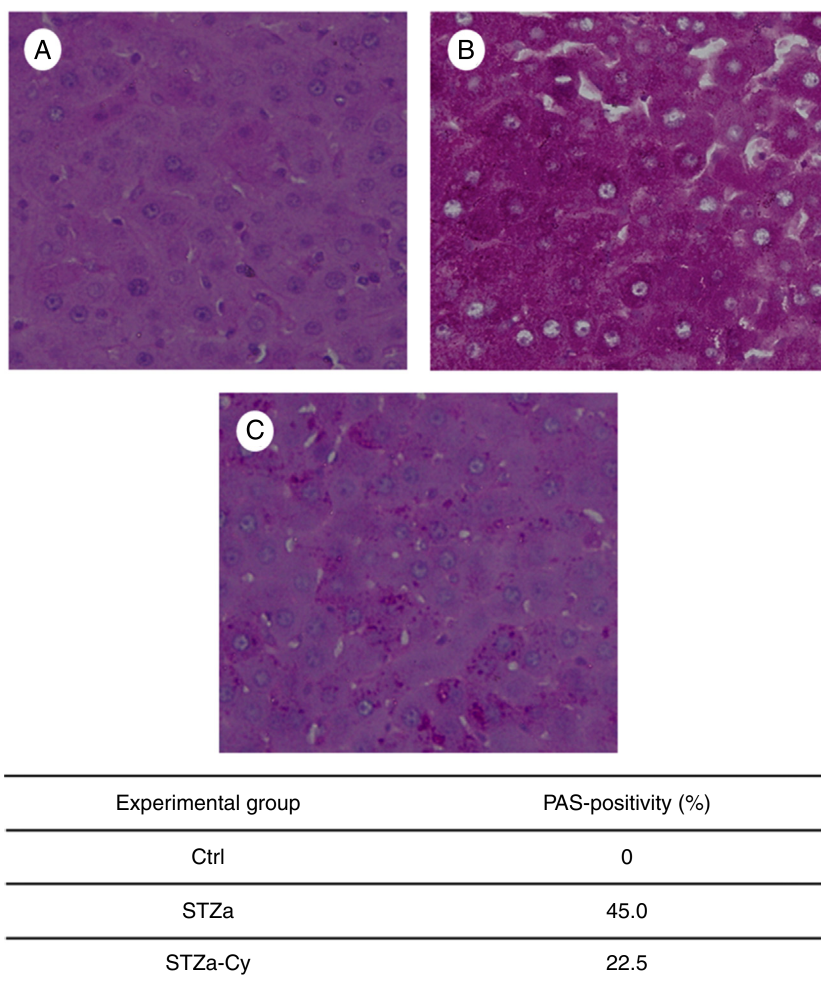


Fig. 4. *Slc2a2* (A) and *Gck* (B) gene expression in hepatic tissue of experimental animals. Data represent the mean ± standard error of the mean. Values with a star are significantly different from the control group at $p < 0.05$. *Abbreviations: Ctrl*, group without diabetes induction; *STZa*, streptozotocin control group; *STZa-Cγ*, streptozotocin group treated with Cγ.

On the other hand, recent reports indicate that the steroid receptor coactivators (SRC) participate in the transcriptional regulation of both, *G6pc* and *Gck* genes. The loss of *Src2* causes down-regulation of *G6pc* and *Gck* gene expression in the liver of *Src2* knockout mice (Fleet et al., 2015). In this respect, the regulation of *Gck* by SRC might be responsible for the low levels observed in this study. Moreover,



Experimental group	PAS-positivity (%)
Ctrl	0
STZa	45.0
STZa-Cy	22.5

Fig. 5. Glycogen content in hepatic tissue of experimental groups. Control group shows a depleted hepatic glycogen reservoir after an overnight fasting period (A, Ctrl). Conversely, hepatic glycogen of diabetes-induced rats was found abundant, homogenously, and completely distributed in hepatocytes of these animals (B, Ctrl). Daily intake of gamma conglutin protein (120 mg/kg) improves hepatic glycogen content of diabetic rats and induces its focal distribution in hepatic cells (C, STZa-Cγ). PAS-positivity was expressed as percentage (%). *Abbreviation: PAS*, periodic acid Schiff stain.

this might explain the *Gck* and *G6pc* down-regulation after Cγ treatment (Gonzalez-Santiago et al., 2017). Whether or not Cγ affects the expression of this group of coactivators remains to be determined.

Altogether, these data indicate a higher hepatic glucose uptake, but whether the glycolysis rate is impacted or alternative glucose metabolism routes are activated is not known and needs further investigation.

Glycogen content in hepatic tissue of Cγ-treated rats

To further explore the metabolic fate of internalized glucose in the hepatic tissue of diabetes-induced rats treated with Cγ, we performed a periodic acid-Schiff staining (PAS) to reveal glycogen deposits. As expected, after an overnight fasting period, glycogen reservoir was depleted in Ctrl rats (0%) (Fig. 5A). On the contrary, STZa animals showed a highly preserved glycogen content after this non-feeding period (45% of positivity to the stain) (Fig. 5B). Similar to the Ctrl rats, STZa-Cγ had lower glycogen content as shown by a decreased PAS positivity after a 7-consecutive-day Cγ treatment (22.5%) (Fig. 5C). In addition, the glycogen distribution was markedly different between hepatocytes from STZa and STZa-Cγ rats. Whereas the glycogen was observed homogenously and completely distributed in the cytoplasm of hepatic cells from STZa animals, glycogen deposits were found focalized inside the hepatocytes of STZa-Cγ rats (Fig. 5A–C).

Accordingly, other authors have reported a significant increase in hepatic glycogen content of animals induced to diabetes with STZ after a 16-h fasting period as compared to non-induced control rats (Ugochukwu and Babady, 2003). Other authors have found that after STZ-induced diabetes, a significant reduction in glycogen phosphorylase is observed, at both mRNA and enzymatic activity levels (Rao et al., 1995). Therefore, our results might be a consequence of the diabetes induced by STZ.

On the other hand, ~80% of total gluconeogenesis is attributed to hepatic glucose production. Additionally, it has been shown that hepatic gluconeogenesis plays a major role in hyperglycemia during the fasting and the feeding states (Sharabi et al., 2015). Thus, an augmented gluconeogenic rate in hepatic tissue is observed in the diabetic state (Lin and Accili, 2011). The increase in hepatic glycogen synthesis in STZa animals without treatment correlates with higher *Gck* mRNA levels, possibly indicating a higher rate of glucose phosphorylation. Nonetheless, G6P can be directed either to glycolysis or glycogen synthesis. Altogether, our data suggest a modulation of glycogen metabolism in STZa-Cγ rats, resembling the findings in the Ctrl group.

The PAS staining has the disadvantage of being unspecific for glycogen detection. Therefore, we performed a diastase-PAS staining to corroborate the presence of glycogen in the histological sections. After a diastase-digestion, we confirmed that the PAS-positive sections corresponded to glycogen (data not shown).

In conclusion, our findings demonstrate that Cγ protein affects genes related to glucose homeostasis and corroborates its glycemia modulatory effect. These data indicate that Cγ induces higher hepatic glucose uptake up-regulating the *Slc2a2* gene in diabetes-induced rats.

Also, we proposed that Cγ may improve β-cell function given the reestablishment of normal GLUT2 content in pancreatic islets of diabetes-induced rats. This argument is supported by the increase of *Pdx-1* and *Slc2a2* gene expression in pancreas. In this study, we used a model that involves selective β-cells' damage as a way to resemble a T2D phenotype. We are aware that this model does not fulfill all the factors involved in T2D such as obesity and/or glucolipotoxicity conditions and that in general STZ induces a phenotype resembling T1D. However, we consider that this model can be used to get an insight into Cγ mechanism of action. Whether Cγ administration improves β-cell function in the diabetic state remains to be determined.

Authors' contribution

All authors contributed substantially to the present work. RdeJSM, BVG, and TJG performed the experimental work and the statistical analysis. PMGL and ALMA contributed in the protein isolation process and critical analysis of the paper. JADR carried out the histological evaluation of the tissues. CMGD contributed in the experimental conception, designing, data interpretation and critical analysis of the paper. CMGD and TJG drafted the manuscript. All authors read and approved the final version of the manuscript before submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest

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