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Response to Reviewers: Reviewer #1:
Most of the points made have been addressed in some way by the authors. It would be helpful if there was some discussion in the manuscript on authors belief that part of actions could not be secondary to effects on islet hormone secretions - insulin and glucagon. Also, comment should be added concerning previous studies on islet morphology.

Accordingly to the reviewer´s suggestion a new paragraph was added to the discussion (Page 13, lines 19-23 and page 14, lines 1-6).

Highlights:

INGAP-PP significantly increases liver glucose metabolism.
INGAP-PP effects were possibly mediated by P-Akt signaling pathway.
INGAP-PP might become an effective pharmacological tool to treat people with T2D.

***Detailed Response to Reviewers**

CENEXA
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Centro Colaborador de la OPS/OMS
en Diabetes

December 22th, 2017

Karl-Heinz Herzig, MD, PhD
Editor
Peptides

Dear Prof. Dr. Herzig:

Thank you for your kind letter of December 18th referred to our manuscript Ms. No. PEPTIDES-D-17-00310R1.

We have revised our manuscript and according to the Editor and reviewers´ suggestions, the following changes have been introduced:

Reviewer #1:
Most of the points made have been addressed in some way by the authors.
It would be helpful if there was some discussion in the manuscript on authors belief that part of actions could not be secondary to effects on islet hormone secretions - insulin and glucagon. Also, comment should be added concerning previous studies on islet morphology.

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We hope this time the manuscript can be suitable for its publication in *Peptides*.

Waiting for your news, we remain

Yours sincerely

Dr. F. Francini, on behalf of all authors
f_francini@yahoo.com

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LIVER CARBOHYDRATES METABOLISM: A NEW ISLET-NEOGENESIS ASSOCIATED PROTEIN PEPTIDE (INGAP-PP) TARGET

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Abbreviations: Glucokinase, GK; glucose-6-phosphatase, G-6-Pase; glyceraldehyde 3-phosphate dehydrogenase, GAPDH; glycogen synthase kinase-3β, GSK3; hepatic insulin sensitivity, HIS; homeostasis model assessment, HOMA-IR; Islet-Neogenesis Associated protein peptide, INGAP-PP; phosphoenolpyruvate carboxykinase, PEPCK; phosphofructokinase-2, PFK-2.

ABSTRACT

Islet-Neogenesis Associated Protein-Pentadecapeptide (INGAP-PP) increases β-cell mass and enhances glucose and amino acids-induced insulin secretion. Our aim was to demonstrate its effect on liver metabolism. For that purpose, adult male Wistar rats were injected twice-daily (10 days) with saline solution or INGAP-PP (250 μg). Thereafter, serum glucose, triglyceride and insulin levels were measured and homeostasis model assessment (HOMA-IR) and hepatic insulin sensitivity (HIS) were determined. Liver glucokinase and glucose-6-phosphatase (G-6-Pase) expression and activity, phosphoenolpyruvate carboxykinase (PEPCK) expression, phosphofructokinase-2 (PFK-2) protein content, P-Akt/Akt and glycogen synthase kinase-3β (P-GSK3/GSK3) protein ratios and glycogen deposit were also determined. Additionally, glucokinase activity and G-6-Pase and PEPCK gene expression were also determined in isolated hepatocytes from normal rats incubated with INGAP-PP (5 μg/ml).

INGAP-PP administration did not modify any of the serum parameters tested but significantly increased activity of liver glucokinase and the protein level of its cytosolic activator, PFK-2. Conversely, INGAP-PP treated rats decreased gene expression and enzyme activity of gluconeogenic enzymes, G-6-Pase and PEPCK. They also showed a higher glycogen deposit and P-GSK3/GSK3 and P-Akt/Akt ratio. In isolated hepatocytes, INGAP-PP increased GK activity and decreased G-6-Pase and PEPCK expression.

These results demonstrate a direct effect of INGAP-PP on the liver acting through P-Akt signaling pathway. INGAP-PP enhances liver glucose metabolism and deposit and reduces its production/output, thereby contributing to maintain normal glucose homeostasis. These results reinforce the concept that INGAP-PP might become a useful tool to treat people with impaired islet/liver glucose metabolism as it occurs in T2D.

Key words: Islet neogenesis associated protein; liver metabolism; carbohydrates metabolism

1. Introduction

Since Type 2 diabetes is a disease resulting from progressive loss of β-cell mass and insulin secretion associated with a decrease in insulin effect on peripheral tissues, therapeutic efforts have been focused on the reversal of these abnormalities. In this context, Islet-Neogenesis Associated protein (INGAP), a protein of the Reg family, has emerged as a reasonable therapeutic alternative tool [30]. Although its presence was initially described only in exocrine pancreatic cells, INGAP mRNA expression and its protein presence were thereafter identified in pancreatic exocrine-, duct- and islet-non-β-cells from normal hamsters and adult rats [15]. Evidence showed that a pentadecapeptide with the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduces the stimulatory effect of the complete molecule upon thymidine incorporation into duct cells and a duct cell line [30].

Several studies have clearly demonstrated that addition of INGAP-PP to cultured islets isolated from either normal neonatal or adult rats induced expression of genes related to β-cell function [2-3,17,28,35] and significantly increased β-cell mass and insulin release in response to glucose and amino acids [2-3,6,20,35]. *In vivo* studies have also demonstrated that transgenic mice expressing INGAP in β-cells (IP-INGAP) normalized blood glucose during intraperitoneal glucose challenge [8] and had significantly delayed development of streptozotocin-induced hyperglycemia, probably due to its pleiotropic effect [8]. Further, Madrid et al. [19] demonstrated that administration of INGAP-PP to hamsters for 10 days increased β-cell replication and neogenesis and decreased β-cell apoptosis rates leading to an increase in their β-cell mass. Complementary *ex vivo* experiments with islets isolated from these animals showed an increase in glucose-induced insulin release [19]. This pentadecapeptide also enhanced β-cell mass and reversed diabetes in C57BL/6J mice [33].

In a clinical trial, INGAP-PP administration increased serum C-peptide levels in people with Type 1 diabetes and improved glycemic control in those with Type 2 diabetes [12], thus suggesting that INGAP-PP could be a potential tool to treat diabetes [29].

Although INGAP-mRNA was identified only in pancreatic tissue from normal hamsters, other members of the Reg protein family such as Reg2, Reg3α, and Reg3β were identified in liver by qPCR [37]. Interestingly, INGAP-PP specifically bound to liver and small intestine [5], thus suggesting a potential role of the peptide in these tissues.

In view of the lack of reported evidence on the effect of INGAP-PP in liver, we evaluated the possible effect of this molecule on hepatic metabolism.

2. Material and methods

2.1 Chemicals and drugs

All reagents of the purest available grade were provided by Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA), while secondary antibody peroxidase-conjugated Affinity Pure donkey anti-rabbit IgG and anti-goat IgG were provided by Dianova (Hamburg, Germany). INGAP-PP (NH2-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH) was kindly provided by Dr. G. Alexander Fleming (Kinexum LLC, Harper's Ferry, West Virginia, USA). Quality control of the peptides (aminoacid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63.

2.2 Animals

Adult male Wistar rats (230 g bw) kept at 23°C on a fixed 12-h light-dark cycle (06:00–18:00 h), were divided into 2 different experimental groups injected with: a) 200 µl of 0.9 % NaCl (W/V)/twice a day (Control [C]) or b) 200 µl of INGAP-PP (250 µg in 0.9 % NaCl/twice a day [INGAP-PP]). Animals in both groups were injected every 12 h (at 8:00 AM and PM) for 10 consecutive days. This peptide dose and time-period was selected on the basis of previous *in vivo* studies that shown that INGAP-PP effectively affects several parameters in islet tissue [19,32-33]. During this period both groups received

a standard commercial diet and tap water *ad libitum*. Water and food intake were measured daily, while individual body weight was recorded once a week. The experiment was replicated 5 times (total: 20 animals per group). At the end of the treatment and after fasting for 4h, blood samples were taken from each animal from the retroorbital plexus under light anesthesia (halothane) for serum determinations. Thereafter, rats were sacrificed by decapitation and the liver medial lobe was dissected for different assays.

Experimental animals were handled following the *Ethical Principles and Guidelines for Experimental Animals* (3rd Edition 2005) of the Swiss Academy of Medical Sciences.

2.3. Serum measurements

Serum glucose was determined by test strips (Accu-Chek Performa, Roche, Manheim, Germany); triglyceride levels by an enzymatic reaction kit (TG color GPO/PAP AA, Wiener lab, Argentina) and immunoreactive insulin levels by radioimmunoassay using an antibody against rat insulin (Sigma Chemical Co.), rat insulin standard (Novo Nordisk Pharma Argentina), and highly purified porcine insulin labeled with ^{125}I.

Insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) using the formula [Serum insulin (μU/ml) x fasting blood glucose (mM)/22.5] [25]. Hepatic insulin sensitivity (HIS) was also calculated with the formula k/ fasting plasma insulin x fasting plasma glucose, where k: 22.5 x 18 [24]. Since HOMA-IR and HIS in rats have no validated cut off values, we compared values obtained in controls *vs.* those recorded for the INGAP-PP group.

2.4. Hepatocyte Isolation and Culture conditions

Hepatocytes were isolated from non-fasting male Wistar rat livers by collagenase perfusion and mechanical disruption as previously described [34] with some modifications [16]. Cell viability was

checked by Trypan blue exclusion. The viability of all cell suspensions used was higher than 85%. Hepatocytes were seeded in Petri dishes with a concentration of 1.5 x 10^6 cell/dish in 8.0 mL of culture medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 µg/ml streptomycin). Cells were incubated at 37° C in a humidified atmosphere of 5% CO_2 for 4 h, allowing cell attachment to plates. After this time-period, medium was changed to fresh one supplemented with 10% FBS and incubated with 5 µg/ml of INGAP-PP. This dose was selected on the basis of previous *in vitro* studies [6]. Hepatocytes were incubated at different times (6h for mRNA analysis, or overnight for GK activity assay).

2.5. Total RNA

RNA was isolated from the liver of C and INGAP-PP rats and from normal hepatocytes cultured in control and INGAP-PP supplemented medium using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) [9]. Agarose-formaldehyde gel electrophoresis and the 260/280 nm absorbance ratio were used to evaluate integrity and quality of isolated RNA, while DNase I (Gibco-BRL) digestion was used to avoid DNA contamination. Reverse transcription-PCR utilized SuperScript III (Gibco-BRL) with total RNA (50 ng) as a template.

2.6. Analysis of gene expression by real-time PCR (qPCR)

We utilized a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad) and SYBR Green I as fluorescent dye for qPCR reactions. For this purpose 10 ng of cDNA was amplified using FastStart SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany) with 40 cycles (denaturation at 95° C for 30 sec, annealing at 65° C for 30 sec and extension at 72° C for 45 sec). Negative controls consisted of replacing samples with the same volume of water. Oligonucleotide primers (Invitrogen) used were: β-actin NM_031144.2, forward primer 5’-

AGAGGGAAATCGTGCGTGAC-3' and reverse primer 5'-CGATAGTGATGACCTGACCGT-3'; glucokinase (GK) NM_012565.1, forward primer 5'-GTGTACAAGCTGCACCCGA-3' and reverse primer 5'-CAGCATGCAAGCCTTCTTG-3'; glucose-6-phosphatase (G-6-Pase) NM_013098.2, forward primer 5'-GATCGCTGACCTCAGGAACGC-3' and reverse primer 5'-AGAGGCACGGAGCTGTTGCTG-3' and phosphoenolpyruvate carboxykinase (PEPCK) NM_198780.3, forward primer 5'-TGCCCCAGGAAGTGAGGAAG-3' and reverse primer 5'-GGTCAGTGAGAGCCAGCCAAC-3'.

All amplicons included fragments with a 90 to 250 bp size range. Reaction specificity was checked by melting curve analysis. Data are expressed as relative gene expression after normalization to the β-actin housekeeping gene using Qgene96 and LineRegPCR software [26,31].

2.7. Western blot analysis

Liver homogenates from C and INGAP-PP animals were used for GK, phosphofructokinase-2 (PFK2), P-Akt/Akt, glycogen synthase kinase-3β (P-GSK-3β/GSK-3β), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin immunodetection. Protein content of the samples was evaluated by Bio-Rad protein assay [7] and afterwards homogenates were treated with dithiothreitol and bromophenol blue (final concentration of 100 mM and 0.1%, respectively). One hundred μg of whole protein from each homogenate were loaded into 10% SDS-PAGE and transferred by electroblotting to PVDF membranes. Nonspecific binding sites were blocked by overnight incubation with non-fat dry milk. Each protein was identified and quantified using specific primary antibodies against GK (1:2,000) for 90 minutes; PFK2 (1:10,000) for 120 minutes; P-Akt and Akt (1:1,000), P-GSK-3B and GSK-3B (1:1,000) and GAPDH (1:1,000) overnight, and β-actin (1:10,000) for 60 minutes. Thereafter, membranes were incubated with the corresponding secondary antibodies. Intensity of specific bands was determined by densitometry using Gel-Pro Analyser software. β-actin density

was used to normalize GK and PFK-2 protein content and GAPDH for P-Akt, Akt, P-GSK-3B and GSK-3B. The ratio target protein content/housekeeping protein level in control animals is expressed as 100%.

2.8. Liver Glycogen content measurement

Liver medial lobe pieces (400 mg) were placed in 1 ml of 33% KOH and incubated for 20 min at 100º C. Then, 1.25 ml of ethanol was added to each tube, the mixture incubated for 48 h at 4º C and finally centrifuged at 700 ×g for 20 min. The pellets were resuspended in 1 ml of distilled water plus 3 ml of Antrone solution (0.1% in 84% H_2SO_4) and incubated for 20 min at 100º C. Absorbance was measured photometrically at 620 nm and results were expressed as μmol of glycogen/mg of tissue [10].

2.9. Glucokinase activity

Liver pieces removed from the animals were homogenized in ice-cold PBS containing 0.1 mM PMSF, 0.1 mM benzamidine, 2 mM DTT, 4 μg/ml aprotinin, and 0.3 M sucrose, pH 7.5. Aliquots of these homogenates were centrifuged at 600 ×g to separate the nuclear fraction. The supernatant was centrifuged twice at 8,000 and 100,000 ×g at 4º C, and the resultant supernatant collected and identified as the cytosolic fraction (c_f), which contains the active functional enzyme. For cultured hepatocytes (either control or INGAP-PP treated conditions), cells were washed and collected in PBS; thereafter, hepatocytes were centrifuged at 30 xg at 4° C and pellets were resuspended in PBS plus inhibitors buffer, disrupted by sonication (5 times 10 seconds) and treated as previously mentioned for liver pieces. Glucose phosphorylation rates were measured at 37º C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay [21]. Glucokinase activity was determined by subtracting activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose. Enzyme activity was expressed as mU/mg of protein. One unit of

enzyme activity was defined as 1 µmol of glucose-6-phosphate formed from glucose and ATP/min at 37º C.

2.10. Liver Glucose-6-phosphatase activity

Homogenization of liver samples and isolation of microsomes were carried out as described by Nordlie & Arion [27] using a 0.25 M sucrose/5 mM Tris-acetate/0.5 mM EDTA, pH 7.4 medium. Microsomes were washed once with the same medium and centrifuged at 100,000 ×g. Fully disrupted microsomes were prepared at 0º C by adding 0.1 ml of 0.75% Triton X-100 to 0.9 ml of untreated microsomes (approximately 10 mg of protein) and allowed to stand on ice for 20 min. Then 50 µl of the mixture and 50 µl of 0.8 M glucose-6-phosphate sodium salt were added and samples incubated for 10 min at 30º C. The reaction was stopped by adding 250 µl of 10% trichloroacetic acid; then, 2 ml of $MoNH_4$ (diluted in 1M H_2SO_4) plus 320 µl of $FeSO_4$ (diluted in 0.15M H_2SO_4) were added to 200 µl of each sample. Glucose-6-phosphatase activity was determined by measuring the release of inorganic phosphate from glucose-6-phosphate. Absorbance was photometrically read against a reagent blank at 660 nm and results were expressed as 'latency', calculated with the following formula: 100 × (activity in disrupted microsomes – activity measured in untreated microsomes)/activity measured in disrupted microsomes.

2.11. Statistical analysis

Results are expressed as mean ± SEM for the indicated number of observations. Differences between groups were considered significant when $p<0.05$ using t-test.

3. Results

3.1. Body weight and food intake

Food intake was similar in C and INGAP-PP treated rats (19.8 ± 0.4 *vs*. 20.6 ± 0.4 g/rat/day) while they also showed a comparable body weight increase (Δ g/rat: 32.3 ± 3.4 *vs.* 36.1 ± 4.0, respectively).

3.2. Serum measurements

C and INGAP-PP treated rats showed no difference in serum glucose (107 ± 2,5 *vs*. 113 ± 2.8 mg/dl), insulin (0.63 ± 0.07 *vs*. 0.57 ± 0.08 ng/ml), and triglyceride (122.7 ± 8.8 *vs.* 115.4 ± 7.9 mg/dl) concentrations. Regarding HOMA-IR, values obtained were 4.01 ± 0.52 *vs*. 4.06 ± 0.58 for C and INGAP-PP animals, respectively. HIS in C and INGAP-PP animals correspondingly showed no statistically significant differences (5.19 ± 0.67 *vs.* 6.74 ± 2.16 respectively).

3.3. GK, PFK-2 and G-6-Pase levels.

C and INGAP-PP rats showed no statistically significant differences either in GK gene expression or in protein level (Fig.1 A and B-C, respectively). However, INGAP-PP animals showed significant higher enzyme activity (Fig. 1 D). This change was associated with an increase in the protein level of PFK-2, the GK citoplasmatic activator (Fig. 1 E-F).

Both G-6-Pase gene expression and enzymatic activity were significantly lower in INGAP-PP rats (Fig. 1 G and H, respectively) as well as PEPCK gene expression (Fig. 1 I).

Complementary, GK activity was also significantly increased in hepatocytes isolated from normal rats and incubated with INGAP-PP 5 μM during 18 hours compared to control cells (Fig. 2A). INGAP-PP also induced in these cultured hepatocytes a significant reduction in G-6-Pase and PEPCK gene expression (Fig. 2 B and C).

3.4. Glycogen content and P-GSK-3β/GSK-3β levels

Liver glycogen content was significantly higher in INGAP-PP treated animals (Fig 3 A). The P-GSK-3β/GSK-3β ratio was also increased in these rats (Fig. 3 B-C).

3.5. P-Akt/Akt protein levels

Although total protein level of Akt was similar in both groups, the phosphorylated form of the enzyme was significantly higher in INGAP-PP-treated rats. Consequently, the P-Akt/Akt ratio was higher in INGAP-PP than in C rats (Fig. 4 A-B).

Discussion

Our results show that INGAP-PP administration to normal rats for ten days induced significant changes in several markers of liver carbohydrate metabolism. In fact, this treatment produced a significant increase of glucokinase activity with a parallel increase in liver glycogen content. Conversely, G-6-Pase, the enzyme involved in the last step of gluconeogenesis pathway, showed significantly decreased gene expression and enzymatic activity in INGAP-PP treated animals. PEPCK, other enzyme involved in gluconeogenesis process, also decrease its gene expression. The combination of these uneven metabolic changes suggests that INGAP-PP could increase glucose entry into liver cells and its deposit as glycogen with a simultaneous decrease in its production/output.

No significant differences were found between control and INGAP-PP treated rats in serum glucose, insulin or triglyceride levels or in HOMA-IR or HIS, which suggests that its administration did not exert any negative effect on normal glucose and overall metabolic homeostasis.

These results were not sufficient to postulate a direct action of INGAP-PP on hepatocytes or a consequence of general endocrine/metabolic changes. The effects on liver metabolism resembled those associated with increased insulin action. In addition, we have previously reported a stimulatory effect of INGAP-PP on β-cell mass and function [19,32], However, no changes were observed in serum insulin level, thus playing against such alternative. Complementary, since glycaemia was also not

modified, changes recorded in glucose liver metabolism cannot be ascribed to a decrease in glucagon serum levels (not determined in the present work). Further, rats treated with INGAP-PP did not show changes in α-cell mass [19,32]. The direct effect of INGAP-PP on hepatic cells was definitely confirmed incubating isolated hepatocytes from normal rats in the presence of the peptide. Under these conditions, INGAP-PP increased GK activity together with a reduction in G-6-Pase and PEPCK gene expression.

In this context, INGAP-PP directly affects glucokinase activity, enzyme that is the rate-limiting step of glucose metabolism, thus playing a pivotal role as a glucose sensor both in pancreatic β-cells and hepatocytes [23]. Further, Maiztegui et al. demonstrated that INGAP-PP also directly increases glucokinase activity in cultured pancreatic islets [20] whereas our current data demonstrated a similar effect in liver. In the liver, this activity is conditioned by an increase in the bifunctional enzyme PFK-2 rather than by an increase in enzyme protein content [22]. In islets this effect was associated with an increase in IR, IRS-2 and PI3K protein concentrations together with an increase in tyrosine phosphorylation of these compounds [20]. We have now shown an increase in the P-Akt/Akt ratio, a downstream target of PI3K activation, which suggests involvement of this pathway in the mechanism by which INGAP-PP induces its effects both in islets and in liver. The report that an active Akt pathway could inhibit hepatic gluconeogenesis in both normal and diabetic animals supports our statement [1,14]. However, since P-Akt is a common end-point of different receptor activated pathways, we cannot completely rule out the possibility that INGAP-PP could activate other receptors in the liver as well.

We also found a significant increase in the P-GSK-3β/GSK-3β ratio which implies inhibition of enzyme activity [11]. GSK-3β is a negative modulator of glycogen synthase activity, a rate-limiting enzyme that promotes glycogen deposit [13,39]. Therefore, its inhibition would lead to an increase of the glycogen synthase activity that coincides with our finding of a significant increase in glycogen liver

content. Since Akt phosphorylates both GSK-3 isoforms on the regulatory serine residues, inhibition of GSK-3 could be mediated by Akt [4,11,18]. This pathway may explain the intracellular mechanism by which INGAP-PP exerts its effect on liver function.

Altogether, these data provide additional mechanistic support for the beneficial effect of INGAP-PP on hyperglycemia either for experimental animal models or for people with diabetes [8,12,33,36].

Our results complete the findings reported by Borelli and colleagues [5] who described that although INGAP (mRNA and protein) is present only in pancreatic islets and exocrine tissue of normal hamsters, ^{125}I-tyrosylated INGAP pentadecapeptide (^{125}I-T-INGAP-PP) specifically binds to the liver and small intestine. We have now provided evidence that this liver binding is associated with significant changes in carbohydrate metabolism in the gland.

After the demonstration that a pentadecapeptide with the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduced the stimulatory effect of the complete molecule on thymidine incorporation into duct cells [30], several reports have shown its enhancing effects on pancreatic β-cell mass and function [2-3,6,19-20,38] together with its modulatory effect on hyperglycemia in experimental and clinical diabetes [8,33,36].

Despite all these well characterized cellular effects of INGAP-PP, identification and characterization of its specific receptor has remained elusive. However, since specific *in vivo* binding of this peptide to liver cells is the closest approximation to the existence of this receptor in this gland [5], it is plausible that INGAP-PP dependent metabolic changes assayed in our treated rats may result from its interaction with this receptor. The direct effects recorded in hepatocytes incubated with INGAP-PP, together with a previous report of these kind of effects at pancreatic islet levels lends support to this assumption [20].

In brief, our results demonstrate for the first time that INGAP-PP significant and directly affected glucose metabolism in liver, possibly through the P-Akt signaling pathway. These changes might result

in higher glucose entry into hepatocytes associated with a potential reduction in its production/output, thus contributing to maintain normal glucose homeostasis. This combination of beneficial effects on liver glucose metabolism as well as on β-cell mass and function reinforce the concept that INGAP-PP might become an effective pharmacological tool to treat diabetic patients with dysfunction of both organs.

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Figure Legends

Figure 1. Liver glucokinase (GK), PFK-2, glucose-6-phosphatase (G-6-Pase) and phosphoenolpiyruvate carboxykinase (PEPCK) levels. Liver GK gene expression (A), protein content (B and C) and activity (D); PFK-2 protein content (E and F), G-6-Pase gene expression and protein level (G and H) and PEPCK gene expression (I). C and F: representative blots showing the bands corresponding to GK and PFK-2 protein or β-actin protein in control and INGAP-PP treated rats. (B and E): band intensities in control (white bar) and INGAP-PP treated animals (black bar). Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 2. Glucokinase (GK) activity and glucose-6-phosphatase (G-6-Pase) and phosphoenolpiyruvate carboxykinase (PEPCK) gene expression in isolated hepatocytes. Hepatocytes GK activity (A), G-6-Pase (B) and PEPCK (C) gene expression in control (white bar) and INGAP-PP treated cells (black bar). Results are mean ± SEM of 2 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 3. Liver glycogen content and P-GSK-3β/GSK-3β level. Liver glycogen content (A) in control (white bar) and INGAP-PP treated animals (black bar). B: relative band intensities in both control and INGAP-PP treated animals. C: representative blots showing the bands corresponding to P-

GSK-3β and GSK-3β proteins in control and INGAP-PP treated rats. Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 4. Liver P-Akt/Akt level. A: P-Akt/Akt relative band intensities in both control and INGAP-PP treated animals. B: representative blots showing the bands corresponding to P-Akt/Akt proteins in control and INGAP-PP treated rats. Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

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LIVER CARBOHYDRATES METABOLISM: A NEW ISLET-NEOGENESIS ASSOCIATED PROTEIN PEPTIDE (INGAP-PP) TARGET

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Abbreviations: Glucokinase, GK; glucose-6-phosphatase, G-6-Pase; glyceraldehyde 3-phosphate dehydrogenase, GAPDH; glycogen synthase kinase-3β, GSK3; hepatic insulin sensitivity, HIS; homeostasis model assessment, HOMA-IR; Islet-Neogenesis Associated protein peptide, INGAP-PP; phosphoenolpyruvate carboxykinase, PEPCK; phosphofructokinase-2, PFK-2.

ABSTRACT

Islet-Neogenesis Associated Protein-Pentadecapeptide (INGAP-PP) increases β-cell mass and enhances glucose and amino acids-induced insulin secretion. Our aim was to demonstrate its effect on liver metabolism. For that purpose, adult male Wistar rats were injected twice-daily (10 days) with saline solution or INGAP-PP (250 μg). Thereafter, serum glucose, triglyceride and insulin levels were measured and homeostasis model assessment (HOMA-IR) and hepatic insulin sensitivity (HIS) were determined. Liver glucokinase and glucose-6-phosphatase (G-6-Pase) expression and activity, phosphoenolpyruvate carboxykinase (PEPCK) expression, phosphofructokinase-2 (PFK-2) protein content, P-Akt/Akt and glycogen synthase kinase-3β (P-GSK3/GSK3) protein ratios and glycogen deposit were also determined. Additionally, glucokinase activity and G-6-Pase and PEPCK gene expression were also determined in isolated hepatocytes from normal rats incubated with INGAP-PP (5 μg/ml).

INGAP-PP administration did not modify any of the serum parameters tested but significantly increased activity of liver glucokinase and the protein level of its cytosolic activator, PFK-2. Conversely, INGAP-PP treated rats decreased gene expression and enzyme activity of gluconeogenic enzymes, G-6-Pase and PEPCK. They also showed a higher glycogen deposit and P-GSK3/GSK3 and P-Akt/Akt ratio. In isolated hepatocytes, INGAP-PP increased GK activity and decreased G-6-Pase and PEPCK expression.

These results demonstrate a direct effect of INGAP-PP on the liver acting through P-Akt signaling pathway. INGAP-PP enhances liver glucose metabolism and deposit and reduces its production/output, thereby contributing to maintain normal glucose homeostasis. These results reinforce the concept that INGAP-PP might become a useful tool to treat people with impaired islet/liver glucose metabolism as it occurs in T2D.

Key words: Islet neogenesis associated protein; liver metabolism; carbohydrates metabolism

1. Introduction

Since Type 2 diabetes is a disease resulting from progressive loss of β-cell mass and insulin secretion associated with a decrease in insulin effect on peripheral tissues, therapeutic efforts have been focused on the reversal of these abnormalities. In this context, Islet-Neogenesis Associated protein (INGAP), a protein of the Reg family, has emerged as a reasonable therapeutic alternative tool [30]. Although its presence was initially described only in exocrine pancreatic cells, INGAP mRNA expression and its protein presence were thereafter identified in pancreatic exocrine-, duct- and islet-non-β-cells from normal hamsters and adult rats [15]. Evidence showed that a pentadecapeptide with the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduces the stimulatory effect of the complete molecule upon thymidine incorporation into duct cells and a duct cell line [30].

Several studies have clearly demonstrated that addition of INGAP-PP to cultured islets isolated from either normal neonatal or adult rats induced expression of genes related to β-cell function [2-3,17,28,35] and significantly increased β-cell mass and insulin release in response to glucose and amino acids [2-3,6,20,35]. *In vivo* studies have also demonstrated that transgenic mice expressing INGAP in β-cells (IP-INGAP) normalized blood glucose during intraperitoneal glucose challenge [8] and had significantly delayed development of streptozotocin-induced hyperglycemia, probably due to its pleiotropic effect [8]. Further, Madrid et al. [19] demonstrated that administration of INGAP-PP to hamsters for 10 days increased β-cell replication and neogenesis and decreased β-cell apoptosis rates leading to an increase in their β-cell mass. Complementary *ex vivo* experiments with islets isolated from these animals showed an increase in glucose-induced insulin release [19]. This pentadecapeptide also enhanced β-cell mass and reversed diabetes in C57BL/6J mice [33].

In a clinical trial, INGAP-PP administration increased serum C-peptide levels in people with Type 1 diabetes and improved glycemic control in those with Type 2 diabetes [12], thus suggesting that INGAP-PP could be a potential tool to treat diabetes [29].

Although INGAP-mRNA was identified only in pancreatic tissue from normal hamsters, other members of the Reg protein family such as Reg2, Reg3α, and Reg3β were identified in liver by qPCR [37]. Interestingly, INGAP-PP specifically bound to liver and small intestine [5], thus suggesting a potential role of the peptide in these tissues.

In view of the lack of reported evidence on the effect of INGAP-PP in liver, we evaluated the possible effect of this molecule on hepatic metabolism.

2. Material and methods

2.1 Chemicals and drugs

All reagents of the purest available grade were provided by Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA), while secondary antibody peroxidase-conjugated Affinity Pure donkey anti-rabbit IgG and anti-goat IgG were provided by Dianova (Hamburg, Germany). INGAP-PP (NH2-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH) was kindly provided by Dr. G. Alexander Fleming (Kinexum LLC, Harper's Ferry, West Virginia, USA). Quality control of the peptides (aminoacid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63.

2.2 Animals

Adult male Wistar rats (230 g bw) kept at 23°C on a fixed 12-h light-dark cycle (06:00–18:00 h), were divided into 2 different experimental groups injected with: a) 200 µl of 0.9 % NaCl (W/V)/twice a day (Control [C]) or b) 200 µl of INGAP-PP (250 µg in 0.9 % NaCl/twice a day [INGAP-PP]). Animals in both groups were injected every 12 h (at 8:00 AM and PM) for 10 consecutive days. This peptide dose and time-period was selected on the basis of previous *in vivo* studies that shown that INGAP-PP effectively affects several parameters in islet tissue [19,32-33]. During this period both groups received

a standard commercial diet and tap water *ad libitum*. Water and food intake were measured daily, while individual body weight was recorded once a week. The experiment was replicated 5 times (total: 20 animals per group). At the end of the treatment and after fasting for 4h, blood samples were taken from each animal from the retroorbital plexus under light anesthesia (halothane) for serum determinations. Thereafter, rats were sacrificed by decapitation and the liver medial lobe was dissected for different assays.

Experimental animals were handled following the *Ethical Principles and Guidelines for Experimental Animals* (3rd Edition 2005) of the Swiss Academy of Medical Sciences.

2.3. Serum measurements

Serum glucose was determined by test strips (Accu-Chek Performa, Roche, Manheim, Germany); triglyceride levels by an enzymatic reaction kit (TG color GPO/PAP AA, Wiener lab, Argentina) and immunoreactive insulin levels by radioimmunoassay using an antibody against rat insulin (Sigma Chemical Co.), rat insulin standard (Novo Nordisk Pharma Argentina), and highly purified porcine insulin labeled with ^{125}I.

Insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) using the formula [Serum insulin (μU/ml) x fasting blood glucose (mM)/22.5] [25]. Hepatic insulin sensitivity (HIS) was also calculated with the formula k/ fasting plasma insulin x fasting plasma glucose, where k: 22.5 x 18 [24]. Since HOMA-IR and HIS in rats have no validated cut off values, we compared values obtained in controls *vs.* those recorded for the INGAP-PP group.

2.4. Hepatocyte Isolation and Culture conditions

Hepatocytes were isolated from non-fasting male Wistar rat livers by collagenase perfusion and mechanical disruption as previously described [34] with some modifications [16]. Cell viability was

checked by Trypan blue exclusion. The viability of all cell suspensions used was higher than 85%. Hepatocytes were seeded in Petri dishes with a concentration of 1.5 x 10^6 cell/dish in 8.0 mL of culture medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 µg/ml streptomycin). Cells were incubated at 37° C in a humidified atmosphere of 5% CO_2 for 4 h, allowing cell attachment to plates. After this time-period, medium was changed to fresh one supplemented with 10% FBS and incubated with 5 µg/ml of INGAP-PP. This dose was selected on the basis of previous *in vitro* studies [6]. Hepatocytes were incubated at different times (6h for mRNA analysis, or overnight for GK activity assay).

2.5. Total RNA

RNA was isolated from the liver of C and INGAP-PP rats and from normal hepatocytes cultured in control and INGAP-PP supplemented medium using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) [9]. Agarose-formaldehyde gel electrophoresis and the 260/280 nm absorbance ratio were used to evaluate integrity and quality of isolated RNA, while DNase I (Gibco-BRL) digestion was used to avoid DNA contamination. Reverse transcription-PCR utilized SuperScript III (Gibco-BRL) with total RNA (50 ng) as a template.

2.6. Analysis of gene expression by real-time PCR (qPCR)

We utilized a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad) and SYBR Green I as fluorescent dye for qPCR reactions. For this purpose 10 ng of cDNA was amplified using FastStart SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany) with 40 cycles (denaturation at 95° C for 30 sec, annealing at 65° C for 30 sec and extension at 72° C for 45 sec). Negative controls consisted of replacing samples with the same volume of water. Oligonucleotide primers (Invitrogen) used were: β-actin NM_031144.2, forward primer 5’-

AGAGGGAAATCGTGCGTGAC-3' and reverse primer 5'-CGATAGTGATGACCTGACCGT-3'; glucokinase (GK) NM_012565.1, forward primer 5'-GTGTACAAGCTGCACCCGA-3' and reverse primer 5'-CAGCATGCAAGCCTTCTTG-3'; glucose-6-phosphatase (G-6-Pase) NM_013098.2, forward primer 5'-GATCGCTGACCTCAGGAACGC-3' and reverse primer 5'-AGAGGCACGGAGCTGTTGCTG-3' and phosphoenolpyruvate carboxykinase (PEPCK) NM_198780.3, forward primer 5'-TGCCCCAGGAAGTGAGGAAG-3' and reverse primer 5'-GGTCAGTGAGAGCCAGCCAAC-3'.

All amplicons included fragments with a 90 to 250 bp size range. Reaction specificity was checked by melting curve analysis. Data are expressed as relative gene expression after normalization to the β-actin housekeeping gene using Qgene96 and LineRegPCR software [26,31].

2.7. Western blot analysis

Liver homogenates from C and INGAP-PP animals were used for GK, phosphofructokinase-2 (PFK2), P-Akt/Akt, glycogen synthase kinase-3β (P-GSK-3β/GSK-3β), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin immunodetection. Protein content of the samples was evaluated by Bio-Rad protein assay [7] and afterwards homogenates were treated with dithiothreitol and bromophenol blue (final concentration of 100 mM and 0.1%, respectively). One hundred µg of whole protein from each homogenate were loaded into 10% SDS-PAGE and transferred by electroblotting to PVDF membranes. Nonspecific binding sites were blocked by overnight incubation with non-fat dry milk. Each protein was identified and quantified using specific primary antibodies against GK (1:2,000) for 90 minutes; PFK2 (1:10,000) for 120 minutes; P-Akt and Akt (1:1,000), P-GSK-3B and GSK-3B (1:1,000) and GAPDH (1:1,000) overnight, and β-actin (1:10,000) for 60 minutes. Thereafter, membranes were incubated with the corresponding secondary antibodies. Intensity of specific bands was determined by densitometry using Gel-Pro Analyser software. β-actin density

was used to normalize GK and PFK-2 protein content and GAPDH for P-Akt, Akt, P-GSK-3B and GSK-3B. The ratio target protein content/housekeeping protein level in control animals is expressed as 100%.

2.8. Liver Glycogen content measurement

Liver medial lobe pieces (400 mg) were placed in 1 ml of 33% KOH and incubated for 20 min at 100º C. Then, 1.25 ml of ethanol was added to each tube, the mixture incubated for 48 h at 4º C and finally centrifuged at 700 ×g for 20 min. The pellets were resuspended in 1 ml of distilled water plus 3 ml of Antrone solution (0.1% in 84% H_2SO_4) and incubated for 20 min at 100º C. Absorbance was measured photometrically at 620 nm and results were expressed as µmol of glycogen/mg of tissue [10].

2.9. Glucokinase activity

Liver pieces removed from the animals were homogenized in ice-cold PBS containing 0.1 mM PMSF, 0.1 mM benzamidine, 2 mM DTT, 4 µg/ml aprotinin, and 0.3 M sucrose, pH 7.5. Aliquots of these homogenates were centrifuged at 600 ×g to separate the nuclear fraction. The supernatant was centrifuged twice at 8,000 and 100,000 ×g at 4º C, and the resultant supernatant collected and identified as the cytosolic fraction (c_f), which contains the active functional enzyme. For cultured hepatocytes (either control or INGAP-PP treated conditions), cells were washed and collected in PBS; thereafter, hepatocytes were centrifuged at 30 xg at 4° C and pellets were resuspended in PBS plus inhibitors buffer, disrupted by sonication (5 times 10 seconds) and treated as previously mentioned for liver pieces. Glucose phosphorylation rates were measured at 37º C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay [21]. Glucokinase activity was determined by subtracting activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose. Enzyme activity was expressed as mU/mg of protein. One unit of

enzyme activity was defined as 1 μmol of glucose-6-phosphate formed from glucose and ATP/min at 37º C.

2.10. Liver Glucose-6-phosphatase activity

Homogenization of liver samples and isolation of microsomes were carried out as described by Nordlie & Arion [27] using a 0.25 M sucrose/5 mM Tris-acetate/0.5 mM EDTA, pH 7.4 medium. Microsomes were washed once with the same medium and centrifuged at 100,000 ×g. Fully disrupted microsomes were prepared at 0º C by adding 0.1 ml of 0.75% Triton X-100 to 0.9 ml of untreated microsomes (approximately 10 mg of protein) and allowed to stand on ice for 20 min. Then 50 μl of the mixture and 50 μl of 0.8 M glucose-6-phosphate sodium salt were added and samples incubated for 10 min at 30º C. The reaction was stopped by adding 250 μl of 10% trichloroacetic acid; then, 2 ml of $MoNH_4$ (diluted in 1M H_2SO_4) plus 320 μl of $FeSO_4$ (diluted in 0.15M H_2SO_4) were added to 200 μl of each sample. Glucose-6-phosphatase activity was determined by measuring the release of inorganic phosphate from glucose-6-phosphate. Absorbance was photometrically read against a reagent blank at 660 nm and results were expressed as 'latency', calculated with the following formula: 100 × (activity in disrupted microsomes – activity measured in untreated microsomes)/activity measured in disrupted microsomes.

2.11. Statistical analysis

Results are expressed as mean ± SEM for the indicated number of observations. Differences between groups were considered significant when $p<0.05$ using t-test.

3. Results

3.1. Body weight and food intake

Food intake was similar in C and INGAP-PP treated rats (19.8 ± 0.4 *vs*. 20.6 ± 0.4 g/rat/day) while they also showed a comparable body weight increase (Δ g/rat: 32.3 ± 3.4 *vs.* 36.1 ± 4.0, respectively).

3.2. Serum measurements

C and INGAP-PP treated rats showed no difference in serum glucose (107 ± 2,5 *vs*. 113 ± 2.8 mg/dl), insulin (0.63 ± 0.07 *vs*. 0.57 ± 0.08 ng/ml), and triglyceride (122.7 ± 8.8 *vs.* 115.4 ± 7.9 mg/dl) concentrations. Regarding HOMA-IR, values obtained were 4.01 ± 0.52 *vs*. 4.06 ± 0.58 for C and INGAP-PP animals, respectively. HIS in C and INGAP-PP animals correspondingly showed no statistically significant differences (5.19 ± 0.67 *vs.* 6.74 ± 2.16 respectively).

3.3. GK, PFK-2 and G-6-Pase levels.

C and INGAP-PP rats showed no statistically significant differences either in GK gene expression or in protein level (Fig.1 A and B-C, respectively). However, INGAP-PP animals showed significant higher enzyme activity (Fig. 1 D). This change was associated with an increase in the protein level of PFK-2, the GK citoplasmatic activator (Fig. 1 E-F).

Both G-6-Pase gene expression and enzymatic activity were significantly lower in INGAP-PP rats (Fig. 1 G and H, respectively) as well as PEPCK gene expression (Fig. 1 I).

Complementary, GK activity was also significantly increased in hepatocytes isolated from normal rats and incubated with INGAP-PP 5 µM during 18 hours compared to control cells (Fig. 2A). INGAP-PP also induced in these cultured hepatocytes a significant reduction in G-6-Pase and PEPCK gene expression (Fig. 2 B and C).

3.4. Glycogen content and P-GSK-3β/GSK-3β levels

Liver glycogen content was significantly higher in INGAP-PP treated animals (Fig 3 A). The P-GSK-3β/GSK-3β ratio was also increased in these rats (Fig. 3 B-C).

3.5. P-Akt/Akt protein levels

Although total protein level of Akt was similar in both groups, the phosphorylated form of the enzyme was significantly higher in INGAP-PP-treated rats. Consequently, the P-Akt/Akt ratio was higher in INGAP-PP than in C rats (Fig. 4 A-B).

Discussion

Our results show that INGAP-PP administration to normal rats for ten days induced significant changes in several markers of liver carbohydrate metabolism. In fact, this treatment produced a significant increase of glucokinase activity with a parallel increase in liver glycogen content. Conversely, G-6-Pase, the enzyme involved in the last step of gluconeogenesis pathway, showed significantly decreased gene expression and enzymatic activity in INGAP-PP treated animals. PEPCK, other enzyme involved in gluconeogenesis process, also decrease its gene expression. The combination of these uneven metabolic changes suggests that INGAP-PP could increase glucose entry into liver cells and its deposit as glycogen with a simultaneous decrease in its production/output.

No significant differences were found between control and INGAP-PP treated rats in serum glucose, insulin or triglyceride levels or in HOMA-IR or HIS, which suggests that its administration did not exert any negative effect on normal glucose and overall metabolic homeostasis.

These results were not sufficient to postulate a direct action of INGAP-PP on hepatocytes or a consequence of general endocrine/metabolic changes. The effects on liver metabolism resembled those associated with increased insulin action. In addition, we have previously reported a stimulatory effect of INGAP-PP on β-cell mass and function [19,32], However, no changes were observed in serum insulin level, thus playing against such alternative. Complementary, since glycaemia was also not

modified, changes recorded in glucose liver metabolism cannot be ascribed to a decrease in glucagon serum levels (not determined in the present work). Further, rats treated with INGAP-PP did not show changes in α-cell mass [19,32]. The direct effect of INGAP-PP on hepatic cells was definitely confirmed incubating isolated hepatocytes from normal rats in the presence of the peptide. Under these conditions, INGAP-PP increased GK activity together with a reduction in G-6-Pase and PEPCK gene expression.

In this context, INGAP-PP directly affects glucokinase activity, enzyme that is the rate-limiting step of glucose metabolism, thus playing a pivotal role as a glucose sensor both in pancreatic β-cells and hepatocytes [23]. Further, Maiztegui et al. demonstrated that INGAP-PP also directly increases glucokinase activity in cultured pancreatic islets [20] whereas our current data demonstrated a similar effect in liver. In the liver, this activity is conditioned by an increase in the bifunctional enzyme PFK-2 rather than by an increase in enzyme protein content [22]. In islets this effect was associated with an increase in IR, IRS-2 and PI3K protein concentrations together with an increase in tyrosine phosphorylation of these compounds [20]. We have now shown an increase in the P-Akt/Akt ratio, a downstream target of PI3K activation, which suggests involvement of this pathway in the mechanism by which INGAP-PP induces its effects both in islets and in liver. The report that an active Akt pathway could inhibit hepatic gluconeogenesis in both normal and diabetic animals supports our statement [1,14]. However, since P-Akt is a common end-point of different receptor activated pathways, we cannot completely rule out the possibility that INGAP-PP could activate other receptors in the liver as well.

We also found a significant increase in the P-GSK-3β/GSK-3β ratio which implies inhibition of enzyme activity [11]. GSK-3β is a negative modulator of glycogen synthase activity, a rate-limiting enzyme that promotes glycogen deposit [13,39]. Therefore, its inhibition would lead to an increase of the glycogen synthase activity that coincides with our finding of a significant increase in glycogen liver

content. Since Akt phosphorylates both GSK-3 isoforms on the regulatory serine residues, inhibition of GSK-3 could be mediated by Akt [4,11,18]. This pathway may explain the intracellular mechanism by which INGAP-PP exerts its effect on liver function.

Altogether, these data provide additional mechanistic support for the beneficial effect of INGAP-PP on hyperglycemia either for experimental animal models or for people with diabetes [8,12,33,36].

Our results complete the findings reported by Borelli and colleagues [5] who described that although INGAP (mRNA and protein) is present only in pancreatic islets and exocrine tissue of normal hamsters, ^{125}I-tyrosylated INGAP pentadecapeptide (^{125}I-T-INGAP-PP) specifically binds to the liver and small intestine. We have now provided evidence that this liver binding is associated with significant changes in carbohydrate metabolism in the gland.

After the demonstration that a pentadecapeptide with the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduced the stimulatory effect of the complete molecule on thymidine incorporation into duct cells [30], several reports have shown its enhancing effects on pancreatic β-cell mass and function [2-3,6,19-20,38] together with its modulatory effect on hyperglycemia in experimental and clinical diabetes [8,33,36].

Despite all these well characterized cellular effects of INGAP-PP, identification and characterization of its specific receptor has remained elusive. However, since specific *in vivo* binding of this peptide to liver cells is the closest approximation to the existence of this receptor in this gland [5], it is plausible that INGAP-PP dependent metabolic changes assayed in our treated rats may result from its interaction with this receptor. The direct effects recorded in hepatocytes incubated with INGAP-PP, together with a previous report of these kind of effects at pancreatic islet levels lends support to this assumption [20].

In brief, our results demonstrate for the first time that INGAP-PP significant and directly affected glucose metabolism in liver, possibly through the P-Akt signaling pathway. These changes might result

in higher glucose entry into hepatocytes associated with a potential reduction in its production/output, thus contributing to maintain normal glucose homeostasis. This combination of beneficial effects on liver glucose metabolism as well as on β-cell mass and function reinforce the concept that INGAP-PP might become an effective pharmacological tool to treat diabetic patients with dysfunction of both organs.

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Figure Legends

Figure 1. Liver glucokinase (GK), PFK-2, glucose-6-phosphatase (G-6-Pase) and phosphoenolpiyruvate carboxykinase (PEPCK) levels. Liver GK gene expression (A), protein content (B and C) and activity (D); PFK-2 protein content (E and F), G-6-Pase gene expression and protein level (G and H) and PEPCK gene expression (I). C and F: representative blots showing the bands corresponding to GK and PFK-2 protein or β-actin protein in control and INGAP-PP treated rats. (B and E): band intensities in control (white bar) and INGAP-PP treated animals (black bar). Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 2. Glucokinase (GK) activity and glucose-6-phosphatase (G-6-Pase) and phosphoenolpiyruvate carboxykinase (PEPCK) gene expression in isolated hepatocytes. Hepatocytes GK activity (A), G-6-Pase (B) and PEPCK (C) gene expression in control (white bar) and INGAP-PP treated cells (black bar). Results are mean ± SEM of 2 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 3. Liver glycogen content and P-GSK-3β/GSK-3β level. Liver glycogen content (A) in control (white bar) and INGAP-PP treated animals (black bar). B: relative band intensities in both control and INGAP-PP treated animals. C: representative blots showing the bands corresponding to P-

GSK-3β and GSK-3β proteins in control and INGAP-PP treated rats. Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure 4. Liver P-Akt/Akt level. A: P-Akt/Akt relative band intensities in both control and INGAP-PP treated animals. B: representative blots showing the bands corresponding to P-Akt/Akt proteins in control and INGAP-PP treated rats. Results are mean ± SEM of 5 different experiments run in triplicate. *$p < 0.05$ vs. C.

Figure

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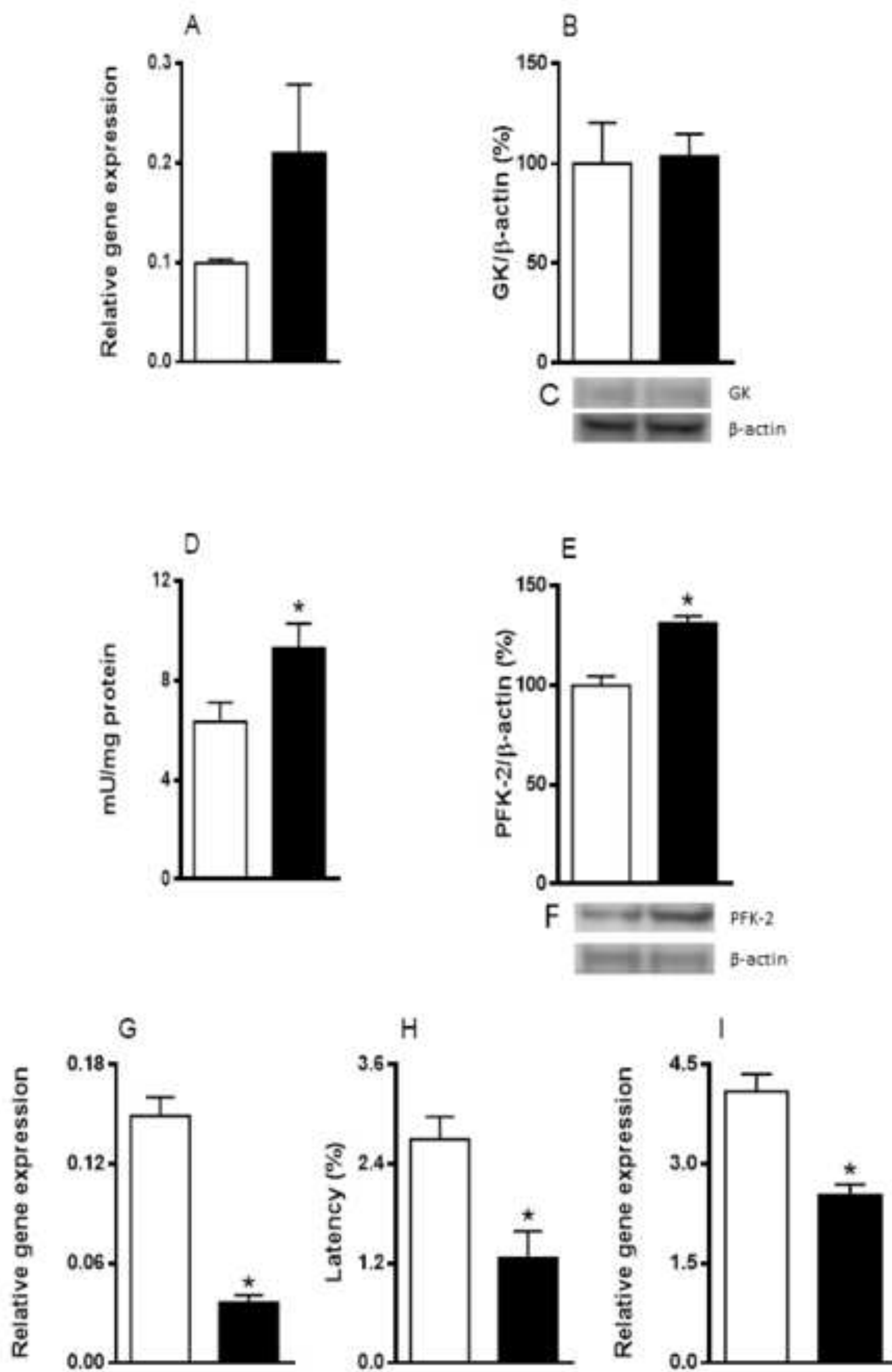

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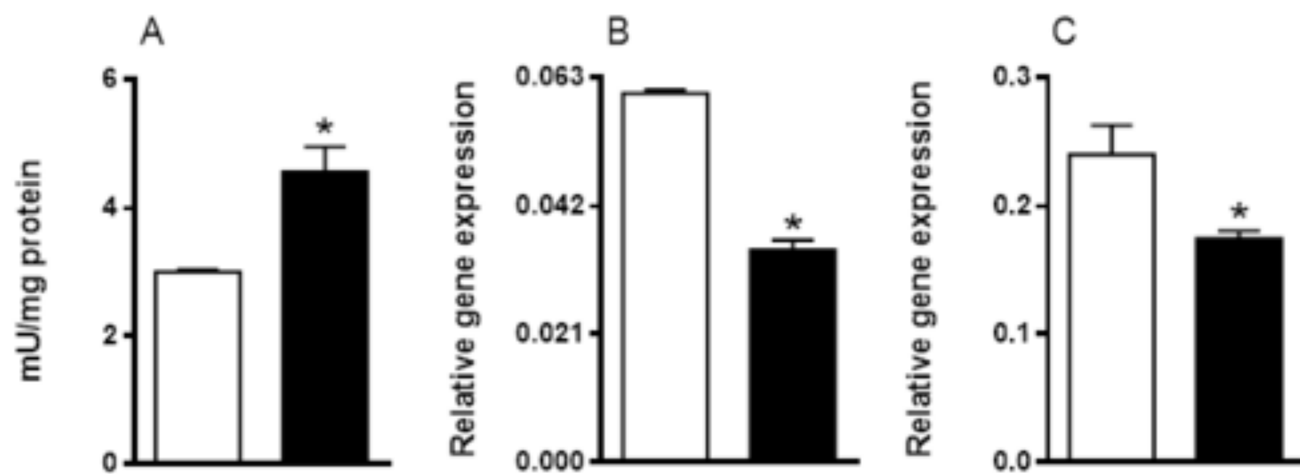

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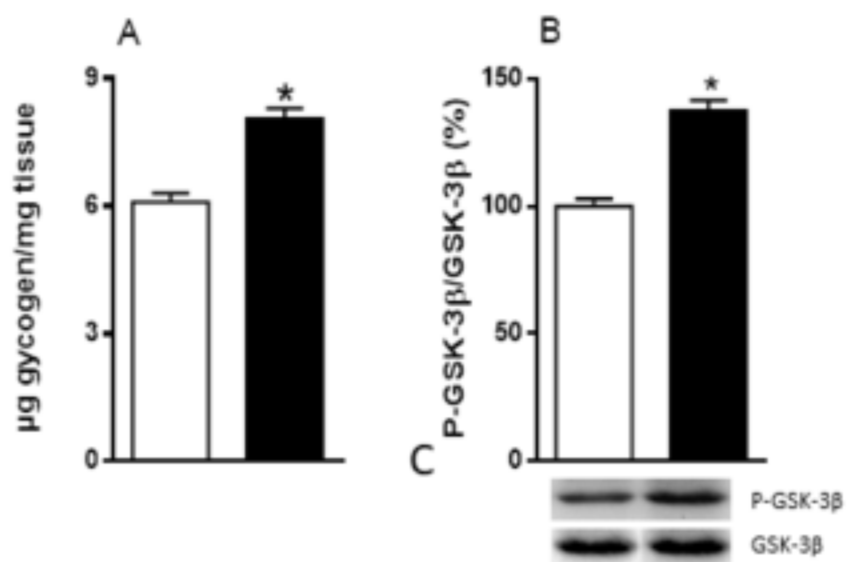

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