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Islet neogenesis-associated protein pentadecapeptide (INGAP-PP): Mechanisms involved in its effect upon β-cell mass and function

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ABSTRACT

The effect of islet neogenesis-associated protein pentadecapeptide (INGAP-PP) administration to normal male hamsters upon serum glucose and triglyceride levels, β-cell mass and function was studied. INGAP-PP (500 μg) or saline was injected twice daily during 10 days. Both groups showed comparable body weight, serum glucose and triglyceride levels. INGAP-PP treated animals had significantly higher HOMA-IR and HOMA-β and their islets released more insulin in response to glucose; they had lower islet DNA content, significantly increased number of islets/unit area, β-cell replication rate and mass, cells co-expressing Pdx-1/INGAP and islets in contact with ducts, and decreased β-cell apoptosis rate. The percentage of cells expressing Pdx-1 alone or together with INGAP or insulin increased significantly in ducts. These animals also showed a significantly higher concentration of Pdx-1 and Ngn-3 mRNA and a lower number of INGAP-positive cells. In conclusion, INGAP-PP promoted a controlled and functionally active increase of β-cell mass; our data demonstrate for the first time the mechanism responsible for such changes; that Ngn-3 would be involved in INGAP-PP-induced neogenesis; and the existence of a negative feedback loop with endogenous INGAP-producing cells. Accordingly, INGAP-PP could be used to induce these effects in people with or at risk of developing diabetes.

1. Introduction

Diabetes mellitus, particularly type 2 diabetes, is a serious and continuously increasing metabolic disorder worldwide [1]. Despite the availability of a large number of new drugs, modern control, treatment devices and evidence-based guidelines, many people with diabetes do not achieve adequate metabolic control to prevent the development and progression of diabetes chronic complications [2], with the consequent increase in treatment costs and decrease in quality of life of people with the disease [3,4].

A key factor in the pathogenesis of type 2 diabetes is the early and progressive decrease of β-cell function and mass [5,6]. Many attempts have been made to reestablish the decreased β-cell mass through pancreas or islet transplantation [7]; however, both approaches are limited by the shortage of available organs and the accompanying need for chronic immunosuppressive therapy. Studies performed with either endogenous adult or exogenous embryonic stem cells have provided a new approach to replacement therapy, showing that these cells can be differentiated into specific cell lineages [8]. Such differentiation can be induced by endogenous or pharmacological compounds either *in vivo* or *in vitro*, providing functional β-cells able to correct the decreased β-cell mass and normalize blood glucose levels in various animal models [9,10].

Among these compounds, a pentadecapeptide having the 104–118 aminoacid sequence of endogenous islet neogenesis-associated protein (INGAP; INGAP-PP) represents an attractive therapeutic alternative. Whereas INGAP-PP could reproduce the stimulatory effect of the intact molecule upon thymidine incorporation into duct cells and a duct cell line [11], administration of pharmacological doses of INGAP-PP to streptozotocin-diabetic mice decreased significantly the percentage of diabetic animals and increased their islet mass [12]. Further, we have demonstrated that INGAP-PP added to the culture media of either neonatal or adult normal rat islets induces the expression of several genes related to β-cell function [13] and increases significantly islet β-cell size and insulin release in response to glucose and aminoacids [14].

In an attempt to deepen the current knowledge of the effect of this peptide upon β-cell mass and its possible mechanism of action, we have currently studied the changes induced by INGAP-PP administration to normal hamsters upon glucose and triglyceride levels as well as on β-cell mass and function.

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2. Materials and methods

2.1. Chemicals and drugs

Collagenase was obtained from Serva Feinbiochemica (Heidelberg, Germany); bovine serum albumin (BSA) fraction V and other reagents were from Sigma Chemical Co (St. Louis, MO, USA). INGAP antibody was kindly provided by Dr. A. Vinik (Strelitz Diabetes Institutes, The Research Institute at Eastern Virginia Medical School Norfolk, USA).

2.2. Pentadecapeptide synthesis

INGAP-PP, a pentadecapeptide with the 104–118 aminoacid sequence of INGAP (NH-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH), and the scrambled peptide (Thr-Ser-Asn-Leu-Ile-Gly-Gly-Asp-Pro-His-Gly-Pro-Leu-Ser-His) – used as negative control to discard any possible general peptide effect upon the animal pancreases – were provided by GenScript (Scotch Plains NJ, USA). Quality control of the peptides (aminoacid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63.

2.3. Experimental groups

Adult male Syrian hamsters (90–110 g bw) were used. They were maintained under controlled conditions of 23 °C and a fixed 12-h light/12-h dark cycle (0600 h–1800 h), with free access to standard commercial food and water. INGAP-PP, the scrambled INGAP-PP (500 μg/0.6 ml) or the same volume of saline solution (C) were *i.p.* injected every 12 h during 10 consecutive days. We included 6 animals per group.

Animal experiments and handling were performed according to the "Ethical principles and guidelines for experimental animals" (3rd ed., 2005) from the Swiss Academy of Medical Sciences (mail@samw.ch).

2.4. Serum glucose, triglyceride and insulin levels

At the time of sacrifice (around 0900 am), blood samples were drawn from non fasted animals under light isoflurane (1-chloro-2,2,2-trifluoroethyldifluoromethyl ether, Abbott Laboratories Company, Bedford, MA, USA) anesthesia from the retroorbital plexus to measure glucose, triglyceride and insulin levels. Glucose levels were measured using Medisense glucometer (Abbott); triglycerides levels were determined with commercial kits (Sea Pack-Bayer) implemented in an automated clinical analyzer [15], and plasma insulin was by radioimmunoassay (RIA) [16] using an antibody against rat insulin, rat insulin standard (Lynco Research Inc., St. Charles, MO, USA) and highly purified porcine insulin labeled with ^{125}I [17].

Insulin resistance was assessed with the HOMA-IR index, calculated as insulin (μU/L) × glucose (mmol/L)/22.5 [18], and β-cell reserve was estimated by HOMA-β, calculated as insulin (μU/L) × 20/glucose (mmol/L) − 3.5 [18].

2.5. Immunohistochemical studies

The whole pancreas was removed and its wet weight recorded. It was then fixed in 10% formaldehyde and embedded in paraffin. Serial sections of fixed pancreas (5 μm) were obtained from three different levels of the blocks with a rotating microtome, and mounted on silanized slides (3-amino-propyltriethoxy-silane; Sigma). Sections were deparaffinized, incubated for 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and rehydrated in a descending ethanol series, followed by an incubation with 2.5% porcine serum to reduce non-specific binding. The slides were then incubated for 24 h at 4 °C in a humidified chamber with different appropriately diluted primary antibodies. For the final staining of all the cell types, we used the streptavidin–biotin complex appropriately diluted (1:40 and 1:20 respectively; Sigma), or alkaline phosphatase (1:40; Sigma). The incubation period for this step-procedure was 30 min with the secondary biotinylated antibodies or alkaline phosphatase [19].

2.6. Cell replication rate

We performed sequential double-staining of sections using these combinations: a) proliferating cell nuclear antigen (PCNA) antibody (Sigma; 1:4000) [20] and β-cells (our own guinea pig-insulin antibody; 1:20,000), b) PCNA and non-β-cells with a mixture of three different rabbit antibodies against somatostatin (DAKO, Glostrup, Denmark; 1:6000), glucagon (Peninsula Laboratories, Belmont, CA, USA, 1:400) and PP (Novo Nordisk A/S, Bagsvaerd, Denmark; 1:10,000), and c) PCNA and INGAP cells with rabbit INGAP antibody (1:600). First, we performed PCNA staining as described above using carbazole as chromogen; the same section was then immunostained for β-, non β- and INGAP-cell identification as described above, except that alkaline phosphatase and fast blue (Sigma) were used as chromogens. Alkaline phosphatase conjugate was applied to each section for 30 min at room temperature; the sections were then washed and alkaline phosphatase substrate was applied for another 30 min. Sections were then washed and mounted in aqueous medium (DAKO). Within a given cell type, the replication rate was quantified and expressed as the percentage of PCNA labeled cells among the total β, non-β and INGAP cells counted (no less than 3000 each). Immunocytochemical staining has been validated in previous studies [21,22]. Further, for every immunostaining, sample controls were done by omitting the primary antibody.

2.7. Islet cell apoptotic rate

We used the propidium iodide technique to identify apoptotic bodies [23]. Deparaffinized and rehydrated pancreas sections were washed in phosphate-buffered saline before incubation for 30 min in a dark humidified chamber with a solution of propidium iodide (4 μg/ml; Sigma) and ribonuclease A (100 μg/ml; Sigma). Thereafter, we used a Zeiss Axiolab epifluorescence microscope equipped with an HBO50 mercury lamp and two different filters to visualize auto-fluorescent apoptotic bodies. Insulin immunofluorescence staining (fluorescein isothiocyanate; FITC) was used to reveal islet cells. Labeled apoptotic endocrine cells in sections obtained from different levels of the blocks were counted under a ×40 objective lens. The number of apoptotic cells was expressed as the percentage of the total number of β-cells counted.

2.8. Cytokeratin (CK) immunostaining

For antigen retrieval, deparaffinized sections were pretreated with 250 ml antigen-retrieval solution (Vector Laboratories, Burlingame, CA, USA) for 10 min in a 500 W microwave oven [24]. To reveal the presence of CK-positive cells, we used a panspecific cocktail of antibodies against human CK clone AE1-AE3 (DAKO) and CK 19 (Sigma clone 4·62).

2.9. Detection of Ngn-3-positive cells

Deparaffinized sections pretreated for antigen retrieval (see above) were incubated for 24 h at 4 °C in a humidified chamber with anti-rabbit Ngn-3 antibody (1:2000, kindly provided by Dr M German, University of California, San Francisco, CA, USA). The final staining was accomplished with the streptavidin–biotin complex (1:40 and 1:20, respectively; Sigma). The incubation period for this step was 30 min with the secondary biotinylated antibodies; cells were revealed using carbazole as chromogen. Sections of normal mouse embryos (gestational age, 15.5 days; E15.5) were used as positive controls.

2.10. Detection of Pdx-1- and INGAP-positive cells

Sequential double-staining was used to identify Pdx-1- and INGAP-positive cells: we first stained Pdx-1 cells with a Pdx-1 specific antibody (kindly provided by Dr C Wright, Department of Cell Biology, Vanderbilt University, Nashville, TN, USA; 1:1200), and revealed positive cells using carbazole as chromogen (see above); the same section was then immunostained with INGAP antibody (1:250), except that alkaline phosphatase and fast blue (Sigma) were used as chromogen. The percentage of cells expressing either one or simultaneously both factors was quantified within each pancreas subsector, *i.e.* islet, extrainsular, and duct cells (no less than 1000 each) [25].

2.11. Morphometric analysis

The morphometric analysis was performed by videomicroscopy with a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera together with the OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA). We measured the following parameters: total pancreatic area excluding connective tissue; exocrine and endocrine pancreatic area; insular, extrainsular and ductal β-cell area and non-β-cell area; number of β- and non-β-cells; number of cells with and without Pdx-1/INGAP co-expression; number of islets per unit area, and single islet area. In each section, we recorded number and area of every islet or small group of endocrine cells. With these data, we defined the composition and frequency of islets and endocrine cell groups. For that purpose, islet areas were previously log-transformed. We also estimated the volume density of β- and non-β-cells, Pdx-1/INGAP and Pdx-1 cells, as well as the individual size of β- and non-β-cells. To estimate islet β-cell, non-β-cell, INGAP/Pdx-1, extrainsular-β cell and ductal-β cell mass, we multiplied the respective volume densities by the weight of the total pancreas [26]. We also measured the relationship between the islets and duct cells, and expressed it as the percentage of the total number of islets in contact with ducts [27].

2.12. Islet isolation

After animal sacrifice, pancreases were removed for the isolation of islets by collagenase digestion [28].

2.13. Quantitative real-time PCR

Total RNA was isolated from pancreatic islets using Trizol reagent (Invitrogen, California, USA) [29]. RNA integrity was checked by agarose–formaldehyde gel electrophoresis; possible contamination with protein or phenol was controlled by measuring the 260/280 nm absorbance ratio [30], while DNA contamination was avoided treating the sample with DNase I (Invitrogen); 1 μg of total RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT. Specific pairs of primers based on hamster cDNA sequences were designed as follows: Pdx-1 (GenBank accession no. U73854) sense primer: 5′aaatccaccaaagctcatgc3′, antisense primer: 5′tgatgtgtctctcggtcagg3′; INGAP (GenBank accession no. GI: 1514683) sense primer 5′aacctgtcctcaaggctctg3′; antisense primer 5′tcagcacattggaactgctc3′ and β-actin (GenBank accession no. VO1217) sense primer, 5′agagggaaattgtgcgtgac3′; antisense primer, 5′ttgccaatggtgatgacctg3′. Since hamster Ngn-3 cDNA sequence is not currently available at the GenBank, a pair of primers was designed based on the mouse Ngn-3 cDNA sequence (GenBank accession no. NM009719 Ngn3 *Mus musculus*) sense primer, 5′cacgaagtgctcagttccaa3′; antisense primer, 5′tctgagtcagtgcccagatg3′. The amplification product was further purified (QIAquick PCR Purification Kit — Qiagen) and sequenced (Megabace 1000, GE Healthcare Life Sciences) showing an 88% of homology with mouse Ngn-3 cDNA sequence. Based on our own hamster Ngn-3 sequence, we designed a new pair of primers to be used in real-time PCR (sense primer, 5′acctcaactcggcactggat3′; antisense primer, 5′gatgtaattgtgggcgaagc3′).

Real-time PCRs were run in triplicate using 1/40th of the cDNA per reaction and 1 μmol/l forward and reverse primers with Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) in the iCycler 5 (BioRad). The cycling profile was as follows: 1 cycle of 2 min at 50 °C (uracil-DNA glycosylase (UDG) activation), 1 cycle of 2 min at 95 °C (DNA denaturation and UDG inactivation), 40 cycles of 15 s at 95 °C and 30 s at 60 °C, 1 cycle of 1 min at 95 °C followed by a melting curve from 55 °C to 90 °C. Quantified values for each gene of interest were normalized against a housekeeping gene (β-actin) using the individual efficiency calculated with a standard curve for each gene. The standard curve was created using a mixture of cDNA samples obtained from islets isolated from various normal hamsters.

2.14. Insulin secretion

Groups of 5 islets isolated from each experimental group were incubated for 60 min at 37 °C in 0.6 ml Krebs–Ringer bicarbonate buffer (KRB), pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), containing 1.0% (w/v) BSA and 3.3, 8 or 16.7 mM glucose. Thereafter, aliquots from the medium were taken and kept frozen for insulin determination by RIA [16].

2.15. Islet DNA content

Islets from each experimental group were homogenized and stored at −70 °C for subsequent measurement of DNA content by the fluorometric assay [31].

2.16. Statistical analysis

Quantitative data are expressed as means ± SEM. The statistical significance was determined by Student's *t*-test. Differences were considered significant when P value <0.05.

3. Results

3.1. Body weight and metabolic parameters

At the end of the study, comparable body weights, serum glucose and triglyceride levels were recorded in INGAP-PP-treated and C hamsters (Table 1).

INGAP-PP treated animals had higher but not significantly different serum insulin levels than those measured in C animals; however, both HOMA-β (47.66 ± 2.89 vs. 28.86 ± 3.40; $P<0.01$) and HOMA-IR (0.97 ± 0.17 vs. 0.51 ± 0.08; $P<0.05$) were significantly higher in INGAP-PP animals (Table 1). These changes were not observed in animals injected with the scrambled INGAP-PP (data not shown).

Table 1
Clinical and metabolic parameters after treatment with INGAP-PP.

	10-day INGAP-PP treatment	
	C	INGAP-PP
Initial body weight (g)	109 ± 7	109 ± 5
Final body weight (g)	114 ± 6	116 ± 6
Pancreas weight (mg)	325 ± 15	320 ± 25
Blood glucose (mg%)	91 ± 4	96 ± 4
Serum triglyceride (mg%)	171 ± 32	204 ± 12
Serum insulin (μU/ml)	2.25 ± 0.30	4.0 ± 0.9
HOMA-β function	28.86 ± 3.40	47.66 ± 2.89**
HOMA-IR	0.51 ± 0.08	0.97 ± 0.17*

Data are means ± SEM; $n = 12$; *$P<0.05$, **$P<0.01$.

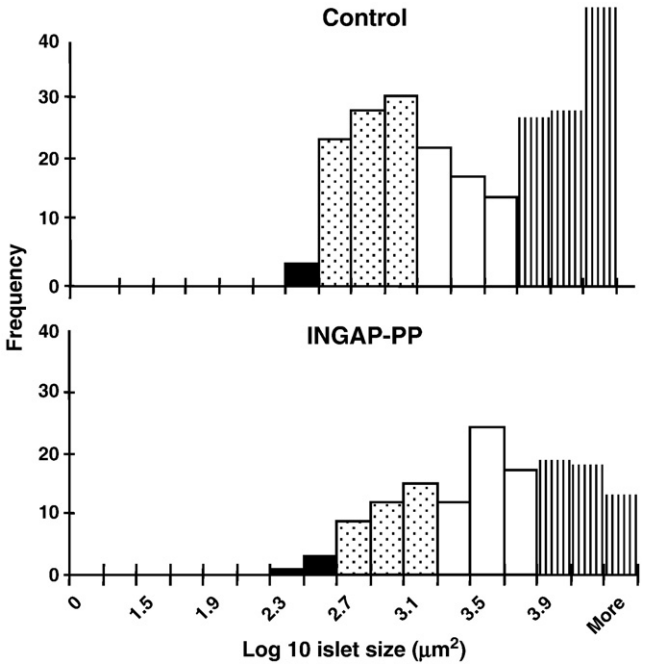


Fig. 1. Frequency distribution of endocrine cell areas in control and INGAP-PP-treated animals. The original data were log-transformed before drawing the histograms (see Materials and methods) ($n=6$ in both groups).

3.2. Morphometrical analysis

Islet size distribution in INGAP-PP treated hamsters is represented in Fig. 1. A marked decrease in the number of large islets can be seen in these animals; consequently, INGAP-PP treated hamsters had a significant decrease in the mean single islet area (68 ± 6.92 vs. $80.83\pm7.85\times10^{-2}$ μ^2) (Fig. 2A and B). They also had an increase in a) the number of islets per unit area (2.8 ± 0.3 vs. 2.1 ± 0.2 islets/mm^2; $P<0.05$) (Fig. 2A and B), b) the number of extrainsular β-cells (2.3 ± 0.5 vs. 1.35 ± 0.3 cells/mm^2; $P<0.05$) (Fig. 2C and D), and c) total β-cell mass (6.6 ± 0.04 vs. 4.1 ± 0.7 mg; $P<0.05$) (Fig. 2A–F).

β-cell replication index was higher (3.0 ± 0.5 vs. 1.7 ± 0.3%; $P<0.05$; Fig. 2G and H), while the number of apoptotic β-cells was significantly lower in INGAP-PP treated animals (0.27 ± 0.03 vs. 0.47 ± 0.05%; $P<0.05$; Fig. 2I and J). These animals also showed a significant increase in a) the mass of Pdx-1 positive cells (2.1 ± 0.22 vs. 1.23 ± 0.26 mg; $P<0.05$), b) the percentage of cells co-expressing Pdx-1/INGAP (13.95 ± 3.45 vs. 5.75 ± 3.25%; $P<0.05$; Fig. 2K and L), and c) the percentage of islets in contact with ducts (77 ± 11 vs. 50 ± 3.3%, $P<0.05$; Fig. 2M and N).

On the other hand, the number of INGAP-positive cells was significantly lower in treated animals (20.33 ± 2.2 vs. 25.5 ± 3.5; $P<0.001$) (Fig. 2K and L); Ngn-3 could not be detected either in treated or in control animals (Fig. 2P) although we were able to detect it in cerebellum from normal hamsters (Fig. 2O).

At the duct cell compartment level, treated animals had a significant increase in the percentage of Pdx-1 positive (3.48 ± 0.30 vs. 1.65 ± 0.60; $P<0.05$) (Fig. 2Q and R) and Pdx-1/INGAP-positive cells (3.35 ± 0.40 vs. 0.75 ± 0.40; $P<0.001$) as well as in the mass of duct cells positively stained with the insulin antibody (0.030 ± 0.008 vs. 0.010 ± 0.004; $P<0.01$) (Fig. 2E and F).

3.3. mRNA level

Pdx-1 mRNA concentration (286.9 ± 29.9 vs. $100.\pm21.3$; $P<0.001$; Fig. 3A) as well as that of Ngn-3 (175.2 ± 12.9 vs. 100.0 ± 2.9; $P<0.01$) (Fig. 3B) was significantly higher in the islets of INGAP-PP treated animals. Conversely, INGAP mRNA concentration was significantly lower in these animals (48.2 ± 4.0 vs. 100.0 ± 8.9; $P<0.01$; Fig. 3C). None of these changes were observed in animals injected with the scrambled INGAP-PP (data not shown).

3.4. DNA content

DNA content was lower in islets isolated from INGAP-PP-treated animals (0.07 ± 0.002 vs. 0.1 ± 0.01 ng/islet; $P<0.05$).

3.5. Insulin secretion

Insulin secretion of islets isolated from both INGAP-PP-treated and C hamsters increased as a function of the glucose concentration in the incubation media (3.3, 8, and 16.7 mM glucose; Fig. 4). No differences were recorded between groups at any glucose concentration tested when insulin secretion was expressed as ng insulin per islet (Fig. 4A). However, a significant increase was observed in INGAP-PP-treated animals in response to 16.7 mM glucose when insulin release was related to islet DNA content ($P<0.05$; Fig. 4B). This difference was not found in animals treated with the scrambled INGAP-PP.

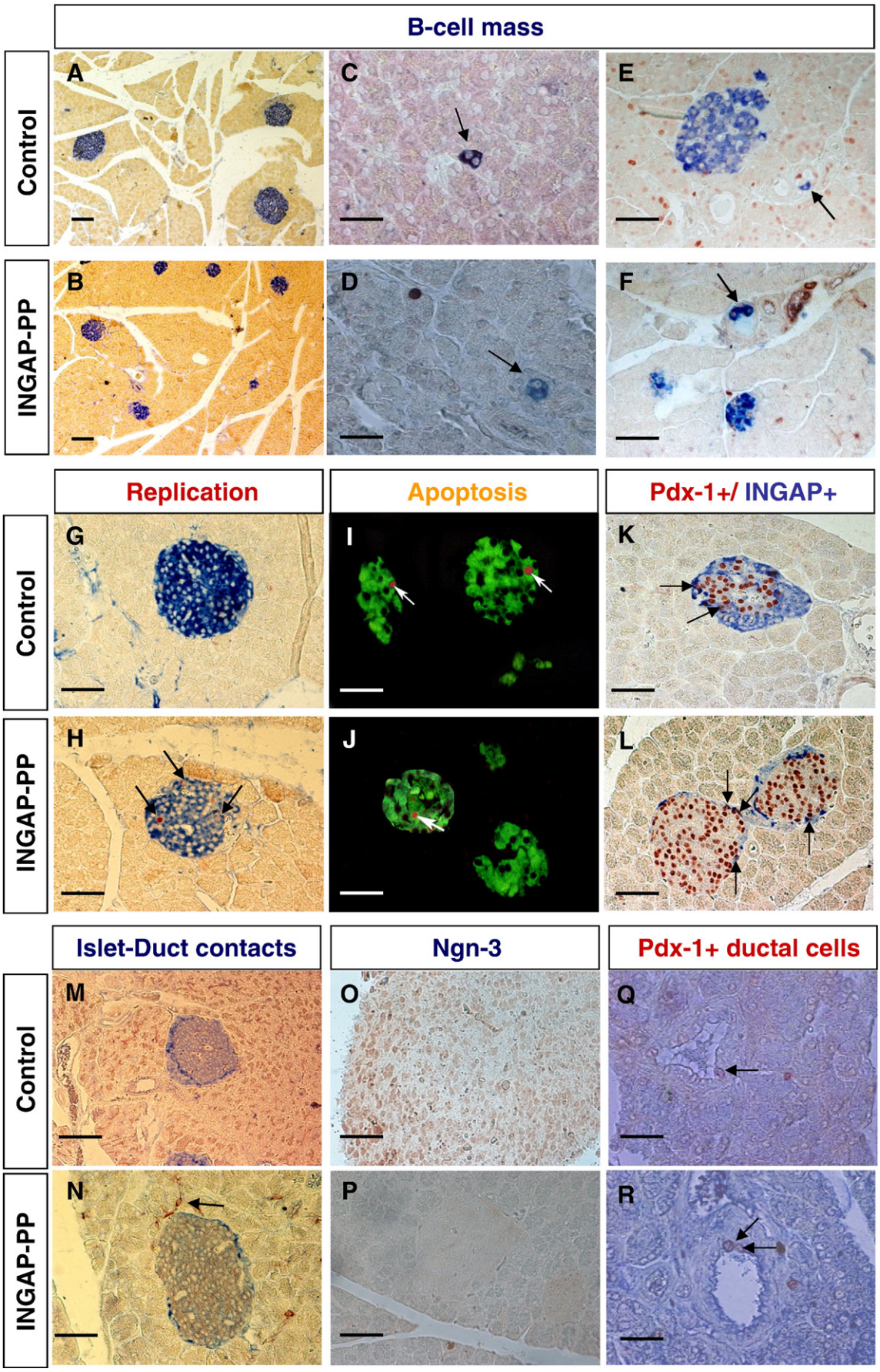
3.6. Insulin content

No differences in insulin content – expressed either per islet or per islet DNA content – were recorded between groups (INGAP-PP vs. C, 82.67 ± 15.51 vs. 99.58 ± 9.96 ng insulin/islet and 1181 ± 236 vs. 995.8 ± 99 ng insulin/ng DNA).

4. Discussion

Our results confirm that daily administration of INGAP-PP to normal hamsters for ten days induced an increase in the number of islets and in β-cell mass [12]. Additionally, we show that these changes occurred without affecting normal serum glucose or triglyceride concentrations, suggesting that the feedback and regulatory mechanism of insulin secretion is similar in both newly formed and mature normal β-cell populations. This would be a specific effect since it is not reproduced using a pentadecapeptide with the same aminoacids as INGAP but in a different sequence (scrambled), which is in agreement with other reports [12,13]. The relationship of peptide structure with its biological effectiveness is supported by the fact that INGAP-PP – but not the scrambled compound – exhibits a stable and predictable structure in solution [32] and by the specific binding of INGAP-PP to pancreatic tissue observed in an *in vivo* model [33].

Fig. 2. A–F Pancreas sections from C (A, C and E) and INGAP-PP-treated (B, D and F) hamsters stained with insulin antibody (blue cytoplasm). Arrows show extrainsular (C and D) and ductal (E and F) insulin-positive cells. G–H Pancreas sections from C (G) and INGAP-PP-treated (H) hamsters stained with insulin (blue cytoplasm) and PCNA (red nucleus) antibodies. Arrows in H show PCNA-/insulin-positive cells. I–J Immunofluorescence of pancreas sections from C (I) and INGAP-PP-treated (J) hamsters stained with insulin antibody (green cytoplasm) and propidium iodide (red nucleus). Arrows in both panels show apoptotic bodies in insulin-positive cells. K–L Pancreas sections from C (K) and INGAP-PP-treated animals (L) stained with INGAP (blue cytoplasm) and Pdx-1 (red nucleus) antibodies. Arrows in both panels show Pdx-1/INGAP co-expressing cells. M–N Pancreas sections from C (M) and INGAP-PP-treated (N) hamsters stained with a mixture of non-β antibodies (glucagon, somatostatin and PP; blue cytoplasm) and CK AE1–AE3 (red cytoplasm) antibody. The arrow in N shows an islet in contact with a duct. O–P Cerebellum section (O) from a one-day postnatal hamster stained with Ngn-3 antibody as positive control. Representative pancreas section (P) from an INGAP-PP-treated hamster stained with Ngn-3 antibody. No positive cells were found in pancreatic sections from either C (data not shown) or treated animals. Q–R Pancreas sections from C (Q) and INGAP-PP-treated (R) hamsters stained with Pdx-1 antibody (red nucleus). Arrows in both panels show Pdx-1 positive ductal cells. A and B, ×100; bar = 100 μm. C-R, ×400; bar = 50 μm ($n=6$ in both groups). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


B-cell mass
Control
INGAP-PP
A
B
C
D
E
F
Replication
Apoptosis
Pdx-1+/ INGAP+
G
H
I
J
K
L
Islet-Duct contacts
Ngn-3
Pdx-1+ ductal cells
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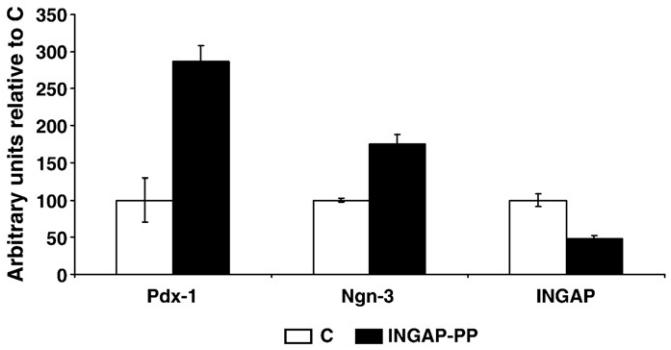

Fig. 3. Comparison of mRNA levels in C and INGAP-PP treated hamsters by quantitative real time PCR. Pdx-1, Ngn-3 and INGAP mRNA levels were measured by real-time PCR in islet cDNA obtained from RNA isolated from C (white bars) and INGAP-PP treated (black bars) hamsters ($n=4$ in both groups). The relative levels of each individual mRNA are referred to the housekeeping gene (β-actin) and their values expressed in arbitrary units relative to C. While in INGAP-PP treated animals there is a significant increase of Pdx-1 and Ngn-3 mRNA levels, INGAP mRNA decreased significantly. *P* between groups <0.01 in all cases.

The current results demonstrate, for the first time, that the increase in β-cell mass induced by INGAP-PP was due to a combination of increased β-cell replication rate and islet neogenesis, with a concomitant decrease in the rate of β-cell apoptosis. This pleiotropic response strongly suggests that appropriate stimuli – in our case INGAP-PP administration – on the pancreas of normal adult hamsters can trigger mechanisms similar to those used for the modulation of β-cell mass during the fetal and newborn periods [34]. The magnitude of the changes caused by each one of these processes was markedly different: islet size decreased by 16%, extrainsular β-cell number increased by 70%, the mass of insulin-immunoreactive duct cells increased by 300%; the percentage of islets in contact with ducts – indicative of β-cell neogenesis – increased by 54%, while β-cell apoptosis decreased by 40%. These facts suggest that all these different mechanisms contributed unevenly to the recorded increase in total β-cell mass. The multiple and synchronized mechanisms triggered by INGAP-PP were similar to those observed in different adult animal models in which pancreatic β-cell mass dynamically reacted to cope with an increased peripheral demand of insulin [29,35,36].

While there is general consensus on the role of β-cell replication and apoptosis as regulators of β-cell mass in adult animals, the role of neogenesis has been seriously questioned [37]. Supporting the occurrence of islet neogenesis in adult animals, we have previously reported a significant increase in β-cell mass, with a parallel increase of several indirect neogenesis markers in newborn hamsters from mothers with sucrose-induced insulin resistance [29]. In these animals we have also recorded an increase of a cell subpopulation co-expressing Pdx-1/INGAP with a high replication rate and without cytoimmunoreactivity for any of the islet hormones. An increase in the number of these cells was also measured in INGAP-PP treated animals. Since it has been considered that cells without measurable insulin but expressing Pdx-1 have reassumed their developmental programs [29], we postulated that Pdx-1/INGAP cells were early precursors of islet cells. Such data would indicate the existence of progenitor cells in adult hamster pancreases which may differentiate into endocrine cells in response to an appropriate stimulus. Reported evidence suggests that the duct cell compartment is the source of such progenitor cells [38,39].

Using a different approach and several modern cell biology techniques, it has recently been shown that islet neogenesis occurred in adult animals submitted to a pancreatic injury (duct ligation) and that Ngn-3 transcription and expression increased under this condition. The authors therefore postulated that Ngn-3 had a key role in this process, becoming a useful neogenesis marker [40]. This concept was challenged by the suggestion that being INGAP a neogenesis stimulator [41], it might be worth testing whether it induced changes in Ngn-3 transcription/expression [41]. In the current study, several indirect markers of neogenesis were significantly modified in the pancreas of INGAP-PP treated animals, namely, islets of smaller size [42], increased percentage of islets in close contact with ducts [31], of duct cell replication rate [43], of Pdx-1-positive cells [44], and of the mass of insulin-positive cells at the duct level [45]. We also found a significant increase in Pdx-1 and Ngn-3 mRNA. The apparent discrepancy between Ngn-3 mRNA (increased) and protein levels (immunoreactively negative) could be the result of a low Ngn-3 protein expression, a low sensitivity of our Ngn-3 antibody, or a combination of both factors. Therefore, our results provide a positive answer to the question of the possible role of Ngn-3 in INGAP-induced β-cell neogenesis, and confirm that in adult animals, this process can be triggered by an appropriate stimulus.

The decreased INGAP-positive cell number and mRNA level recorded in INGAP-treated hamsters would represent a negative feedback mechanism between INGAP-PP- and INGAP-producing cells. The increased Pdx-1 transcription/expression observed in these animals might participate in the mechanism responsible for such inhibitory feedback [46]. This fact would imply that: a) INGAP-producing cells are permanently active, having a potential self-regulated function, and b) the effect of INGAP-PP would be identical to that of the intact protein not only upon thymidine incorporation [11] but also upon INGAP production and on other pancreatic cell functions as well. Possibly, the negative feedback of INGAP-PP mentioned above would be part of a control homeostatic mechanism for islet mass expansion, preventing the development of an abnormal neoplastic reaction [47,48].

Opposed to that reported by Rosenberg et al. using a similar design and animal model [12], we found no significant differences in islet insulin content between control and INGAP-PP-treated animals. We additionally recorded a significant increase in β-cell secretory function both *in vivo* (higher HOMA-β index) and *in vitro* (higher release of insulin in response to 16.6 mM glucose) in INGAP-PP treated animals. Such increased secretory response may be ascribed to the enhancing effect of INGAP-PP upon the expression of islet genes involved in the mechanism of insulin release [13,49]. The recorded increased HOMA-IR value probably reflects an adaptative negative feedback between insulin and its receptors (down-regulation) which contributes to maintain glucose homeostasis and prevent hypoglycemia. Although not significantly, serum insulin levels were higher in INGAP-PP-treated animals. Therefore, we might assume that such difference was sufficient to affect insulin-receptor feedback.

In brief, we have demonstrated that INGAP-PP administration to normal adult hamsters for ten days induced an increase in β-cell mass. The results provide novel evidence on the production mechanism by which INGAP-PP causes its effect, namely: 1) the increase in β-cell mass

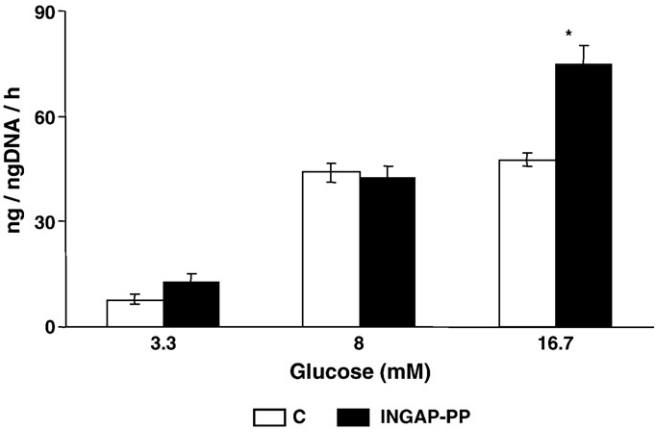

Fig. 4. Insulin release of isolated islets from C and INGAP-PP-treated hamsters in response to glucose. Bars represent means ± SEM of C (white) and INGAP-PP treated (black) animals from 3 independent experiments. Values are expressed as ng/ng DNA/h. *P* values *<0.05 ($n=4$ in both groups).

is ascribed to a complex mechanism of increased replication of preexisting cells and differentiation from progenitor cells probably present in duct epithelium [38–40], together with a significant decrease in the rate of β-cell apoptosis; 2) Ngn-3 would play a role in the INGAP-PP-induced neogenesis developed in adult animals; 3) INGAP would exert a sustained tonic effect upon β-cell mass controlled by a feedback mechanism; 4) the changes in β-cell mass were accompanied by an enhanced release of insulin in response to high glucose both *in vivo* and *in vitro*. The ability of INGAP-PP to promote a controlled and functionally active increase of islet mass represents a potential novel approach for the treatment of diabetes. In fact, its effects could be used to a) induce the differentiation of progenitor cells *in vivo* (regenerative medicine) or *in vitro*, b) enhance the insulin secretory response of islets before their transplant, c) provide a source of new β-cells obtained from xenogenic, allogenic and fetal tissues for further transplant in patients with diabetes, and d) decrease the β-cell apoptotic rate reported in people with impaired fasting glucose and type 2 diabetes [5,6]. The fact that our results were achieved using small amounts of the peptide would increase the potential therapeutic use of INGAP-PP or its analogues in the prevention and treatment of diabetes.

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