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INGAP-PP effects on β-cell mass and function are related to its positive effect on islet angiogenesis and VEGFA production

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ABSTRACT

Our aim was to determine whether islet angiogenesis and VEGFA production/release participate in the mechanism by which INGAP-PP enhances β-cell function and mass. We used two models: a) *in vivo* (normal rats injected with INGAP-PP for 10 days) and b) *in vitro* (normal islets cultured for 4 days with INGAP-PP, VEGFA, Rapamycin, and the specific VEGF-Receptor inhibitor, SU5416). INGAP-PP administration enhanced insulin secretion, β-cell mass, islet vascularization, and angiogenesis without affecting glucose homeostasis. Normal islets cultured with INGAP-PP and VEGFA increased insulin and VEGFA secretion while apoptosis decreased. INGAP-PP-induced effects were prevented by both Rapamycin and SU5416. INGAP-PP effects on β-cell mass and function were significantly associated with a positive effect on islet angiogenesis and VEGFA production/release. VEGF-A possibly potentiates INGAP-PP effect through mTORC pathway.

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1. Introduction

Although in recent years many new prevention and treatment options have been developed for type 2 diabetes (T2D), its individual and social burden continues to grow (Kahn and Buse, 2015). Therefore, several studies are underway in order to identify new agents or approaches able to solve this problem. INGAP (islet neogenesis associated protein), a peptide produced by pancreatic exocrine-, duct- and islet-non-β-cells from normal hamsters might be one of them (Flores et al., 2003). A pentadecapeptide with its 104–118 amino acid sequence (INGAP-PP) reproduces most of the pleiotropic effects of the intact molecule (Borelli et al., 2005; Barbosa et al., 2006; Silva et al., 2008; Madrid et al., 2009; Kapur et al., 2012; Maiztegui et al., 2015; Chang et al., 2011), and has been used as a key component of an *in vitro* β-cell differentiation protocol (Xu et al., 2011). INGAP-PP enhances glucose stimulated insulin secretion (GSIS) and β-cell mass by increasing β-cell glucose metabolism, β-cell replication/neogenesis and reducing its apoptosis rate (Borelli et al., 2005; Barbosa et al., 2006; Silva et al., 2008; Madrid et al., 2009; Kapur et al., 2012; Maiztegui et al., 2015; Chang et al., 2011; Rosenberg et al., 2004). Since these mechanisms are impaired in T2D, this small peptide might represent an attractive therapeutic alternative (Weyer et al., 1999; Butler et al., 2003; Kahn, 2003). In fact, a short preliminary clinical trial in which INGAP-PP was administered to people with type 1 and T2D yielded promising results (Dungan et al., 2009).

Although the participation of the above mentioned impairment of β-cell mass and function in T2D pathogenesis is widely accepted, other factors also contribute to this process. Consequently, the physical and functional relationship between endothelial and β-cells affects their function and proliferation through the production of several angiogenic and growth factors (Nikolova et al., 2006; Zanone et al., 2008). VEGFA is the main angiogenic factor expressed in β-cells, and is necessary for correct pancreatic growth and development of islet-specific capillary fenestrations (Inoue et al., 2002; Lammert et al., 2003; Brissova et al., 2006; Iwashita et al., 2007); it is also a key modulator of β-cell mass: a continuous provision of exogenous VEGFA in a hypoglycemic state prevents β-cell mass reduction (Xiao et al., 2013). Therefore, β-cell

Abbreviations: T2D, Type 2 diabetes; INGAP, islet neogenesis associated protein; GSIS, glucose stimulated insulin secretion; GTT, glucose tolerance test; AUC, area under the curve; TBARS, Thiobarbituric acid reactive substances; IR, insulin resistance; Vvi, volume density.

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mass could be regulated through a controlled release of VEGFA from β-cells depending on physiological needs (Xiao et al., 2013; Reinert et al., 2013).

In this context we assume that it could be important to test the potential participation of islet VEGFA in the modulatory effect of INGAP-PP on β-cell function and mass. For this purpose we studied the effect of this angiogenic factor on insulin secretion and β-cell apoptosis by two different experimental approaches: one *in vivo* in which normal rats were injected with INGAP-PP for ten days and other i*n vitro* in which islets isolated from normal rats were cultured with or without INGAP-PP.

2. Material and methods

2.1. Animal treatment

40 adult male Wistar rats (230–260 gbw) were kept under controlled conditions (23 °C and a fixed 12-hour light-dark cycle), with free access to a standard commercial diet and water. Animals were injected every 12 h (at 8:00 am and pm) for 10 consecutive days with INGAP-PP (500 μg/400 μl/day/intraperitoneally; group I-PP; n = 20) or the same volume of saline (group C; n = 20). Water and food intake were measured daily, while individual body weight was recorded once a week. Experiments were performed according to the "Ethical Principles and Guidelines for Experimental Animals" (3rd. Edition, 2005) of the Swiss Academy of Medical Sciences (www.aaalac.org).

2.2. Glucose tolerance test

Glucose tolerance test (GTT) was performed in 12-h fasting rats from each experimental group. Glucose (1.1 g/kg of body weight in saline solution) was injected intraperitoneally and blood samples were obtained from the retro-orbital plexus under ketamine (80 mg/kg body weight) and midazolam (5 mg/kg body weight) anesthesia, 0, 15, 30, 60 and 120 min following the glucose load. In these samples, glucose concentration was measured with test strips (Accu-Chek Performa, Roche, Manheim, Germany). Results were expressed as the area under the glucose curve (AUC).

2.3. Plasma measurements

At the end of the treatment period, blood samples from non-fasting animals in each experimental group were collected from the retro-orbital plexus under light halothane anesthesia at 9:00 am to measure plasma glucose, triacylglycerol, lipid peroxidation (thiobarbituric acid reactive substances [TBARS]) and insulin levels.

Glucose was measured as described above and the triacylglycerol level was determined with commercial kits (Bio-Systems S.A., Buenos Aires, Argentina) implemented in an automated clinical analyzer.

TBARS were determined by fluorimetric assay and results expressed as pmol of malondialdehyde (MDA)/mg of plasma protein (Bio-Rad Protein Assay kit, Bio-Rad Lab, RC, USA).

Plasma insulin was measured by radioimmunoassay (RIA) (Herbert et al., 1965) using a specific antibody against rat insulin (Sigma Chemical Co.), rat insulin standard (Novo Nordisk Pharma Argentina), and highly purified porcine insulin labeled with ^{125}I (Linde et al., 1983).

Insulin resistance (IR) was determined by homeostasis model assessment-IR (HOMA-IR), applying the formula [serum insulin (μU/mL) × fasting blood glucose (mM)]/22.5.

β-cell function was quantified by HOMA-β [serum insulin (μU/mL) × 20/glucose (mM) − 3.5] (Matthews et al., 1985). Since no validated cut off values exist for HOMA-IR/HOMA-β in rats, we compared control values to the I-PP group.

2.4. Immunohistochemical studies

The whole pancreas from three animals of each experimental group was carefully dissected and removed at the time of euthanasia; thereafter, a piece of the tail of each pancreas was fixed in 10% formaldehyde and embedded in paraffin. Serial sections of each of the fixed pancreases (5 μm each) were obtained from three different depths of the blocks with a rotatory microtome and mounted on silanized slides (3-amino-propyltriethoxy-silane; Sigma). Sections were deparaffinized, incubated for 30 min in 3% (v/v) hydrogen peroxide in methanol to block the endogenous peroxidase activity, and rehydrated in a descending ethanol series, followed by incubation in 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 our own guinea pig anti-insulin antibody (1:20,000 dilution). Thereafter, they were incubated for 30 min with a goat anti-guinea pig secondary antibody (1:30 dilution; Santa Cruz Biotechnology Inc sc-2440). Insulin staining was performed by incubating the slides for 30 min with streptavidin alkaline phosphatase conjugate (1:60 dilution; Sigma).

For CD34 immunostaining, the slides were incubated for 24 h at 4 °C in a humidified chamber with mouse monoclonal antibody CD34 (QBEnd/10, Leica Biosystems Newcastle); and then, they were incubated for 30 min with anti mouse secondary antibody (1:30 dilution). Carbazole (Sigma) was used as chromogen for CD34 immunostaining, (1:60 dilution). Thereafter, all sections were stained with haematoxylin.

2.5. Cell replication

Sequential double-staining of sections was performed using a combination of PCNA (proliferating cell nuclear antigen) antibody (1:4000 dilution; Sigma); (Connolly and Bogdanffy, 1993), and our own guinea pig anti-insulin antibody (1:20,000 dilution). Thereafter, they were incubated for 30 min with an anti mouse secondary antibody (1:30 dilution, Sigma) or goat anti-guinea pig secondary antibody, respectively (1:30 dilution). Carbazole and alkaline phosphatase plus Fast Blue (Sigma) were used as chromogens for PCNA and insulin immunostaining, respectively (1:60 dilution). Within a given cell type, the replication rate was quantified and expressed as the percentage of PCNA-labeled cells of total β-cells counted (no less than 3000 cells of each type). This immunocytochemical staining was previously validated in our laboratory (Del Zotto et al., 2000). For every immunostaining performed, simple controls were run simultaneously by omitting the primary antibody.

2.6. Morphometric analysis

Morphometric analysis was done by videomicroscopy on a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera, together with OPTIMAS software (Bioscan). With these tools we measured: total pancreatic area (excluding connective tissue), insulin-positive cells, islet β-cell area [Vvi (volume density)], and extrainsular β-cell area: clusters of 6 or less β cells which cannot be considered islets since they are not associated to glucagon, somatostatin, or PP cells (Bouwens and Pipeleers, 1998).

2.7. Islet DNA content

At the time of euthanasia, the whole pancreas from each animal was removed to isolate islets by collagenase digestion (Lacy and Kostianovsky, 1967). Thereafter, 150 islets from each experimental

condition were hand-picked and randomly divided into 15 groups of ten islets. They were homogenized in 100 μl buffer TNE (Tris 10 mM, EDTA 1 mM, NaCl 2 M), and islet DNA content was measured by its absorbance at 260 nm in the Multi-mode Microplate Reader-Synergy HT (Biotek Instruments, Inc) with Gen5 software.

Islet pick-up was performed by a "blind operator" who did not know which experimental group the islets belonged.

2.8. β cell apoptosis

Apoptosis rate was determined by a colorimetric kit APO-PercentageTM (Biocolor) (Wang et al., 2011). Briefly, after collagenase digestion of the pancreas (Lacy and Kostianovsky, 1967), groups of 250 freshly isolated islets hand-picked from each experimental group were homogenized following manufacturer's instructions. Absorbance at 550 nm of each sample was registered in a Multi-mode Microplate Reader-Synergy HT with Gen5 software (Biotek Instruments, INC).

2.9. Quantitative real-time PCR

Total RNA was obtained from isolated islets using an Rneasy mini kit (Qiagen), and its integrity was tested by agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was controlled by measuring the 260:280 nm absorbance ratio, while DNA contamination was avoided by 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. Real-time PCRs were run in triplicate using FastStart SYBR Green Master (Roche) in the iCycler 5 (BioRad). The cycling profile used was: 1 cycle of 1 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C followed by a melting curve from 55 °C to 90 °C. Quantified values were normalized against the housekeeping gene β *actin*, using the individual efficiency calculated with a standard curve for each gene.

Specific pairs of primers based on rat cDNA sequences were designed as follows:

Insulin (NM_019130) forward primer 5′-TGTGGTTCTCACTTGG TGGA-3′, reverse primer 5′-CAGTGCCAAGGTCTGAAGGT-3′;
VegfA (NM_031836.2) forward primer 5′-GCTTTACTGCTG-TACCTCCAC-3′, reverse primer 5′-GTATATCTTCAAGCCGTCCTG-3′;
Ang1 (NM_053546) forward primer 5′-ACGGGGACAGCAGGCA AACAG-3′, reverse primer 5′-CACAGGCATCAAACCACCAACC-3′;
Ang2 (BC161931) forward primer 5′-CCTGTCCGGCGAG-GAGTCCA-3′, reverse primer 5′- GCGTCGAACCACCAGCCTCC-3′;
Integrin β1 (NM_017022.2) forward primer 5′-GAGAGA-GATTACTTCAGAC-3′, reverse primer 5′-AGCAGTCGTGTTA-CATTC-3′;
Bcl2 (L14680) forward primer 5′-CGGGAGAACAGGGTATGA-3′, reverse primer 5′-CAGGCTGGAAGGAGAAGAT-3′;
Caspase3 (NM_012922.2) forward primer 5′-CAAGTCGATG-GACTCTGGAA-3′, reverse primer 5′-GTACCATTGCGAGCTGACAT-3′;
CK19 (NM_199498.1) forward primer 5′-AGTAACGTGCGTGCT-GACAC-3′, reverse primer 5′-ACCTTGCTACCAGTGCGACT-3′;
Ngn3 (NM_021700.1) forward primer 5′-GGCGCCTCATCCCTTG-GATG-3′, reverse primer 5′-CAGTCACCCACTTCTGCTTCG-3′;
β *Actin* (NM_031144.3) forward primer, 5′-AGAGGGAAA TCGTGCGTGAC-3′; reverse primer, 5′-CGATAGTGATGACCTG ACCGT-3′.

2.10. Western blotting

Islets were homogenized in 80 mM Tris (pH 6.8), 5 mM EDTA, 5% SDS, 5% dithiothreitol, 10% glycerol, and protease inhibitors (1 mM phenyl-methylsulfonyl-fluoride and 4 mg aprotinin). Samples were then fractionated under reducing conditions by SDS/PAGE and electroblotted to polyvinylidene difluoride transfer membrane (Amersham Hybond-P, GE Healthcare, UK). The amount of protein loaded onto the gel was quantified by Bio-Rad protein assay. Nonspecific binding sites were blocked with non-fat milk solution at 4° C for 90 min for Caspase3, BCL2, Integrin β1 and VEGFA, and overnight for β Actin. The membranes were then incubated with specific antibodies against BCL2 (rabbit polyclonal, Santa Cruz Biotechnology Inc sc-492; 1:2000 dilution), active Caspase3 (rabbit Sigma Aldrich C8487; 1:1000 dilution), Integrin β1 (rabbit polyclonal, Santa Cruz Biotechnology Inc sc-8978; 1:2000 dilution) and VEGFA (rabbit polyclonal, Santa Cruz Biotechnology Inc sc-152; 1:2000) at 4 °C overnight, or for 90 min with antibody against β Actin (mouse monoclonal, Sigma Aldrich; 1:10,000 dilution). After rinsing with T-TBS, the blots were incubated with anti-rabbit IgG-HRP for 1 h at room temperature. For β Actin, horseradish-peroxidase-conjugated anti-mouse IgG-HRP was used as secondary antibody. Proteins were revealed by an enhanced chemiluminescence detection system (ECL Prime, Amersham, GE Healthcare, UK). Finally, the bands were quantified by Image Studio Digits 22 3.1 software.

2.11. Glucose-stimulated insulin secretion (GSIS)

10 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, 8.3 or 16.7 mM glucose. Thereafter, aliquots from the medium were taken and kept frozen for insulin determination by RIA (Linde et al., 1983) as described above. Insulin released to the incubation medium was expressed as ng of insulin/μg of islet DNA/hour in order to avoid bias due to individual islet size.

2.12. Insulin content

After pancreas digestion by collagenase (Lacy and Kostianovsky, 1967), 120 islets isolated from each experimental condition were hand-picked and randomly divided into groups of ten islets. They were homogenized by ultrasound in 200 μl water and kept frozen for insulin determination by RIA (Linde et al., 1983).

Islet pick-up was performed by a "blind operator" who did not know to which experimental group the islets belonged.

2.13. Islet culture

Pancreatic islets were isolated from 18 normal rats and cultured in RPMI-1640 medium (Microvet SRL, Argentina), pH 7.4 containing 2 g/L $NaHCO_3$, 5% (v/v) fetal bovine serum, 1% penicillin/streptomycin, and 10 mM glucose at 37 °C in a humid atmosphere (5% CO_2/95% O_2). Islets were cultured for 4 days in the absence (control C) or presence of 10 μg/ml INGAP-PP (I-PP), 10 ng/ml VEGFA (V), 10 ng/ml Rapamycin (R) as mTORC inhibitor, 10 μM SU5416 (Semaxanib ab145056) as selective VEGFR2 inhibitor (SU) (Kim et al., 2016), and the combination of INGAP-PP + Rapamycin (I-PP+R), INGAP-PP + Rapamycin + VEGFA (I-PP+R+V), INGAP-PP + SU5416 (I-PP+SU) and VEGFA + SU5416 (V+SU). Since 0.1% DMSO was used as Rapamycin and SU5416 vehicle, the same amount of DMSO was added to the medium of C, I-PP and V groups. Medium was renewed every 2 days. After culture, islets were pre-incubated in KRB buffer,

Table 1
Plasma parameters and glucose tolerance test.

	Glucose	Insulin	TG	TBARs	HOMA-IR	HOMA-β	GTT (AUC)
C	5.94 ± 0.14	14.25 ± 2.00	122.6 ± 8.8	50.9 ± 6.8	4.01 ± 0.50	52.4 ± 6.2	2898 ± 649
I	6.28 ± 0.16	15.75 ± 1.75	115.4 ± 7.9	47.8 ± 5.2	4.06 ± 0.60	41.7 ± 7.1	2854 ± 525

Values represent the mean ± SEM of serum concentrations of glucose (mg/dL), insulin (μU/mL), triacylglycerol (mg/dL), and TBARS (pmol/mg prot) from five independent experiments with 4 animals each. None of the parameters determined in I-PP animals showed significant differences against those in C. Area under the glucose curve (AUC) during glucose tolerance test (GTT) is expressed as mM glucose/120 min.

pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), containing 1% (w/v) BSA and 3.3 mM glucose at 37 °C for 45 min.

Quantitative real-time PCR, western blotting, DNA content, and GSIS assays were run on cultured islets as described above (See sections 2.7; 2.9; 2.10 and 2.11).

2.14. VEGFA release

At the end of the culture period, aliquots of medium were collected for quantitative VEGF measurement using a VEGF ELISA kit (RayBio, Inc) following the manufacturer's instructions. Reaction was quantified by measuring absorbance at 450 nm in a Multimode microplate reader (BioTek, Inc.).

2.15. Statistical data analysis

Experimental data were statistically analyzed using SPSS program (15.0 version, SPSS, Inc, 25 Chicago, IL); ANOVA was applied for independent samples with normal distribution, followed by Tukey or Tamhane test for similar variance samples. Results are expressed as mean ± SEM. Differences between groups were considered significant when p values were <0.05.

3. Results

3.1. In vivo model (normal rats treated with INGAP-PP)

3.1.1. Body weight, food intake and plasma parameters

Food intake was comparable in INGAP-PP (I-PP group) and control rats (C group); C: 19.8 ± 0.4 *vs.* I-PP: 20.6 ± 0.4 g/rat/day. Consequently, both experimental groups showed a similar body weight gain (Δ g/animal: 32.3 ± 3.4 *vs.* 36.1 ± 4.0, respectively).

INGAP-PP administration did not significantly modify any of the plasma parameters measured (glucose, insulin, triacylglycerol and TBARS); therefore, HOMA-IR and HOMA-β values were comparable in both experimental groups (Table 1).

3.1.2. Glucose tolerance test

Plasma glucose values measured at 0, 15, 30, 60, and 120 min after glucose load were comparable in both experimental groups (C and I-PP) and consequently, no significant differences were found between their areas under the curve (AUC; Table 1).

3.1.3. Pancreas morphometric analysis

INGAP-PP administration significantly increased the number of pancreatic islets per area unit ($p < 0.05$; Fig. 1A-C). Mean islet area recorded in I-PP rat pancreas was lower - though not significantly - than in C rats (C: 8235.65 ± 1212.37 *vs.* I-PP: 5718.29 ± 1027.53 μm^2). Additionally, the islet size distribution (represented in Fig. 1E), showed that the number of small islets (those $\leq$5000.00 μm^2) was significantly higher in I-PP (75.51%) than in C rats (45.23%; $p < 0.05$).

INGAP-PP also increased β-cell volume density (Vvi) at both insular and extrainsular level ($p < 0.05$; Fig. 1F-G). Conversely, INGAP-PP administration modified neither insular α-cell Vvi nor the individual size of β- and α-cells (data not shown).

3.1.4. Islet DNA content

Islets isolated from I-PP rats showed a significantly lower DNA content than in C animals ($p < 0.05$), demonstrating that I-PP islets presented a lower number of cells than those in C (Fig. 1D).

3.1.5. Replication and neogenesis

β-cell replication rate (PCNA immunolabeling, Fig. 2A-C), was significantly higher in I-PP than in C rats ($p < 0.05$). PCNA-stained nuclei were found in islets of different sizes from both experimental groups, suggesting that there would be no direct relationship between the size of the islets and the presence of dividing cells (data not shown).

Islet neogenesis was also increased in I-PP animals: in addition to the increase of extrainsular β-cell Vvi shown in Fig. 1G, we also detected single insulin-positive cells among duct-cells (Fig. 2D) and several islets in close contact with ducts (Fig. 4C) and budding from them. mRNA levels of classical neogenesis markers as citokeratin 19 (CK19) (Bouwens et al., 1994) and Ngn3 (Xu et al., 2008) were also significantly increased ($p < 0.05$) in islets isolated from INGAP-PP treated rats (Fig. 2E-F).

3.1.6. Apoptosis

Islets isolated from I-PP rats showed a significantly lower apoptotic rate (Fig. 2G) than those isolated from C. Concomitantly, I-PP animals showed a significant increase in mRNA and protein levels of Bcl-2 (anti-apoptotic gene; Fig. 2H and I). Concordantly, INGAP-PP induced a significant decrease in both mRNA (Fig. 2J) and protein levels of the active form of Caspase3 (cleaved protein; Fig. 2K).

3.1.7. Insulin mRNA levels and GSIS

Islets isolated from I-PP rats showed a significant increase of insulin mRNA level ($p < 0.05$; Fig. 3A). They also released a higher amount of insulin in response to 3.3, 8.3 and 16.7 mM glucose than those isolated from C (Fig. 3B).

3.1.8. Islet insulin content

Insulin content of islets isolated from I-PP rats was not significantly different from that measured in islets isolated from C (4.30 ± 0.43 *vs.* 4.27 ± 0.47 ng insulin/islet, respectively).

3.1.9. Angiogenesis

INGAP-PP administration induced a significant increase of insular angiogenesis ($p < 0.05$) as determined by CD34 (Fina et al., 1990) immunostaining (Fig. 4A-C). Islets from I-PP rats showed a significantly larger CD34-positive area than those from C rats (Fig. 4D). mRNA levels of pro-angiogenic markers (*VegfA, Ang1* and *Ang2*) were also significantly ($p < 0.05$) higher in islets from INGAP-PP treated animals than in those from C rats (Fig. 4E). However, the increment recorded of VEGFA protein level in islets from INGAP-PP treated rats was not statistically significant (Fig. 4F).

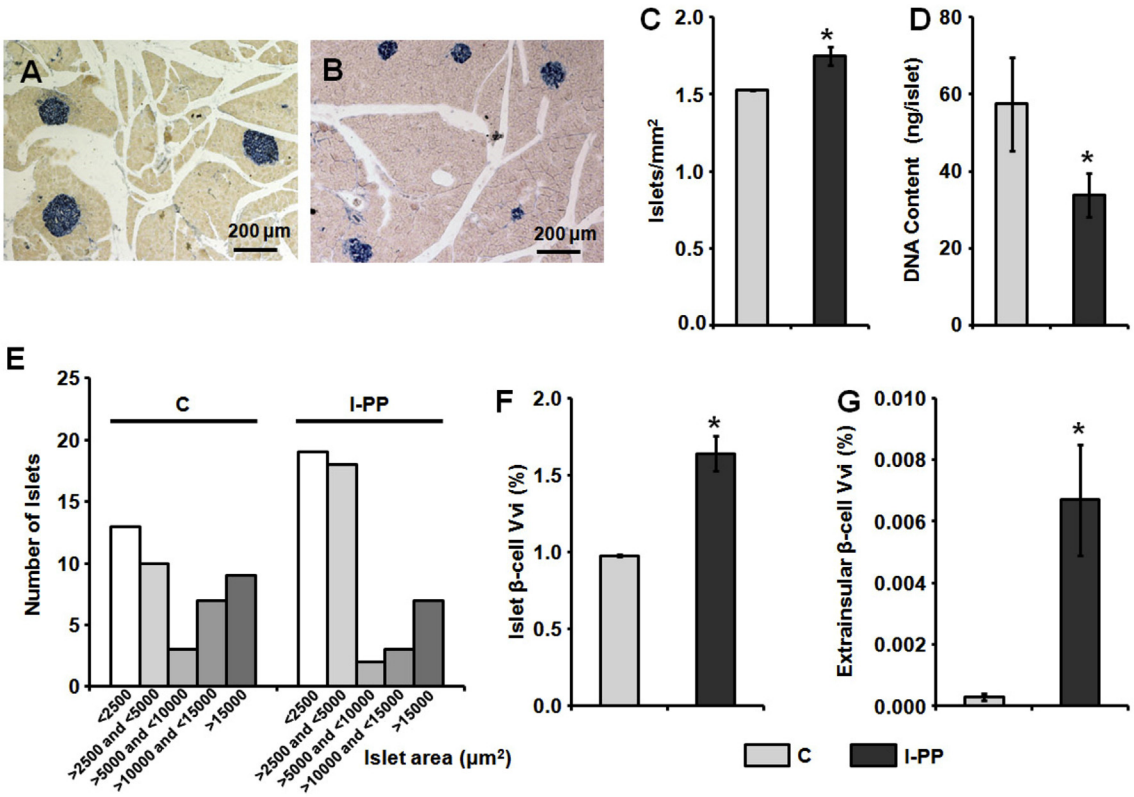

Fig. 1. Size, number of islets and β-cell volume-density.
A-C: Islet number: panels **A and B** show representative images of control **(A)** and I-PP **(B)** pancreas stained with haematoxylin (10×). **C:** islets per *area* unit (islets/mm^2) of C and I-PP islets. Bars represent mean values ± SEM from 3 different levels.
D: Islet-DNA content: measured in islets isolated from C (grey bars) and I-PP (black bars) rats and expressed as ng/islet. Bars represent mean values ± SEM from 3 independent experiments.
E. Number of islets per islet size category in C (left group of bars) and I-PP (right group of bars) rats. Bars represent the number of islets counted in each size category from 3 different levels.
F and G: Vvi of islet β-cell **(F)** and extrainsular β-cell **(G).** Bars represent mean values ± SEM from 3 levels.
Bar color code is the same in all panels. $p < 0.05$ *vs.* C in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.10. β- and endothelial cell cross-talk

INGAP-PP administration increased *Integrin β1* mRNA and protein levels (Fig. 4G and H), thereby suggesting a closer physical and functional relationship between islet β- and endothelial-cells.

3.2. "In vitro" model (islets isolated from normal rats cultured with or without INGAP-PP)

In this approach normal isolated islets were cultured for 4 days with or without INGAP-PP or VEGFA and/or Rapamycin, thus resulting in 6 different experimental conditions: C, I-PP, V, R, I-PP+R and I-PP+R+V respectively (see Material and Methods section).

3.2.1. Apoptosis

Islets cultured in the presence of VEGFA (V) significantly increased their *Bcl2* mRNA level ($p < 0.05$ *vs.* C) whereas the increase was not significant in islets cultured in the presence of INGAP-PP (I-PP; Fig. 5A). Addition of Rapamycin to the culture medium (R, I-PP+R and I-PP+R+V) significantly reduced islet *Bcl2* mRNA level ($p < 0.05$). Bcl2 protein concentration was increased by V ($p < 0.05$) whereas neither I-PP nor R reproduced this effect (Fig. 5B).

Caspase3 mRNA level was significantly lower in I-PP islets (46%) but not in V islets. Rapamycin alone significantly increased *Caspase3* mRNA level either in the presence or absence of INGAP-PP and VEGFA (Fig. 5C). The active form of Caspase3 protein level was not modified by either I-PP or by V, but was significantly increased by Rapamycin. Addition of INGAP-PP (I-PP+R) abolished this effect (Fig. 5D).

3.2.2. Insulin mRNA level and GSIS

I-PP and V islets had significantly higher levels of *Insulin* mRNA than C islets ($p < 0.05$). Rapamycin blunted this effect and further addition of VEGFA failed to recover I-PP effect (Fig. 6A).

I-PP and V islets released significantly larger amounts of insulin in response to 16.7 mM glucose ($p < 0.05$; 37% and 29% for I-PP and V, respectively, compared to C). This effect was not observed in the presence of 3.3 mM glucose. Addition of Rapamycin to the culture medium (R or I-PP+R islets) significantly decreased GSIS; however, the enhancing effect of INGAP-PP was still evident (I-PP+R was 39% higher than R). I-PP+R+V islets significantly released larger amounts of insulin than I-PP+R ones, thus VEGFA significantly rescued INGAP-PP effect on insulin secretion; (Fig. 6B).

3.2.3. VEGFA release

I-PP islets released a significantly larger amount of VEGFA into the culture medium than C islets (C *vs.* I-PP: 164.9 ± 14.0 *vs.* 209.6 ± 17.4 pg/100 islets/day; $p < 0.05$). Rapamycin alone decreased VEGFA secretion and blunted the enhancing effect of

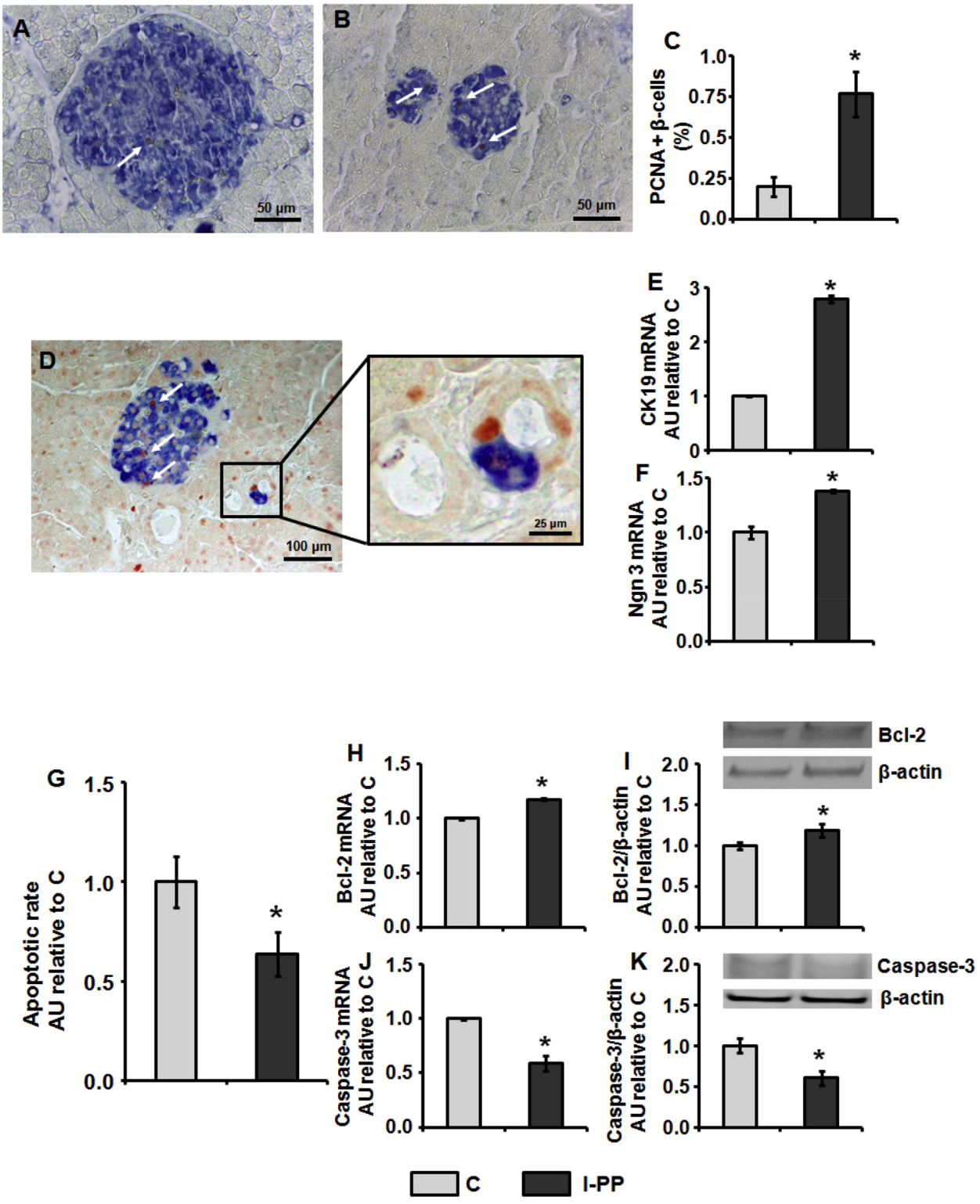

Fig. 2. Replication, neogenesis, and apoptosis.

A-C. Replication: A and B: Images show representative islets of C **(A)** and I-PP **(B)** rats (40×) immunostained with PCNA (brown nucleus indicated by arrows). **C:** Islet-cell replication rate is expressed as the percentage of PCNA-labeled cells of the total number of β-cells counted (see material and methods section).

D-F. Neogenesis: D. Insulin and PCNA immunostaining of I-PP pancreas (20×). Inset shows higher magnification (4×) of an insulin positive cell in a pancreatic duct. **E and F**: CK-19 **(E)** and Ngn3 **(F)** mRNA levels (RT qPCR) in islets isolated from C and I-PP rats. In all RT qPCR experiments β-actin was used as internal standard. Values were expressed in arbitrary units (AU) compared to mRNA level measured in C islets.

G-K: Apoptosis: G: Apoptosis rate determined by colorimetric kit (see material and methods section). **H and J**: Bcl-2 **(H)** and Caspase-3 **(J)** mRNA level (RT qPCR) in islets isolated from C and I-PP rats. **I and K:** Bcl-2 **(I)** and active Caspase-3 **(K)** protein level determined by western blot in islet homogenates from both experimental groups. A representative blot from 3 independent experiments is shown in each case. Bars below the blot represent mean values ± SEM expressed in arbitrary units (AU) as the ratio between the protein of interest and β-actin band intensity compared to C islets.

Bars represent mean rate ± SEM of C (grey) and I-PP (black) islets from three independent experiments in all panels. $p < 0.05$, *vs.* C in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

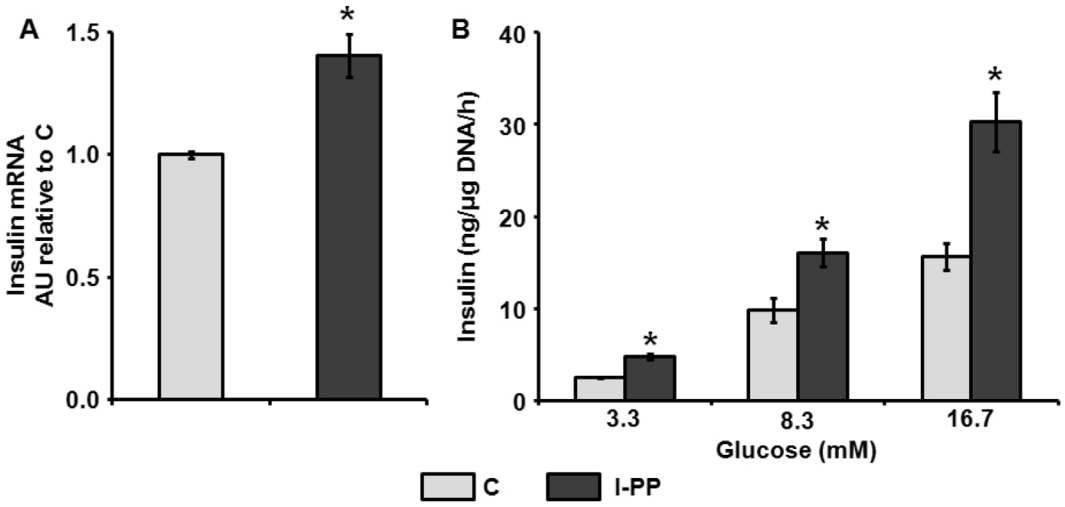

Fig. 3. Insulin gene expression and GSIS.
A. Insulin mRNA level (RT qPCR) in C (grey bar) and I-PP (black bar) isolated islets. β-actin was used as internal standard. Values are expressed in arbitrary units (AU) compared to mRNA level determined in C islets. Bars represent mean values ± SEM from three independent experiments.
B. Insulin secretion in response to different glucose concentrations by islets isolated from C and I-PP rats. Bars represent mean values ± SEM from 5 independent experiments. Bar color code is the same in all panels. $p < 0.05$, *vs.* C in both panels.

INGAP-PP on this release (I-PP *vs.* I-PP+R; $p < 0.05$; Fig. 7A).

3.2.4. VEGFA, Ang1, Ang2 and integrin β1 gene expression

I-PP and V islets showed a significantly higher VEGFA mRNA level than C islets (43% and 94%, respectively; $p < 0.05$ *vs.* C). Although the effect of exogenous VEGFA was greater than that of INGAP-PP, the difference was not statistically significant. Rapamycin blunted this enhancing effect either in the presence or absence of INGAP-PP (Fig. 7B). However addition of INGAP-PP + VEGFA to the culture medium recovered I-PP effect on VEGFA mRNA. Islet VEGFA protein concentration was also significantly increased by INGAP-PP and exogenous VEGFA (37% and 89%, respectively; $p < 0.05$). The enhancing effect of INGAP-PP was blocked by Rapamycin. (Fig. 7C).

I-PP and V islets showed significantly higher *Ang1* and *Ang2* mRNA levels compared to C islets, however, Rapamycin decreased this effect only in the case of Ang1 (Fig. 7D and E).

Neither I-PP nor V islets showed significant changes in islet *Integrin β1* mRNA or protein levels (Fig. 7F and G).

3.2.5. Effect of inhibition of VEGFR2

Normal isolated islets were cultured for 4 days with or without INGAP-PP or VEGFA and/or SU5416 (a specific VEGFR2 inhibitor) (Kim et al., 2016), thus resulting in 6 different experimental conditions: C, I-PP, V, SU, I-PP+SU and V+SU respectively. Addition of SU5416 to the culture medium did not modify insulin secretion in response to 16.7 mM glucose in none of the experimental groups tested (SU; I-PP+SU and; V+SU islets; Fig. 8A). SU islets significantly decreased mRNA level of insulin compared to C ones ($p < 0.05$), however, the enhancing effect of INGAP-PP was still evident (I-PP+SU was 22% higher than SU; Fig. 8B).

Islets cultured in the presence of the VEGFR2 inhibitor (SU and I-PP+SU) significantly increased VEGFA mRNA level compared to C and I-PP islets respectively (Fig. 8C), blocked INGAP-PP and VEGFA effect on Ang1 and Ang2 ($p < 0.05$; Fig. 8D and E) and did not modify integrin β1 mRNA levels (Fig. 8F). INGAP-PP and VEGFA significantly attenuated the inhibitory effect of SU5416 upon mRNA level of pro-angiogenic factor Ang2 ($p < 0.05$; Fig. 8E).

In all cases, simultaneous addition of VEGF-A and SU5416 (V+SU) did not affect the inhibitory effect of SU546 (Fig. 8).

4. Discussion

Our current data confirm that administration of INGAP-PP to normal rats for ten days enhances β-cell mass and function (Barbosa et al., 2006; Silva et al., 2008; Madrid et al., 2009; Kapur et al., 2012; Chang et al., 2011). Pancreas from INGAP-PP treated rats had an increased β-cell Vvi and a higher number of islets than untreated animals. These islets were smaller in size and in number of cells (islets with lower DNA content), data consistent with an increased number of newly formed islets (Bonner-Weir et al., 2010). In I-PP animals, β-cell mass was enhanced by decreasing apoptosis and increasing replication (PCNA immunostaining) and β-cell neogenesis. In a recent review, S. Bonner Weir et al. (Bonner-Weir et al., 2012) have summarized different neogenesis indicators used in the literature, such as the presence of insulin-positive cells found in the ductal epithelium, insulin/CK double-positive cells, and small clusters of 1–6 insulin-positive cells scattered in the parenchyma in the adult human pancreas. Most of these indicators were present in the pancreas of our I-PP rats (higher extrainsular β-cell Vvi and increased number of both, insulin positive ductal cells and islets close to ducts) together with an enhanced mRNA level of CK19 (Bouwens et al., 1994) and Ngn3 (Xu et al., 2008). Although the CK19 mRNA detected in isolated islets could reflect the presence of both, ductal cells associated to small islets and islet-cells expressing CK19, its simultaneous increase with Ngn3 mRNA level can be considered an additional marker of neogenesis that should be added to those previously mentioned.

Islets isolated from these rats also showed a significant increase in their insulin gene expression and their GSIS at every glucose concentration tested resembling a dose-response curve. However, the enhanced GSIS did not affect the glucose homeostasis in the intact animals since comparable values were recorded in control and I-PP rats at serum glucose, TBARS, insulin, and triglyceride levels, as well as glucose tolerance and HOMA −IR and −β. Altogether, these data suggest that INGAP-PP administration to normal rats did not affect their metabolic homeostasis.

Islets from INGAP-PP treated animals also had greater vascular density as demonstrated by the enhancement of CD34-positive areas (Fina et al., 1990) and gene expression of pro-angiogenic factors (VegfA, Ang1 and Ang2) and Integrin β1. Integrin β1 is a

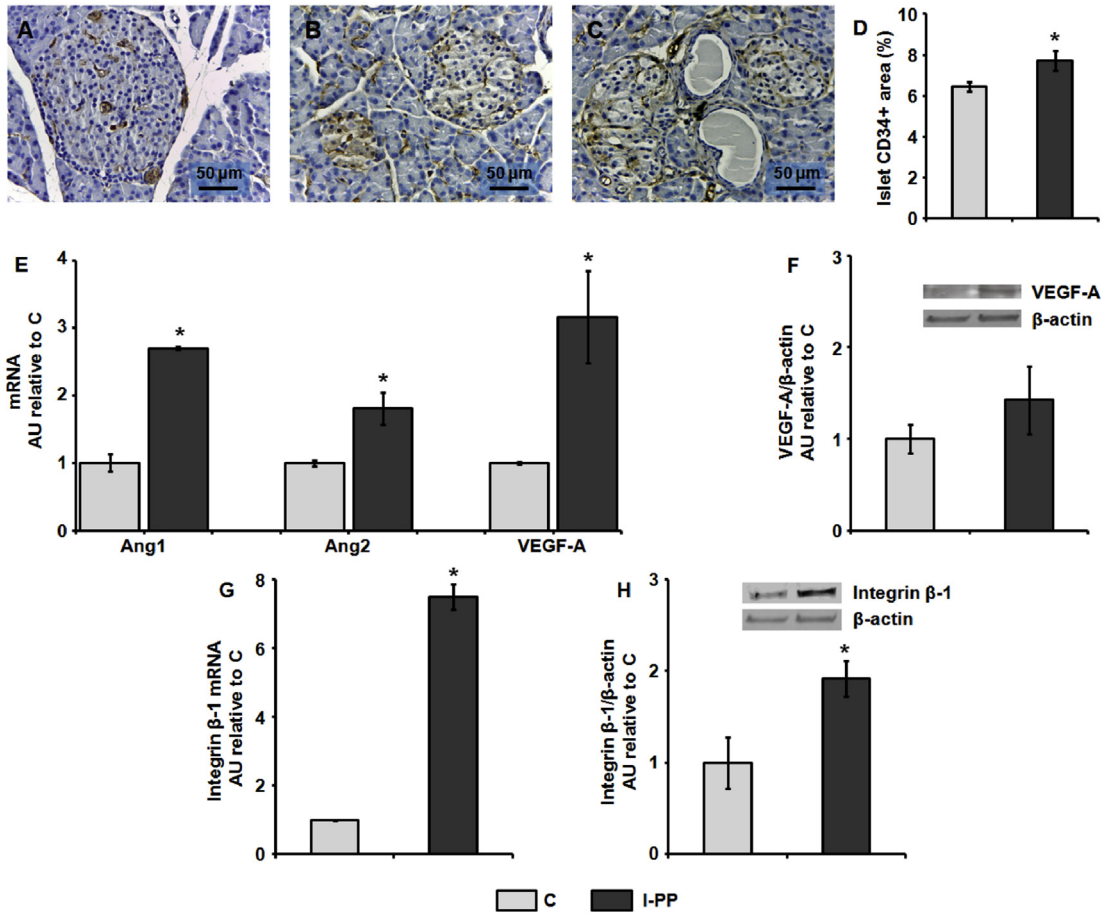

Fig. 4. Islet Angiogenesis and integrin β1 mRNA level.
A-G Islet Angiogenesis. CD34 immunostaining in pancreas sections (40×) from C **(A)** and I-PP rats **(B and C).** Islet CD34-positive area (%) in C (grey bars) and I-PP (black bars) rats **(D).** Ang1, Ang2 and VEGFA **(E)** mRNA level (RT qPCR) in islets isolated from C and I-PP rats. β-actin was used as internal standard. Values were expressed in arbitrary units (AU) compared to mRNA level determined in C islets. **F:** VEGF-A protein level measured by western blot in islet homogenates from both experimental groups (C and I-PP). A representative blot from three independent experiments is shown in each case. Values were expressed in arbitrary units (AU) as the ratio between the protein of interest and β-actin band intensity compared to C islets.
G and H: Integrin β-1 gene expression: mRNA **(G)** and protein **(H)** level in islets isolated from C or I-PP rats as described above.
Bars represent mean values ± SEM from 3 independent experiments; bar color code is the same in all panels. $p < 0.05$, *vs. C in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein involved in the interdependent physical and functional relationship between islet β- and endothelial cells that modulates insulin secretion and β-cell proliferation rate (Zanone et al., 2008). These data suggest that INGAP-PP might enhance this interaction and endogenous VEGFA production/release. All together, these effects might play an important modulatory role in cross-talk between islet β- and endothelial cells in a glucose-dependent manner (Xiao et al., 2013). The demonstration that islet endothelial cells increase integrin β1 expression in β cells, which in turn leads to improved insulin secretion and β cell function (Spelios et al., 2015), lends support to this assumption. Further, integrin β1-deficient mice display impaired glucose tolerance and insulin secretion associated with a reduction in β-cell mass and proliferation (Riopel et al., 2011); these data further argue in favor of a regulatory role of integrin β1 on glucose metabolism and maintenance of β-cell survival and function.

Concomitantly, β cells promote endothelial cell recruitment, proliferation, growth, and islet vascularization through angiogenic factors such as VEGFA or angiopoietins 1 and 2 (Ang1 and Ang2), while endothelial cells reciprocally promote islet development and maintenance of β-cell homeostasis (Lammert et al., 2003; Cleaver and Melton, 2003; Cleaver and Dor, 2012). Ang1 has also been proposed to participate in vessel stabilization (Suri et al., 1998), whereas Ang2 plays a dual function: it inhibits Ang1 signaling at low levels of VEGFA but stimulates neovascularization in the presence of high VEGFA levels (Lobov et al., 2002). Our results showed that INGAPP-PP administration induced an increase of VEGFA, Ang1 and Ang2 mRNA levels.

In our *in vitro* assay while INGAP-PP significantly increased VEGFA release, VEGFA also potentiated GSIS resembling the enhancing effect of INGAP-PP, thereby suggesting that endogenous VEGFA might mediate the enhancing INGAP-PP effect on GSIS. Addition of VEGFA to Rapamycin+INGAP-PP-cultured islets significantly recovered INGAP-PP effect on insulin secretion supporting our assumption.

Regarding β-cell mass, INGAP-PP and VEGFA showed similar effects on the expression of factors involved in β-cell apoptotic pathway: INGAP-PP induced a significant decrease in mRNA levels of *Caspase3* while VEGFA significantly enhanced *Bcl2* gene expression at both mRNA and protein levels. Rapamycin (an mTOR inhibitor) inhibited both effects, suggesting that mTORC pathway participates in the mechanism of β-cell apoptosis. Further, INGAP-PP might also exert its antiapoptotic effect by blocking the positive effect of Rapamycin upon active Caspase3 protein levels (Zhang

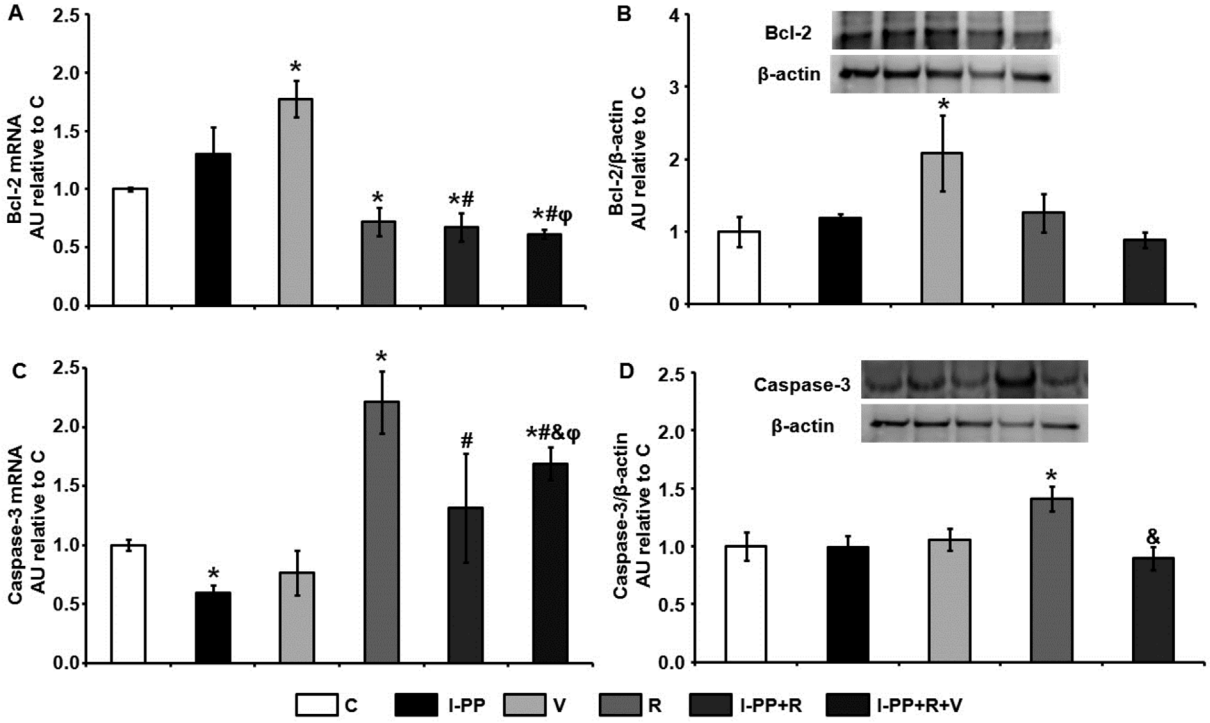

Fig. 5. Apoptosis.
Bcl-2 **(A)** and Caspase-3 **(C)** mRNA level (RT qPCR) in islets previously cultured for 4 days in the absence (C islets; white bars) or presence of 10 μg/ml INGAP-PP (I-PP islets; black bars), 10 ng/ml VEGF-A (V islets, light grey bars), 10 ng/ml Rapamycin (R islets; grey bars), and the combinations: I-PP+R islets (dark grey bars) and I-PP+R+V (very dark grey bars). In both cases β-actin was used as internal standard and values were expressed in arbitrary units (AU) compared to mRNA level determined in C islets.
Bcl-2 **(B)** and active Caspase-3 **(D)** protein level determined by western blot in islet homogenates from all culture conditions using β-actin as housekeeping protein. A representative blot from 3 independent experiments is shown. Values were expressed in arbitrary units (AU) as the ratio between the protein of interest and β-actin band intensity compared to C islets.
Bars represent mean values ± SEM from three independent experiments. In all panels, bar color code is the same and $p < 0.05$, *vs. C; #vs I-PP, φ vs. V and & vs. R islets.

et al., 2007). Addition of INGAP-PP and/or VEGFA to Rapamycin-cultured islets blunted only partially it effect, maybe due to the doses used in the assays.

The islet VEGFA effect on GSIS has been proposed to act through the PI3K pathway (Gerber et al., 1998). Concurrently, we recently demonstrated that PI3K/AKT pathway participates in the mechanism by which INGAP-PP increases islet glucokinase activity, glucose metabolism and tyrosine phosphorylation of insulin receptor and also insulin receptor substrates (IRS1 and IRS2) (Chang et al., 2011). Downstream effectors of PI3K cascade also modulate mTORC pathway, which in turn up-regulates multiple processes including angiogenesis (Dibble and Manning, 2013; Cella et al., 2015; Ersahin et al., 2015). The fact that Rapamycin significantly decreased insulin and VEGFA secretion in the presence or absence of INGAP-PP strongly suggests that mTORC pathway could actively participate in the mechanism by which INGAP-PP enhances

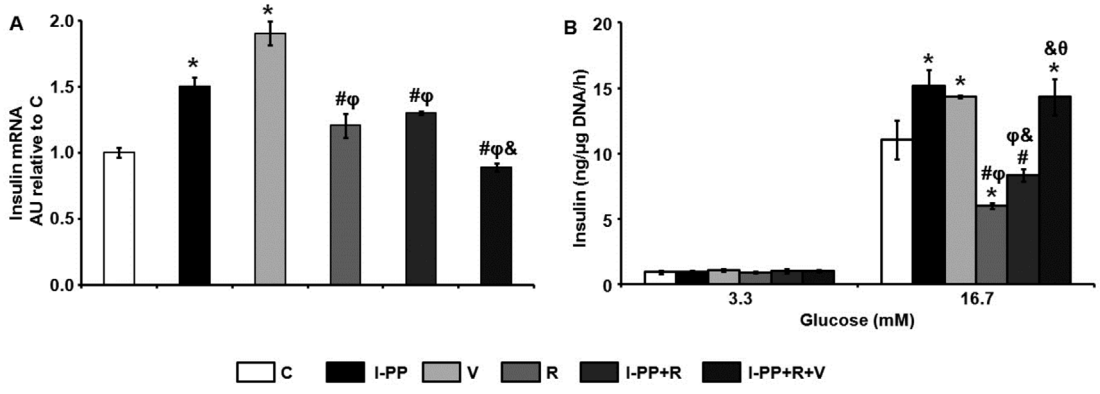

Fig. 6. Insulin gene expression and GSIS.
A. Insulin mRNA level (RT qPCR) in islets from all culture conditions: C (white bar), I-PP (black bar), V (light grey bar), R (grey bar), I-PP+R (dark grey bar) and I-PP+R+V (very dark grey bars). β-actin was used as internal standard. Values are expressed in arbitrary units (AU) compared to mRNA level in C islets.
B. Insulin secretion in response to 3.3 and 16.7 mmol/L glucose by islets from all culture conditions. Insulin released to incubation media was expressed as ng of insulin per μg of islet DNA/1 h. Bars represent mean values ± SEM from five independent experiments. Insulin secreted at 16.7 mmol/l vs. 3.3 mmol/L glucose, $p < 0.05$ for each experimental group. Bar color code is the same in all panels. $p < 0.05$, *vs. C islets; #vs. I-PP, φvs. V, &vs. R and θvs. I-PP+R islets.

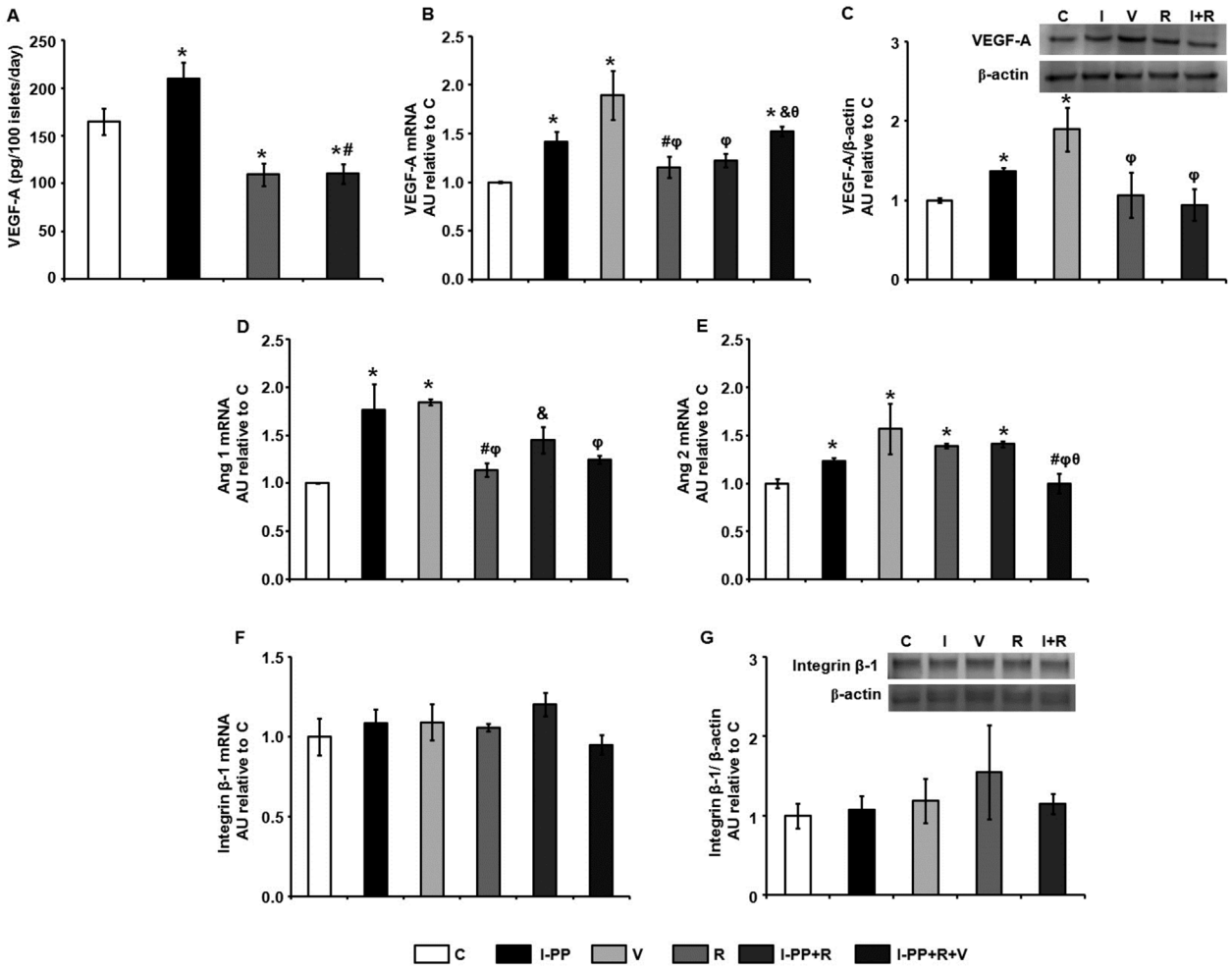

Fig. 7. Islet VEGF-A, Ang1, Ang2 and integrin β-1.
A: VEGFA released by C (white bar), I-PP (black bar), R (grey bar), and I-PP+R (dark grey bar) cultured-islets. VEGF-A released into culture medium was expressed in pg/100 islets/day.
VEGFA **(B),** Ang1 **(D)**, Ang2 **(E)** and Integrin β-1 **(F)** mRNA levels (RT qPCR) in islets from all culture conditions (C, I-PP, R, I-PP+R and I-PP+R+V [very dark grey bars]). β-actin was used as internal standard. Values are expressed in arbitrary units (AU) with respect to mRNA level determined in C islets.
VEGF-A **(C)** and integrin β1 **(G)** protein concentration (western blot) in islet homogenates using β-actin as housekeeping protein. A representative blot from 3 independent experiments is shown. Values were expressed in arbitrary units as the ratio between the protein of interest and β-actin band intensity.
Bars represent mean values ± SEM from 3 independent experiments. Bar color code is the same in all panels. $p < 0.05$, *vs.* C; #*vs.* I-PP, φ*vs.* V; &*vs.* R and θ*vs.* I-PP+R islets.

VEGF-A secretion. Therefore, we postulate that INGAP-PP could interact with a specific receptor (not yet structurally identified) and activate PI3K/AKT-mTORC pathway, promoting interaction between endothelial and β-cells. Consequently, this network might be the common path by which INGAP-PP exerts a beneficial effect on islet metabolism, insulin secretion, β-cell mass, and, probably, islet angiogenesis. However, since other pathways (i.e. Ras-Raf-Erk) could be involved in INGAP-PP transduction signaling (Petropavlovskaia et al., 2012), further studies might be conducted in order to confirm our assumption.

When inhibition of VEGFR2 by SU5416 triggered a dramatic inhibition of the angiogenic factors Ang1 and Ang2 gene expression, it enhanced mRNA level of VEGFA, probably as a reactive response to the blockage of its own receptor. The positive modulation of INGAP-PP effect by VEGFA was further confirmed by the fact that in presence of SU5416, INGAP-PP was unable to completely increase insulin secretion, insulin, Ang1 and Ang2 mRNA levels.

Further addition of VEGF-A did not completely reversed the effect of the inhibitor, however, we cannot discard that it could be needed higher VEGF-A concentrations to compete with SU5416.

In brief, our results showed that the beneficial effect of INGAP-PP on β-cell mass and function previously shown either *in vitro* or *in vivo* (Borelli et al., 2005; Barbosa et al., 2006; Silva et al., 2008; Madrid et al., 2009; Kapur et al., 2012; Maiztegui et al., 2015; Chang et al., 2011) could be partly mediated through the production and release of islet VEGFA. Although Reinert and colleagues reported that the absence of islet VEGFA in adult mice slightly reduces islet vascularization without affecting β-cell mass (Reinert et al., 2013), our current results showed a strong correlation between islet VEGFA production/release together with an increase in vascularization and β-cell mass. Our data are further supported by the fact that the rise of the apoptotic rate of endothelial cells during sustained hypoglycemia precedes the increase of β-cell apoptosis while VEGFA administration avoids β-cell mass reduction (Xiao et al., 2013). Since INGAP-PP blocks the stimulatory effect of Rapamycin on Caspase3 but VEGFA does not reproduce this effect, the latter could not be the only modulator of INGAP-PP effects.

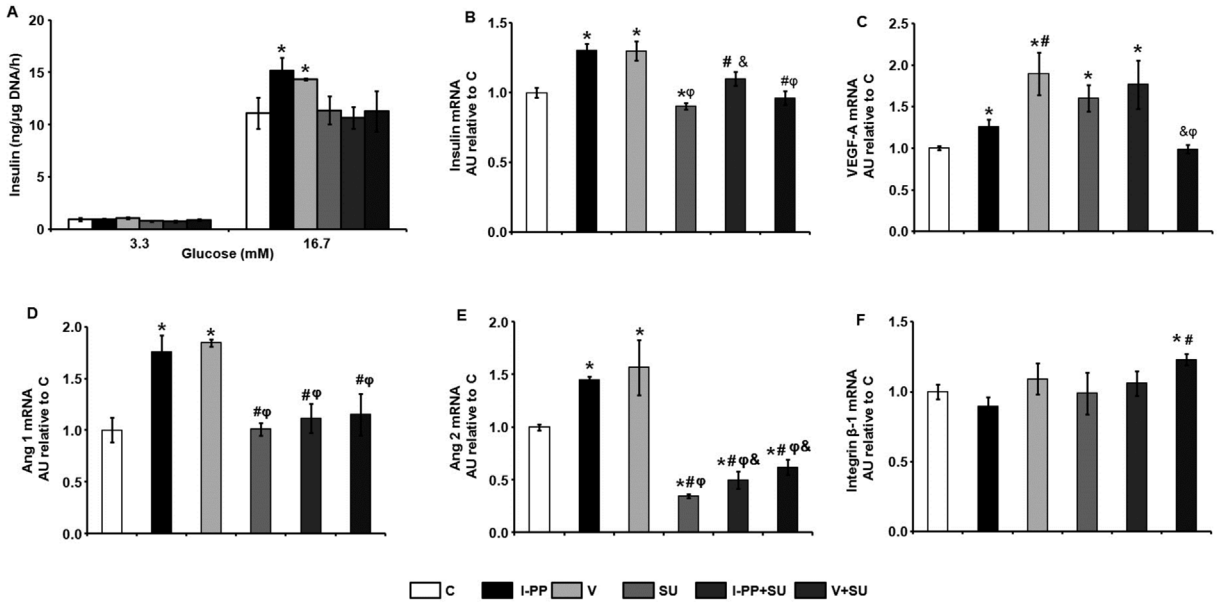

Fig. 8. Effect of SU5416 on GSIS, insulin, VEGFA, Ang1, Ang2 and integrin β1 mRNA levels.
(A) Insulin secretion in response to 3.3 and 16.7 mmol/L glucose by islets from all culture conditions. Insulin released to incubation media was expressed as ng of insulin per µg of islet DNA/1 h. Bars represent mean values ± SEM from three independent experiments. Insulin secreted at 16.7 mmol/l vs. 3.3 mmol/L glucose, $p < 0.05$ for each experimental group. Insulin **(B)** VEGF-A **(C),** Ang1 **(D),** Ang2 **(E),** and Integrin β1 **(F)** mRNA levels (RT qPCR) in islets from all culture conditions. β-actin was used as internal standard. Values are expressed in arbitrary units (AU) compared to mRNA level in C islets. Bars represent mean values ± SEM from three independent experiments.
In all panels C (white bar), I-PP (black bar), V (light grey bar), SU (grey bar), I-PP+SU (dark grey bar) and V+SU (very dark grey bars). Bar color code is the same in all panels. $p < 0.05$, *vs. C; #vs. I-PP; φvs. V and &vs. SU islets.

5. Conclusions

Human T2D is characterized by an early and progressive decrease of β-cell mass and function (Weyer et al., 1999; Butler et al., 2003), as well as disturbances in islet capillary integrity (Brissova et al., 2015). The present work demonstrates that INGAP-PP simultaneously enhanced β-cell mass and function and promoted islet angiogenesis and VEGF-A production/release. We also showed that VEGF-A potentiated the positive effect of INGAP-PP on β-cells, probably through mTORC pathway. Since INGAP-PP increased insulin secretion in a glucose-concentration-dependent fashion, it could represent a promising therapeutic agent for T2D without hypoglycemic risk. However, further clinical studies are necessary to confirm its possible use in the treatment of T2D.

Conflict of interest

The authors declare that they have no conflict of interest.

Fundings

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Author contribution

LEF and JJG conceived and designed the study and drafted the manuscript; CLR, BM, HDZ and LEF carried out the experiments and statistical analyses. All authors read and approved the final manuscript. BM, LEF, HDZ, and JJG are members of the research career of CONICET and CLR is a fellow of CONICET.

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