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**EFFECTS OF ISLET NEOGENESIS ASSOCIATED PROTEIN DEPEND ON
VASCULAR ENDOTHELIAL GROWTH FACTOR GENE EXPRESSION
MODULATED BY HYPOXIA-INDUCIBLE FACTOR 1-ALPHA**

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Highlights:

- **-INGAP-PP administration enhances β -cell mass and function.**
- **-Angiogenesis is stimulated by INGAP-PP through HIF-1 α -mediated VEGF-A secretion.**

- **-INGAP-PP acts through PI3K/Akt/mTOR pathways**
- **-This peptide could represent a therapeutic alternative for diabetes treatment.**

Abstract

Background: Pharmacology has provided efficient tools to improve insulin effect/secretion but the decrease in β -cell mass remains elusive. INGAP-PP could provide a therapeutic alternative to meet that challenge.

Aim: To further understand the mechanism that links INGAP-PP effects upon β -cell mass and function with islet angiogenesis.

Methodology: Normal male Wistar rats were divided into 2 groups and injected with a single dose of 100 mg/Kg suramin or saline. Both groups were divided into 2 subgroups that received daily doses of 2mg/kg INGAP-PP or saline for ten days. Plasma glucose, triacylglycerol, TBARS, and insulin levels were measured. Pancreas immunomorphometric analyses were also performed. Pancreatic islets were isolated to measure glucose-stimulated insulin secretion (GSIS). Specific islet mRNA levels were studied by qRT-PCR. Statistical analysis was done using ANOVA.

Results: No differences were recorded in body weight, food intake, or any other plasma parameter measured in all groups. Islets from INGAP-PP-treated rats significantly increased GSIS, β -cell mass, and mRNA levels of Bcl-2, Ngn-3, VEGF-A, VEGF-R2, CD31, Ang1 and Ang2, Laminin β -1, and Integrin β -1, and decreased mRNA levels of Caspase-8, Bad, and Bax. Islets from suramin-treated rats showed significant opposite effects, but INGAPP-PP administration rescued most of the suramin effects in animals treated with both compounds.

Conclusion: Our results reinforce the concept that INGAP-PP enhances insulin secretion and β -cell mass, acting through PI3K/Akt/mTOR pathways and simultaneously activating angiogenesis through HIF-1 α -mediated VEGF-A secretion. Therefore, INGAP-PP might be a suitable antidiabetic agent able to overcome two major alterations present in T2D.

Abbreviations:

INGAP: Islet neogenesis associated protein; GSIS: Glucose stimulated insulin secretion; TBARS: thiobarbituric acid reactive substances; T2D: Type 2 diabetes; VEGF-A: Vascular endothelial growth factor A; HIF-1 α : Hypoxia-Inducible Factor 1-alpha ; IR: Insulin resistance; HOMA: homoeostasis model assessment; HOMA- β : HOMA for β -cell function; HOMA-IR: HOMA for insulin resistance.

Keywords:

INGAP-PP, angiogenesis, β -cells, HIF-1 α , mTOR pathway.

1. Introduction

Type 2 Diabetes (T2D) presents a serious public health problem due to its increasing prevalence and the high cost of care which derives mainly from the development of its chronic complications [1], facilitated by the low level of metabolic control of the disease [2]. T2D is characterized by early and progressive decrease in β -cell mass and function [3, 4]. Although modern pharmacology has provided efficient tools to improve insulin effect/secretion, the problem of β -cell mass decrease remains elusive [5].

INGAP (islet neogenesis associated protein), a peptide produced by normal pancreatic exocrine, duct, and islet-non- β -cells [6], might help to close the above-mentioned

treatment gap. A pentadecapeptide with its 104-118 amino acid sequence (INGAP-PP) reproduces most of the pleiotropic effects of the intact molecule [7-14].

INGAP-PP administration to normal rats simultaneously enhances glucose-stimulated insulin secretion (GSIS) and increases β -cell mass by reducing their apoptosis rate and promoting replication and neogenesis [8-16]. A preliminary clinical trial in which INGAP-PP was administered to people with type 1 and T2D also showed promising results [17].

Although many factors are involved in the mechanism leading to impairment of β -cell mass and function in T2D, consistent evidence has proved that endothelial cells significantly affect β -cell function and proliferation through several angiogenic and growth factors [18, 19]. In this context, vascular endothelial growth factor-A (VEGF-A) is the main angiogenic factor that increases vascular permeability and endothelial cell proliferation, migration, and invasion into surrounding tissues [20]. VEGF-A gene expression strongly depends on the presence of Hypoxia-Inducible Factor 1- α (HIF-1 α), a basic helix-loop-helix factor which is able to induce transcription of several genes (including VEGF-A) involved in angiogenesis [21-26]. VEGF-A is also expressed in islet β -cells, and its presence is necessary for correct pancreatic growth and development of islet-specific capillary fenestrations [27-30].

VEGF-A exerts its effects through a tyrosine kinase receptor that activates PI3K [31] and mTOR pathways [32-34]. Also, we recently demonstrated that INGAP-PP effects on β -cell mass and function are significantly associated with islet angiogenesis and VEGF-A production/release. Based on these data we proposed that VEGF-A might potentiate INGAP-PP effect by acting through mTORC pathway [16]. However, evidence on how VEGF-A participates in the mechanism by which INGAP-PP enhances β -cell mass and function was insufficient.

Aiming to provide this evidence, we currently studied insulin secretion, β -cell apoptosis, and angiogenesis in normal rats treated with INGAP-PP alone or together with suramin, a compound that acts as a functional VEGF-A antagonist by binding to its receptor [35].

2. Materials and methods

2.1. Animals: 88 normal male Wistar rats (230–260 g body weight) were kept in controlled conditions (23° C and a fixed 12-h light–dark cycle), with free access to standard commercial chow and water. Animals were divided into 2 different experimental groups: on day 0 of the treatment period, animals from one group were injected (i.p.) with suramin in a single dose of 100 mg/Kg in a volume of 200 μ l (S), whereas animals in the other group received an injection with the same volume of saline. Both experimental groups were thereafter divided into 2 subgroups receiving either INGAP-PP (I-PP) in a daily dose of 2 mg/kg or saline for ten days. Daily doses of INGAP-PP and saline were administered in two i.p. injections per day every 12 hours (at 8:00 a.m. and 8:00 p.m.).

INGAP-PP dose employed was selected based on its effectiveness as shown in a previous study [15], whereas in the case of the suramin dose we considered its half-life (50 days) [36].

This experimental design resulted in 4 different groups, each with 22 animals (Figure 1).

Water and food intake were measured daily, and 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. Chemicals and drugs: Collagenase was obtained from Serva Feinbiochemica (Heidelberg, Germany). Suramin sodium salt, bovine serum albumin (BSA) fraction V, and other reagents were from Sigma-Aldrich.

2.3. Plasma measurements: At the end of the treatment period, blood samples from non-fasting animals from each experimental group were collected from the retro-orbital plexus under light halothane anaesthesia to measure plasma glucose, triacylglycerol, lipid peroxidation (TBARS), and insulin.

Glucose was measured with test strips (Accu-Chek Performa Nano System, Roche Diagnostics, Mannheim, Germany) and the triglyceride was determined using commercial kits (BioSystems S.A., Buenos Aires, Argentina).

TBARS were measured by fluorimetric assay and results expressed as pmol of malondialdehyde (MDA)/mg of plasma protein.

Plasma insulin was determined by validated specific radioimmunoassay (RIA) [37], using rat insulin standard (Novo Nordisk Pharma Argentina), a specific antibody against rat insulin (Sigma Chemical Co.) and highly purified porcine insulin labelled with ^{125}I . Insulin resistance (IR) was determined by homeostasis model assessment-IR (HOMA-IR) using the formula $[\text{insulin } (\mu\text{U/L}) \times \text{glucose (mmol/L)}] / 22.5$. β -cell function was quantified by HOMA- β $[\text{insulin } (\mu\text{U/L}) \times 20 / \text{glucose (mmol/L)}] - 3.5$ [38]. Since these indexes were validated in humans but not in rodents, we compared values measured in C rats with the other experimental groups instead of using a cut-off threshold value.

2.4. Immunohistochemical studies: the whole pancreas from three animals in each experimental group was carefully dissected, removed, and immediately weighed;

thereafter, a piece of the pancreas tail of each pancreas was fixed in 10% formaldehyde and embedded in paraffin. Serial sections of each of the fixed pancreases (6 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 deparaffinised, incubated for 30 min in 3% (v/v) hydrogen peroxide in methanol to block 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 insulin antibody (1:20,000 dilution). Then, 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). Final staining was performed by incubating the slides for 15 min with Fast Blue (Sigma).

2.5. Morphometric analysis: Morphometric analysis was performed by videomicroscopy using a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera, together with OPTIMAS software (Bioscan). Using these tools, we measured: total pancreatic area (excluding connective tissue), insulin-positive cells, and islet β -cell area [Vvi (volume density)]. To estimate islet β -cell mass, we multiplied its volume density by the weight of the total pancreas [39].

2.6. Pancreatic islet isolation: At the time of euthanasia, the whole pancreas from each animal was removed and islets were isolated by collagenase digestion [40].

2.7. Glucose-stimulated insulin secretion (GSIS): Pancreatic islets freshly isolated from all experimental groups were hand-picked and randomly divided into 10 groups of 5 islets. They 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 CO₂/O₂ (5/95%) containing

1% (w/v) BSA and 3.3 or 16.7 mM glucose (n=10 for each experimental condition and glucose concentration). Islet pick-up was performed by a “blind operator” who did not know to which experimental group the islets belonged. At the end of the incubation period, aliquots from the medium were taken for insulin measurement by RIA [41] as described above. Insulin released into the incubation medium was expressed as ng of insulin/islet/hour.

2.8. Quantitative real-time PCR: Total RNA was obtained from isolated islets using a commercial kit (Rneasy mini kit. Qiagen, Hilden, Germany), 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, whereas DNA contamination was avoided by treating the samples with DNase I (Invitrogen). Total RNA (1 µg) 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 step of 1 minute at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Cycling profile finished with 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 shown (Table 1).

2.9. 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 the Tukey or Tamhane test for similar variance samples. Results are expressed as mean $\pm$ SEM. Differences between groups were considered significant when p values were <0.05 .

3. Results

3.1. Body weight, food intake, and plasma parameters: Food intake was comparable in all groups and, consequently, all the experimental groups showed a similar body weight gain (Table 2).

None of the plasma parameters studied showed any significant difference; HOMA-IR and HOMA- β were also comparable in all experimental groups (Table 2).

3.2. Glucose-Stimulated Insulin Secretion: Isolated islets from I-PP rats released more insulin in response to glucose than C islets ($p < 0.05$). Suramin administration significantly decreased GSIS at 16.7 mM glucose; however, the simultaneous presence of INGAP-PP and suramin reversed the inhibitory effect of the latter (I-PP+S was 34% higher than S) (Figure 2).

3.3. β cell mass: Whereas INGAP-PP treated rats showed a significant increase in β cell mass ($p < 0.05$), those treated with suramin significantly decreased that mass ($p < 0.05$; Figure 3A, B, C and E). However, the lowering effect of suramin on β cell mass was not evident when S rats were treated with INGAP-PP (S vs. I-PP+S $p < 0.05$; Figure 3D and E).

3.4. Apoptosis: Islets isolated from I-PP rats showed a significant increase in mRNA levels of the anti-apoptotic marker, Bcl-2 ($p < 0.05$ vs. C). The presence of suramin did not significantly affect Bcl-2 expression in C or in I-PP islets (Figure 4A).

Conversely, mRNA levels of intrinsic apoptosis markers (Caspase-8, Bad, and Bax) were significantly decreased in I-PP islets (Figure 4B, C and D; $p < 0.05$ vs. C). Levels of Bad mRNA were decreased in S islets, whereas Bax mRNA levels were increased by suramin treatment (Figure 4C and D). INGAP-PP was unable to attenuate the enhancing effect of suramin on Caspase-8 mRNA level when both compounds were administered

simultaneously to normal rats (Figure 4B); however, the lowering effect of INGAP-PP upon Bax mRNA levels was still evident in I-PP+S islets (Figure 4D).

3.5. Neogenesis and Angiogenesis: INGAP-PP increased islet neogenesis: the mRNA level of Ngn-3, a traditional neogenesis marker, was significantly increased ($p<0.05$) in islets isolated from I-PP- treated rats, whereas the presence of suramin decreased it. However, addition of INGAP-PP (I-PP+S group) partially reversed this effect (Figure 5A).

mRNA levels of the pro-angiogenic marker VEGF-A were significantly higher in I-PP compared to those found in C islets. Suramin administration significantly decreased VEGF-A mRNA levels; once again, simultaneous administration of INGAP-PP and suramin partially overcame this inhibitory effect (Figure 5B).

INGAP-PP administration also significantly increased mRNA levels of VEGF-R2, CD31, Ang1, and Ang2 compared to C rats. Suramin affected none of these mRNA levels (Figure 5C, D, E and F). However, the enhancing effect of INGAP-PP was still evident in I-PP+S islets. (Figure 5C, D, E and F).

When we studied two major markers of β - and endothelial-cell crosstalk, Laminin β -1 and Integrin β -1, we found that I-PP islets showed a significant increase in both mRNA levels ($p<0.05$; Figure 5G and H). Suramin administration decreased only laminin- β -1 mRNA levels; however, this effect was not reversed by simultaneous administration of INGAP-PP (Figure 5G).

3.6. Insulin intracellular pathway mediators: I-PP islets increased mRNA level of IR, IRS1/2, Akt-1, and PI3K ($p<0.05$ vs. C; Figure 6) whereas suramin administration decreased IR and IRS1 mRNA levels (Figure 6A and B). Simultaneous administration of both compounds (I-PP+S group) partly reversed the lowering effect of suramin only in the case of IRS 1/2 and Akt-1 mRNA levels (Figure 6B, C and D).

3.7. mTORC signalling: mRNA level of mTOR and HIF-1 α were significantly increased in I-PP islets ($p < 0.05$ vs. C); conversely, INGAP-PP administration decreased Tsc-2 mRNA levels (Figure 7). Suramin did not significantly affect Tsc-2 mRNA levels but significantly decreased those of mTOR and HIF-1 α . (Figure 7). Simultaneous administration of INGAP-PP and suramin reversed the inhibitory effect of the latter upon HIF-1 α (Figure 7B).

4. Discussion

Our current results confirm and provide additional evidence that administration of INGAP-PP to normal rats enhanced β -cell mass and function with VEGF-A playing an active role in this mechanism [9-13, 16]. In fact, islets isolated from INGAP-PP-treated rats showed a significant increase in their GSIS at every glucose concentration tested. However, this effect did not affect metabolic homeostasis in the intact animals since INGAP-PP treated rats showed no significant differences in any of the serum parameters tested (glucose, triglyceride, and TBARS levels) or on HOMA-IR and $-\beta$ indexes.

Administration of a VEGF-A antagonist, suramin [35], to normal rats, significantly decreased GSIS at 16.7 mM glucose, suggesting an active role of VEGF-A in the mechanism by which glucose stimulates insulin secretion. However, since suramin blocks specific cell surface binding of other factors, such as PDGF, EGF, TGF β , IGF-1, and FGF [42], we cannot discard the involvement of some of these factors. The fact that the enhancing effect of INGAP-PP on insulin release was still present in I-PP+S rats, suggests that a) suramin inhibition on GSIS can be overcome, at least, by exogenous administration of INGAP-PP and b) the VEGF-A pathway is probably not the only

mechanism involved in modulating the enhancing effect of INGAP-PP on glucose-induced insulin secretion.

We previously demonstrated that the positive modulatory effect of INGAP-PP on insulin release is based on its effect upon GK activity and glucose metabolism as well as on cross-talk between β - and endothelial-cells through the PI3K/AKT pathway [14, 16]. The latter assumption is supported by the demonstration that the PI3K/Akt signal transduction pathway actively participates in the process by which VEGF-A promotes endothelial cell survival through inhibition of their apoptosis rate [31]. In this regard, stimulation of insulin intracellular mediators –including PI3K/AKT- by glucose was reported earlier [43], and subsequent studies confirmed their modulatory role in glucose-induced insulin secretion/synthesis [9, 14, 44] as well as in β -cell growth and survival [45]. Thus, INGAP-PP could use a common path to enhance insulin secretion and β -cell mass.

Our current results support this hypothesis: in accordance with our previous reports, INGAP-PP administration to normal rats increased β -cell mass [11,16] and suramin significantly decreased it. Similar to reports on GSIS, INGAP-PP prevented the lowering effect of suramin on β -cell mass when it was administered to S rats. Also, mRNA levels of some pro-apoptotic markers of the intrinsic pathway (Caspase-8, Bax, and Bad) were significantly lower with INGAP-PP treatment whereas gene expression of Ngn-3, a neogenesis marker, was increased, suggesting an improvement in β -cell mass in these animals. INGAP-PP also stimulated factors related to angiogenesis (VEGF-A, VEGF-R2, and Angiopoietin1/2) and insulin signalling pathway (IR, IRS1/2, PI3K, and Akt) in both absence and presence of suramin. The lack of a direct effect of suramin upon angiogenesis related factors' gene expression, could be attributed to the

fact that suramin not only blocks VEGF-A receptor but also interferes in the binding of other factors to their receptors [42].

Downstream effectors of PI3K cascade also modulate mTORC pathway, which in turn up-regulates multiple processes including angiogenesis [34]. Tsc2, a negative regulator of mTOR signalling, is inhibited by phosphorylation mediated by Akt [46]. Our results showed that INGAP-PP treatment decreased Tsc2 mRNA levels and significantly increased those of mTOR and HIF-1 α , suggesting that INGAP-PP could positively modulate mTORC pathway.

VEGF-A gene expression is modulated by various transcription factors including HIF-1 α [21]. Binding of HIF-1 α to the VEGF-A promoter is essential for maximal transcription of VEGF-A mRNA [22-26], and this effect could be potentiated by INGAP-PP administration. Also, whereas suramin alone had an inhibitory effect on mTOR and HIF-1 α gene expression, its co-administration with INGAP-PP was able to revert its enhancing effect upon HIF-1 α , thereby providing additional support for this proposed sequence of intracellular cascade.

In brief, we postulate that INGAP-PP could produce its enhancing effect on insulin secretion and β -cell mass through the following sequence of events: by interacting with a specific receptor (not yet identified) it would act at intracellular level through PI3K/Akt/mTOR pathways [26], simultaneously activating angiogenesis through HIF-1 α -mediated VEGF-A secretion [16].

4.1. Conclusion: Our results provide new evidence to improve our understanding of the role of angiogenesis in the mechanism by which INGAP-PP affects β -cell mass and function. They also lend further support to the potential use of INGAP-PP as an

antidiabetic agent able to improve these two major alterations found in all patients with type 2 diabetes.

Author Contributions

LEF, BM, and JJG conceived and designed the study. Drs. LEF, BM, CLR, MMV, AL, DZH and AM carried out the experiments and statistical analysis, and all the authors contributed to develop and approve the final manuscript.

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Figures and tables

Figure 1: Experimental design. All animals were injected with the same volume (200µl) of either saline, suramin, or INGAP-PP solution at the final dose described in the figure. INGAP-PP and saline were administered daily (from day 1 to 10), divided into two injections (at 8:00 am and 8:00 pm). These conditions resulted in 4 experimental groups: Control (C group, white box); I-PP with 2mg/Kg/day of INGAP-PP every 12 hs for ten consecutive days (black box); S with a single dose of 100 mg/Kg of suramin at outset of treatment (day 0; light grey box) and I-PP+S (dark grey box) where suramin and INGAP-PP were administered in the same doses as in S and I-PP rats, respectively. We included 22 animals per group.

Table 1: Primer sequences. Fw, forward primer and Rv, reverse primer.

Gene	GeneBank Accession number	Sequences
β actin	NM_031144.3	Fw 5'-TGTCACCAACTGGGACGATA-3' Rv 5'-ACCCTCATAGATGGGCACAG-3'
Ngn-3	NM_021700.1	Fw 5'-GGCGCCTCATCCCTTGATG-3' Rv 5'-CAGTCACCCACTTCTGCTTCG-3'
Vegf-A	NM_031836.2	Fw 5'-GCTTTACTGCTGTACCTCCAC-3' Rv 5'-GTATATCTTCAAGCCGTCCTG-3'
Vegf-A R2	NM_013062.1	Fw 5'-AGCGCTGTGAACGCTTGCCT-3' Rv 5'-ACACACAGCGAGCACCAGC-3'
Angiopoietin 1	NM_053546	Fw 5'-ACGGGGACAGCAGGCAAACAG-3' Rv 5'-CACAGGCATCAAACCACCAACC-3'
Angiopoietin 2	BC161931	Fw 5'-CCTGTCCGCGAGGAGTCCA-3' Rv 5'-GCGTCGAACCACCAGCCTCC-3'
CD31	NM_031591.1	Fw 5'-AGGTGACCGTGGACAAAAAG-3' Rv 5'-CTGTTAGTGTTTCGCTGCCA-3'
Integrin β -1	NM_017022.2	Fw 5'-GAGAGAGATTACTTCAGAC-3' Rv 5'-AGCAGTCGTGTTACATTC-3'
Laminin β -1	NM_001106721.1	Fw 5'-GAGCGGTCTTCTGACTTTGG-3' Rv 5'-AAGCAGGATCTAAGGCACGA-3'
Insulin receptor	NM_017071	Fw 5'-ATATTGACCCGCCCCAGAGG-3' Rv 5'-TAGGTCCGGCGTTCATCAGA-3'
IRS-1	NM_012969	Fw 5'-TGTGCCAAGCAACAAGAAAG-3' Rv 5'-ACGGTTTCAGAGCAGAGGAA-3'
IRS-2	NM_001168633.1	Fw 5'-CTACCCACTGAGCCCAAGAG-3' Rv 5'-CCAGGGATGAAGCAGGACTA-3'
PI3K	NM_053481	Fw 5'-TGCCCCATTTATCCTTGTG-3' Rv 5'-GGTTGTTGTTGCCCCAGAC-3'
Akt-1	NM_033230.2	Fw 5'-GGACCACCGCCATTCAGAC-3' Rv 5'-GGTGCCCTTGCCCAGTAG-3'
Caspase-8	NM_022277.1	Fw 5'-TAAAAAGCAGCCCAGAGGAA-3' Rv 5'-ATCAAGCAGGCTCGAGTTGT-3'
Caspase-9	NM_031632.1	Fw 5'-CCAGATGCTGTCCCATACC-3' Rv 5'-ATTGGCGACCCTGAGAAG-3'
Bad	NM_022698.1	Fw 5'-CAGGCAGCCAATAACAGTCA-3' Rv 5'-CCCTCAAATTCATCGCTCAT-3'
Bax	NM_017059	Fw 5'-CGAGCTGATCAGAACCATCA-3' Rv 5'-CTCAGCCCATCTTCTTCCAG-3'
Bcl-2	L14680.1	Fw 5'-CGGGAGAACAGGGTATGA-3' Rv 5'-CAGGCTGGAAGGAGAAGAT-3'
Tsc-2	NM_012680.3	Fw 5'-CTTCGCCGCAGAGCAGTATG-3' Rv 5'-GGAACGCAGACCCCTAGTGA-3'
mTOR	NM_019906.1	Fw 5'-GAGGCCATCCGAGTGTTAGG-3' Rv 5'-CTCGTCCAGTGGCAGGTTT-3'
HIF-1 α	NM_024359.1	Fw 5'-AGCCGGGGGAGGACGATGAA-3' Rv 5'-CACACTGCGGCTGGTTAC-3'

Table 2: Plasma parameters. Values represent the mean $\pm$ SEM from 22 animals per experimental group. None of the parameters determined showed significant differences against C.

Group	Control	INGAP-PP	Suramin	INGAP-PP+ Suramin
Body weight gain (g)	61.7 $\pm$ 3.37	63.8 $\pm$ 3.29	57.3 $\pm$ 2.65	58.15 $\pm$ 3.30
Food intake (g)	20.5 $\pm$ 1.09	20.5 $\pm$ 0.84	21 $\pm$ 1.34	19.5 $\pm$ 0.70
Glucose (mg%)	107.3 $\pm$ 2.58	111.4 $\pm$ 2.49	110.6 $\pm$ 2.04	113.5 $\pm$ 2.3
Insulin (ng/ml)	0.33 $\pm$ 0.02	0.35 $\pm$ 0.03	0.33 $\pm$ 0.02	0.40 $\pm$ 0.04
Triglyceride (mg/dl)	82.85 $\pm$ 13.43	89.52 $\pm$ 9.87	82.15 $\pm$ 9.73	82.06 $\pm$ 9.49
TBARS (pmoles/mg)	72.3 $\pm$ 4.64	73.1 $\pm$ 5.5	75.2 $\pm$ 7.02	77.2 $\pm$ 5.09
HOMA-IR	1.93 $\pm$ 0.19	1.94 $\pm$ 0.16	1.85 $\pm$ 0.12	2.20 $\pm$ 0.24
HOMA- β	24.13 $\pm$ 1.64	22.2 $\pm$ 2.08	25.15 $\pm$ 2.41	25.15 $\pm$ 2.57

Figure 2: Glucose stimulated insulin secretion (GSIS). Insulin secretion in response to 3.3 and 16.7 mmol/L glucose by islets isolated from C (white bar), I-PP (black bar), S (light grey bar) and I-PP+S (dark grey bar) rats. Insulin released into incubation media

is expressed as ng of insulin per islet/1h. Bars represent mean values $\pm$ SEM from five independent experiments. * $p < 0.05$ vs. C; ^ $p < 0.05$ vs. S.

Figure 3: β cell mass: A–D Pancreas sections from C (A), I-PP (B), S (C) and I-PP+S (D) rats stained with insulin antibody (blue cytoplasm). Morphometric analysis (E), bars represent mean values $\pm$ SEM from three levels. C (white bar), I-PP (black bar), S (light grey bar) and I-PP+S (dark grey bar) rats. * $p < 0.05$ vs. C; ^ $p < 0.05$ vs. S; # $p < 0.05$ vs. I-PP.

Figure 4: mRNA levels of apoptosis markers. A) Bcl-2, B) Caspase-8, C) Bad, and D) Bax mRNA level in isolated islets from C group (white bars), I-PP (black bars), S (light grey bars), and I-PP+S (dark grey bars). β actin was used as internal standard and values are expressed in arbitrary units compared to mRNA determined in C islets.

* $p < 0.05$ vs. C, # $p < 0.05$ vs. I-PP, ^ $p < 0.05$ vs. S.

Figure 5: mRNA levels of neogenesis and angiogenesis markers. (A) Ngn-3; (B) VEGF-A; (C) VEGF-R2; (D) CD31; (E) Angiopoietin 1; (F) Angiopoietin 2; (G) Laminin β -1; (H) Integrin β -1. mRNA level in isolated islets from C group (white bars), I-PP (black bars), S (light grey bars), and I-PP+S (dark grey bars). β actin was used as internal standard and values are expressed in arbitrary units compared to mRNA level determined in C islets. * $p < 0.05$ vs. C, # $p < 0.05$ vs. I-PP, ^ $p < 0.05$ vs. S.

Figure 6: mRNA levels of intracellular insulin pathway mediators. (A) Insulin receptor (IR), (B) IRS-1; (C) IRS-2; (D) Akt-1; (E) PI3K. mRNA level in isolated islets from C group (white bars), I-PP (black bars), S (light grey bars), and I-PP+S (dark grey bars). β actin was used as internal standard and values are expressed in arbitrary units compared to mRNA level determined in C islets. * $p < 0.05$ vs. C, # $p < 0.05$ vs. I-PP, ^ $p < 0.05$ vs. S.

Figure 7: mRNA levels of mTORC mediators. (A) mTOR, (B) HIF-1 α , (C) Tsc-2 mRNA level in isolated islets from C group (white bars), I-PP (black bars), S (light grey bars), and I-PP+S (dark grey bars). β actin was used as internal standard and values are expressed in arbitrary units compared to mRNA levels determined in C islets. * $p < 0.05$ vs. C, # $p < 0.05$ vs. I-PP, ^ $p < 0.05$ vs. S.