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Maturação da resposta secretória à glicose pelo INGAP (*Islet Neogenesis Associated protein*) em ilhotas de Langerhans de ratos neonatos.

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Tese apresentada ao Instituto de Biologia para obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Fisiologia.

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Lista de abreviaturas

4-DAMP – *4-diphenylacetoxy-N-methylpiperidine methiodide*

Akt – *thymoma viral oncogene homolog 1*

Akt$^{-Ser473}$ – Akt fosforilada no resíduo serina 473

AP1 – *jun proto-oncogen transcription factor; jun oncogene*

ATP – *adenosine triphosphate*

BSA – *bovine serum albumin*

cAMP – *cyclic adenosine monophosphate*

Cch – *carbachol*

EDTA – *ethylenediamine tetraacetic acid*

eIF-2a – *eukaryotic translation initiation factor 2A*

eIF-2Be – *eukaryotic translation initiation factor 2B*

eIF-2α – *eukaryotic translation initiation factor 2*

eIF-5 – *eukaryotic translation initiation factor 5*

ERK1/2 – *mitogen-activated protein kinase 1/2*

ERK1/2$^{-Thr202/Tyr204}$ – ERK1/2 fosforiladas nos resíduos treonina 202 e tirosina 204, respectivamente

EtBr – *ethidium bromide*

FGFs – *fibroblast growth factors*

FGR – *Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homolog*

FOXA2 – *hepatocyte nuclear factor 3β*

GLP-1 – *glucagon-like peptyde*

HEPES – *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

Hlxb9 – *homeobox gene HB9*

HNF's – *hepatocyte nuclear factors*

HNF1β – *hepatocyte nuclear factor 1β*

HNF3β – *hepatocyte nuclear factor 3β*

IAPP – *Islet amyloid polypeptide*

INGAP – *islet neogenesis associated protein*

Isl1 – *islet 1*

Kir6.2 – *ATP-sensitive inwardly rectifying K^+ channel*

KRB – *Kreb's buffer*

MAP3K1 – *mitogen-activated protein kinase kinase 1 (MKK1)*

MAPK – *mitogen-activated protein kinase*

MIN6 – *mouse insulinoma cells*

mTOR – *mammalian target of rapamycin*, *FK506 binding protein 12-rapamycin associated protein 1; Frap1*

NaCl – cloreto de sódio

NeuroD – *neurogenic differentiation*

Ngn3 – *neurogenin 3*

Nkx2.2 – *NK2 transcription factor related, locus 2*

Nkx6.1 – *NK6 transcription factor related, locus 1*

P70S6K – *protein 70 S6 kinase*

Pax4 – *paired box gene 4*

Pax6 – paired box gene 6

Pdx1 – *pancreatic duodenal homeobox*

PHAS-I - *eukaryotic initiation factor 4E-binding protein (4EBP1)*

PI3K – *phosphatidylinositol 3'-kinase*

PKA – *protein kinase A*

PLC-β2 – *phospholipase C β2*

Ptf1a – *pancreas specific transcription factor 1a*

RNA – *ribonucleic acid*

RPMI 1640 – *Roswell Park Memorial Institute*

RPS-29 – *ribosomal protein S-29*

RT-PCR – *reverse transcription-polymerase chain reaction*

SDS–PAGE – *sodium dodecyl sulfate polyacrylamide gel electrophoresis*

SNAP25 – *synaptosomal-associated protein*

STZ - *streptozotocin*

SUR1 – *sulphonylurea receptor 1*

UFS1 – *upstream stimulatory factor*

(α-^{33}P) dATP – *alpha ^{33}P 2'-deoxyadenosine 5'-triphosphate*

Resumo

Islet Neogenesis Associated Protein (INGAP) aumenta a massa das células β e potencializa a secreção de insulina induzida por glicose. Neste projeto, estudamos os efeitos de um pentadecapeptídeo contendo a seqüência 104 a 118 de aminoácidos do INGAP (INGAP-PP) sobre a expressão de genes das células insulares, expressão e fosforilação de componentes das vias PI3K e MAPK, sinalização colinérgica, bem como secreções dinâmica e estática de insulina, em ilhotas isoladas de ratos neonatos. Ilhotas cultivadas com INGAP-PP por 4 dias secretaram significativamente mais insulina em resposta a glicose, comparado às ilhotas controle. Análise do padrão da expressão, por *macroarray*, de ilhotas cultivadas com INGAP-PP, mostrou que de 2.352 genes fixados na membrana de *nylon* 210 apresentaram expressão aumentada e apenas 4 diminuída. Dentre os genes modulados positivamente pelo INGAP-PP vários estão relacionados com o metabolismo das células insulares, mecanismo de secreção de insulina, crescimento, maturação, manutenção da massa celular e exocitose. Exposição aguda de ilhotas neonatais ao INGAP-PP aumentou significativamente a fosforilação de $Akt^{-Ser473}$ e $ERK1/2^{-Thr202/Tyr204}$ bem como a secreção dinâmica de insulina frente a 2 e 20 mM de glicose. Ilhotas tratadas durante 4 dias com INGAP-PP também apresentaram aumento da expressão do receptor muscarínico M3 e da PLC-β2. Essas ilhotas, quando expostas agudamente ao Cch tiveram fosforilação de $P70S6K^{-Thr389}$ e ERK1/2 aumentada. Ainda, essas ilhotas secretaram mais insulina frente a estímulo colinérgico, comparado às ilhotas controle. Nossos resultados mostram que o INGAP-PP aumenta a secreção de insulina, a transcrição de vários genes importantes para a funcionalidade do pâncreas endócrino e a fosforilação de proteínas envolvidas nas vias PI3K e MAPK. O aumento da secreção de insulina bem como fosforilação de P70S6K e ERK1/2 pelo Cch sugere participação também da via colinérgica nos efeitos mediados pelo INGAP-PP.

Abstract

The Islet Neogenesis Associated Protein (INGAP) increases pancreatic β-cell mass and potentiates glucose-induced insulin secretion. Here, we have studied the effects of the pentadecapeptide having the 104-118 amino acid sequence of INGAP (INGAP-PP) on the expression of genes related to the pancreatic islets, the expression and phosphorylation of components of the PI3K and MAPK pathways, the cholinergic signaling, and static and dynamic insulin secretion in neonatal rat islet. Islets cultured with INGAP-PP released significantly more insulin in response to glucose than controls. The macroarray analysis showed that 210 out of 2,352 genes, spotted in the nylon membranes, were up-,regulated while only 4 were down-regulated by INGAP-PP-treatment. The main categories of genes modulated by INGAP-PP 4-days cultured islet include several genes related with islet metabolism, insulin secretion mechanism, growth, maturation, maintenance of islet-cell mass, and exocytosis. Short-term exposure of neonatal islets to INGAP-PP significantly increased Akt$^{-Ser473}$ and ERK1/2$^{-Thr202/Tyr204}$ phosphorylation as well as insulin secretion from islets perifused with 2 and 20 mM glucose. Four-days cultured islets with INGAP-PP also showed increased expression of M3 receptor subtype and PLC-β2 proteins. In addition, brief exposure of INGAP-PP-treated islets to Cch significantly increased P70S6K$^{-Thr389}$ and ERK1/2 phosphorylation and these islets released more insulin when challenged with Cch. In conclusion, these data show that INGAP-PP enhances insulin secretion and transcription of several islet genes, and also increases the expression and phosphorylation of proteins involved in PI3K and MAPK pathways. The increased insulin secretion in response to Cch as well as P70S6K and ERK1/2 proteins phosphorylation, also suggest the participation of the cholinergic pathway in INGAP-PP-mediated effects.

Introdução

A conservação de massa adequada e funcional das células secretoras de insulina é fundamental para o indivíduo responder à demanda desse hormônio nos vários períodos da vida e assim manter a normoglicemia. Vários fatores exercem funções fundamentais na célula β pancreática, seja estimulando ou suprimindo crescimento, sobrevivência, diferenciação ou secreção de insulina. As mudanças adaptativas que ocorrem durante o crescimento bem como a diferenciação e a maturação do pâncreas endócrino fetal são mediadas por uma variedade de sinais (Nielsen *et al*. 2001). Durante o desenvolvimento embrionário, a diferenciação em células pancreáticas produtoras de insulina, glucagon, somatostatina e outros polipeptídeos é determinada pela expressão de uma série de fatores de transcrição (HNF's; Pdx1; Ngn3; NeuroD; Pax4; Nkx2.2; Nkx6.1 entre outros) (Jensen *et al*. 2000, Gasa 2005). O desenvolvimento pancreático ocorre em células da região endodérmica onde o fator de transcrição Pdx1 é expresso. Ptf1a, um fator de transcrição do tipo *helix-loop-helix,* é necessário para o desenvolvimento do pâncreas ventral, enquanto Hlxb9 desencadeia o desenvolvimento do pâncreas dorsal (Edlund *et al*. 2002). Já é bem estabelecido que tanto células endócrinas quanto exócrinas são originárias de um *pool* de células endodérmicas que expressam os fatores de transcrição Pdx1 (Gu *et al*. 2002) e Ptf1a (Kawaguchi *et al*. 2002). Essas células progenitoras têm sua proliferação estimulada por FGFs (Elghazi *et al*. 2002), enquanto o Isl1 é essencial para o desenvolvimento do mesênquima da porção pancreática dorsal e pela diferenciação deste em células endócrinas (Ahlgren *et al*. 1997). Durante o desenvolvimento embrionário de camundongo as células endócrinas se originam a partir de células ductais que expressam HNF1β e Ngn-3, entre E14 e E17. Dentre células positivas para Ngn-3, aquelas que expressam Pax4 originarão células β, sendo que altas concentrações de Pdx-1, Nkx6.1, Nkx2.2 e Pax6 são observadas em células β já diferenciadas (Jensen 2004). Nesta fase, as células param de se dividir e entram em estado "pós-mitótico", voltando a se replicar durante o crescimento fetal tardio, quando ocorre aumento rápido da massa de célula β, com duplicação da população celular num período de 24 horas, a partir do 16° dia após a fecundação, em ratos (McEvoy *et al*. 1980). Desse

aumento, 80% é atribuído à neogênese a partir da diferenciação e proliferação rápida de células precursoras. Evidências tanto de replicação quanto de neogênese de células β também são observadas após o nascimento, sendo que a neogênese ocorre a partir de células ductais encontradas nas proximidades das ilhotas, e com taxa proliferativa maior que aquela registrada nas células β já existentes (Bouwens *et al*. 1994). Após a primeira semana do nascimento, a massa de células β aumenta devido à duplicação de células já diferenciadas, conforme experimentos em camundongos *knockout* para a ciclina D2, um regulador positivo do ciclo celular. Nesses animais observou-se redução significativa do número de células β (Georgia & Bhushan 2004). Finalmente, durante a vida adulta a manutenção de massa insular adequada depende da reposição das células β perdidas e, embora ainda controverso, acredita-se que esse processo se deve à replicação de células β pré-existentes, diferenciação de novas células a partir de precursores presentes nas células ductais e/ou extra-ilhotas, alem de redução da apoptose (Bouwens *et al*. 1994; Butler *et al*. 2003, Dor *et al*. 2004; Rosenberg *et al*. 2004; Dor *et al*. 2008).

Dessa maneira, durante o desenvolvimento embrionário e fetal, o aumento da massa insular seria, em maior grau, dependente do processo de neogênese de células β, contudo após nascimento, a neogênese teria pouca relevância. Apesar dessa pouca relevância, neogênese considerável pode ser observada em pâncreas de animais adultos em situações especiais onde ocorre regeneração induzida por processos tais como: injeção de estreptozotocina (Fernandes *et al*. 1997), pancreatectomia parcial (Lee *et al*. 1989), ligação do ducto pancreático (Wang *et al*. 1995, Xu *et al*. 2008) ou empacotamento da cabeça do pâncreas em papel celofane (Rosenberg *et al*. 1983).

INGAP e o pâncreas endócrino

Um polipeptídeo denominado INGAP, considerado um membro da família de proteínas relacionadas à regeneração (*Reg3*) (Taylor-Fishwick *et al*. 2003) foi identificado no pâncreas durante a

neogênese de ilhotas a partir de células do ducto pancreático. Os primeiros registros da existência do INGAP foram feitos em pâncreas de *hamsters* cuja cabeça do pâncreas fora previamente embrulhada em papel celofane (Pittenger *et al*. 1992) e também em camundongos normais ou diabéticos por STZ (Rosenberg *et al*. 1996, 2004). O empacotamento do pâncreas por papel celofane é capaz de induzir novos ninhos de ilhotas a partir do ducto no período de duas semanas (Bonner-Weir *et al*. 1993, Leahy 1996). INGAP, originalmente obtido em *hamsters,* consiste em uma proteína contendo 175 aminoácidos, relacionado às lectinas do tipo C, e sua expressão parece ser restrita ao pâncreas e ao duodeno (Rafaeloff *et al*. 1997, Flores *et al*. 2003, Borelli *et al*. 2007). Através de técnicas de clonagem e seqüenciamento foi identificado um pentadecapeptídeo compreendendo a seqüência 104-118 de resíduos de aminoácidos que compõem o INGAP, denominado INGAP-PP ou INGAP$^{104\text{-}118}$ (Rosenberg *et al*. 1996, Rafaeloff *et al*. 1997), capaz de reproduzir o efeito da molécula intacta e que tem sido utilizado nos estudos dos efeitos do INGAP. Administração de INGAP-PP tanto a camundongos quanto cães normais adultos resultou em aumento de massa da célula β e sinais de neogênese (Rosenberg *et al*. 2004, Pittenger *et al*. 2007), sendo as mesmas alterações observadas quando da administração de INGAP-PP a camundongos tornados diabéticos por STZ. Por outro lado, em *hamsters* jovens a administração de água contendo sacarose aumentou a massa da célula β pancreática, com simultâneo aumento na taxa de replicação de células β bem como aparecimento de novas ilhotas (Del Zotto *et al*. 1999, 2000), sugerindo a participação do INGAP também em processos neogênicos induzidos pela referida dieta.

A presença de Pdx-1 é essencial para o desenvolvimento do pâncreas endócrino sendo mais expresso nas fases intra-útero e neonatal, comparado à fase adulta (Stoffers *et al.* 2000, McKinnon & Docherty 2001). No entanto, número significativamente maior de células pancreáticas positivas para Pdx-1 ou INGAP foi observado em descendentes de ratas normais que receberam 10% de sacarose na água durante a gravidez. Essas células também apresentaram altas taxas de replicação, porém sem a

presença de insulina, glucagon, somatostatina ou peptídeo P. Associado a isso, houve aumento de massa de células positivas para INGAP nas ilhotas e nos ductos pancreáticos, além de considerável aumento da massa de células positivas para Pdx-1, particularmente naquelas positivas para INGAP e localizadas nos ductos pancreáticos (Gagliardino *et al*. 2003). Neogênese de ilhota e aumento de Pdx-1 também foram observados 3 dias após pancreatectomia parcial em ratos normais (Sharma *et al*. 1999), acompanhados por aumento na taxa de replicação de células ductais. Esses achados sugerem que as células positivas para Pdx-1/INGAP representam uma subpopulação de precursores de ilhotas em um estágio inicial de desenvolvimento, indicando que o INGAP deve estar envolvido na regulação desses processos neogênicos, uma vez que o crescimento e diferenciação celular foram acompanhados por alterações relacionadas à massa de células positivas para INGAP. Ilhotas humanas, cultivadas em meio RPMI 1640 por 10 dias, adquirem característica de células ductais e, quando tratadas com INGAP por 4 dias, voltam a apresentar características estruturais semelhantes a ilhotas normais (Jamal *et al*. 2005). Essas estruturas, quando estimuladas por glicose secretam insulina em quantidades comparáveis a ilhotas frescas. Ainda, demonstrou-se que a ação do INGAP na reconstituição das ilhotas depende da ativação da via da PI3K.

A falha em se adaptar a mudanças associadas à obesidade, gravidez, sensibilidade à insulina em tecidos periféricos ou lesão pancreática podem levar à hiperglicemia crônica. O aumento global do número de indivíduos diabéticos tem estimulado o desenvolvimento de estratégias terapêuticas no sentido de repor a massa de células β (transplante de ilhotas isoladas ou de pâncreas), reduzir sua destruição ou estimular sua regeneração. O uso de insulina exógena e/ou de agentes hipoglicemiantes disponíveis se mostra insatisfatório, já que esses medicamentos não curam a síndrome e não previnem complicações secundárias associadas ao diabetes. Nesse sentido, o transplante de células β parece uma alternativa interessante (Street *et al*. 2004), porém a escassez de doadores, as dificuldades técnicas do isolamento de um grande número de ilhotas viáveis bem como a necessidade de imunossupressão

tornam esse procedimento praticamente inviável como terapia para um grande número de pacientes. Dentre as possibilidades para contornar esses problemas, alternativas tais como: geração de células β, melhora da funcionalidade das ilhotas de doadores disponíveis ou, ainda, aumento ou regeneração da massa de células β através de fatores de crescimento têm sido estudadas. Nesse sentido, o uso do INGAP para promover redução da hiperglicemia (Rosenberg *et al*. 2004, Ratner *et al*. 2005a, Ratner *et al*. 2005b), aumento da massa de célula (Lipsett *et al*. 2006), recuperação *in vitro* (Jamal *et al*. 2005) e melhora da função da ilhota em resposta a glicose e estímulo colinérgico (Barbosa *et al*. 2006, Barbosa *et al*. 2008 – submetido), parece ser uma ferramenta promissora no tratamento do diabetes. Apesar do número relativamente grande de trabalhos sobre as potencialidades em manter e mesmo melhorar a atividade das ilhotas pancreáticas, os mecanismos de ação utilizados pelo INGAP na indução desses efeitos não estão completamente elucidados.

Assim, durante o desenvolvimento deste projeto de Doutoramento, estudamos os mecanismos de ação do INGAP, mais precisamente o INGAP-PP, sobre vários parâmetros relacionados à fisiologia das ilhotas pancreáticas de ratos neonatos. Inicialmente, traçamos um perfil dos genes modulados pela ação do polipeptídeo, através da técnica de *macroarray*, utilizando membranas de *nylon* comerciais, contendo 2.352 genes fixados. Observamos que o tratamento com INGAP aumentou a expressão de vários genes envolvidos em processos relacionados com crescimento, manutenção e secreção de insulina. Numa segunda etapa, confirmamos que as ações deste polipeptídeo sobre as ilhotas pancreáticas envolvem a ativação das cascatas de sinalização tais como PI3K e MAPK, com participação da via colinérgica.

Objetivos

Os objetivos deste trabalho foram avaliar em ilhotas pancreáticas de ratos neonatos:

1. Os efeitos do tratamento crônico do INGAP sobre a modulação da expressão gênica e protéica;
2. A participação das vias MAPK, PI3K e colinérgica nos mecanismos de ação do INGAP;
3. Os efeitos dos tratamentos agudo e crônico com o INGAP sobre a secreção de insulina.

Os resultados alcançados durante a realização deste trabalho de Tese estão apresentados a seguir sob a forma de dois artigos:

1. ***Islet Neogenesis Associated Protein (INGAP) modulates gene expression in cultured neonatal rat islets (Regulatory Peptides 136 78–84, 2006)*****;**

2. ***Islet Neogenesis Associated Protein signaling in neonatal pancreatic rat islets: involvement of the cholinergic pathway*** **(Submetido,** ***Journal of Endocrinology, 2008*****).**

Artigo 1

Regulatory Peptides 136 (2006) 78–84

Islet Neogenesis Associated Protein (INGAP) modulates gene expression in cultured neonatal rat islets

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Abstract

The Islet Neogenesis Associated Protein (INGAP) increases pancreatic β-cell mass and potentiates glucose-induced insulin secretion. We currently studied the effects of a pentadecapeptide having the 104-118 aminoacid sequence of INGAP (INGAP-PP) on insulin secretion and on transcript profile expression in 4-day-cultured normal pancreatic neonatal rat islets. Islets cultured with INGAP-PP released significantly more insulin in response to 2.8 and 16.7 mM glucose than those cultured without the peptide. The macroarray analysis showed that 210 out of 2,352 genes spotted in the nylon membranes were up-regulated while only 4 were down-regulated by INGAP-PP-treatment. The main categories of genes modified by INGAP-PP included several related with islet metabolism, insulin secretion mechanism, β-cell mass and islet neogenesis. RT-PCR confirmed the macroarray results for nine selected genes involved in growing, maturation, maintenance of pancreatic islet-cells, and exocytosis, i.e., Hepatocyte nuclear factor 3beta (HNF3β), Upstream stimulatory factor 1 (USF1), K^+-channel proteins (SUR1 and Kir6.2), PHAS-I protein, Insulin 1 gene, Mitogen-activated protein kinase 1 (MAP3K1), Amylin (IAPP), and SNAP-25. INGAP-PP also stimulated PDX-1 expression. The expression of three transcripts (HNF3β, SUR1, and SNAP-25) was confirmed by Western blotting for the corresponding proteins. In conclusion, our results show that INGAP-PP enhances specifically the secretion of insulin and the transcription of several islet genes, many of them directly or indirectly involved in the control of islet metabolism, β-cell mass and islet neogenesis. These results, together with other previously reported, strongly indicate an important role of INGAP-PP, and possibly of INGAP, in the regulation of islet function and development.

Introduction

At the embryonic period, pancreas development and islet cell differentiation are controlled by the expression of several transcription factors, such as pancreatic duodenal homeobox-1 (PDX-1), neurogenin3 (ngn3), Nkx-1, as well as many others (1). During the fetal and neonatal periods, adaptative changes of β-cell mass in response to different stimuli are mediated by a variety of hormonal, chemical and neural signals (2). In both cases the majority of new β-cells are formed by neogenesis (3).

Neogenesis is also observed in adult animals after pancreatic injury provoked by streptozotocin injection (4), partial pancreatectomy (5), duct pancreatic ligation (6), cellophane wrapping of the pancreas head (7) and in insulin resistance state induced by sucrose feeding to normal hamsters (8, 9).

INGAP was identified as the active part of a pancreatic protein complex isolated from normal hamsters, whose pancreas heads were previously wrapped in cellophane (10). The INGAP gene expressed both in normal hamster islets and exocrine cells (11). It has been shown that a pentadecapeptide having the 104-118 aminoacid sequence of INGAP (INGAP-PP) reproduces the effect of the intact molecule upon thymidine incorporation into ductal cells and a ductal cell line (12). We have reported that offspring from normal hamsters fed a sucrose diet during pregnancy have an increase in the mass of β-cells, PDX-1 and INGAP-positive cells together with the appearance of a small population of cells that co-express PDX-1/INGAP. Since these cells have a high replication rate and do not stain with insulin-, glucagon-, somatostatin-, or PP-antibodies, we postulated that these cells would be early precursors of islet-cells (11). On the other hand, it was reported an increase in β-cell mass and signs of neogenesis after intraperitoneal injection of this peptide to either normal or streptozotocin-induced diabetic mice (13). More recently, we demonstrated that neonatal and adult normal rat islets cultured with INGAP-PP presented an increase in the size of their β-cells and released significantly more insulin in response to glucose (14). Although these results indicate that INGAP may

be involved in the regulation of islet function and neogenesis, its mechanism of action is not clear as yet.

In an attempt to answer this question, we have analyzed the changes occurred in insulin secretion and gene expression of neonatal islets cultured with INGAP-PP for 4 days using two nylon arrays containing 2,352 spotted genes. Our results showed that INGAP-PP treatment increases simultaneously insulin secretion and the expression of several genes involved in the control of growth and development of islets as well as in their secretory function.

Materials and Methods

Animals and islets

Islets of neonatal Wistar rats were isolated by collagenase digestion and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10 mM glucose, 100 IU of penicillin/ml, 100 μg of streptomycin/ml at 37ºC in a 5% CO_2/air atmosphere for 7 days. At day 3 of culture, we added 5 μg/ml of INGAP-PP to half of the plates, using the remaining ones as control (no INGAP-PP). The medium was renewed every other day. All animal experiments were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas, SP (CEEA/IB/UNICAMP).

Insulin secretion

Cultured neonatal isolated islets were rinsed in HEPES-bicarbonate buffer, pH 7.4, previously gassed with a mixture of CO_2/O_2 (5/95%), and preincubated in 1.0 ml of HEPES-bicarbonate containing 1.5% (w/v) BSA and 5.6 mM glucose at 37ºC for 45 min. After this period, groups of 5 islets were incubated in 1.0 ml HEPES-bicarbonate with the addition of 2.8 or 16.7 mM glucose for 1h. At the end of the incubation period, aliquots of the medium were collected for insulin determination by radioimmunoassay.

Macroarray analysis

The arrays used were the Atlas Rat 1.2 and 1.2 II, Clontech Labs (Palo Alto, CA), containing 1,176 spotted genes each one. Total RNA was obtained from approximately 1000 cultured islets using the Trizol method followed by DNase I (Invitrogen, USA) treatment. The RNA integrity was checked by electrophoresis in agarose denaturating gel and lately quantified by spectrophotometry (GeneQuant, Amersham Biosciences). Radiolabeled cDNA ([α-^{33}P] dATP) was obtained using 5-10 μg of total RNA and a reaction containing a gene-specific CDS primer mix (Clontech Labs, Palo Alto, CA) performed according to the manufacturer's recommendations. Membranes were submitted to an overnight hybridization in a solution containing the radiolabeled cDNA, followed by exposition to a phosphorImager screen (Molecular Dynamics, San José, CA), and finally scanned with a Storm 840 Scanner (Molecular Dynamics). The images obtained were analyzed (Quantity One software, BioRad, USA), the spots were normalized by membrane housekeeping genes and the results expressed as the INGAP-PP-treated/control ratio. Only results higher than the cut-off of two fold or lower than a half, in two independent experiments, were considered as significant.

RT-PCR analysis

Semi-quantitative RT-PCR was done using specific primers to confirm the differential expression of eight up-regulated genes detected in the macroarray analysis. Reverse transcription was done with 3 μg of total RNA using Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers, according to manufacturer's instructions (Invitrogen, USA). RT-PCR assays were done in quadruplicate using recombinant *Taq* DNA polymerase (Invitrogen, USA) and 10 pmol of each primer in a master mix of 50 μl. The primer sets used in RT-PCR analysis are shown in Table 1. The number of cycles for each gene was defined after titration using 20 to 42 cycles and was within the logarithmic phase of amplification. PCR products were separated on 1.5% EtBr-agarose gels and the

band intensities were determined by digital scanning (GelDoc 2000, BioRad) followed by quantification using Scion Image analysis software (Scion Corp., Frerderick, MD). The results were expressed as a ratio of target to RPS-29 signals. The RNAs used for RT-PCR analysis were obtained from three to four sets of experiments.

Western blotting

Cultured islets were homogenized in 100 µl of solubilization buffer (10% Triton-X 100, 100 mM Tris, pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, and 2 mM PMSF) for 30 s using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY) and boiled for 5 min. The extracts were then centrifuged at 12,600 g at 4°C for 20 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method and the BioRad reagent. The proteins were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min. Seventy µg of protein from each sample was applied to a 10% polyacrylamide gel and separated by SDS–PAGE in a BioRad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose was done at 120 V for 50 min or 1 h in a BioRad miniature transfer apparatus. Before incubation with the primary antibody, the nitrocellulose filters were treated with a blocking buffer (5% non-fat dried milk, 10 mM Trizma, 150 mM NaCl, and 0.02% Tween 20) for 2 h at 22°C. The membranes were incubated for 4 h at 22°C with antibodies against, HNF3β (1:500), SUR1 (1:1000) or SNAP-25 (1:1000) (Santa Cruz, CA, USA) diluted in blocking buffer with 3% non-fat dried milk, and then washed for 30 min in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated second antibody for 1 h. Specific protein bands were revealed using commercial enhanced chemiluminescence reagents with exposure to photographic film. The band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

Statistical analysis

Results are shown as means ± SEM, where appropriate statistical comparisons between INGAP-treated islets and the respective control groups were done using the Student´s unpaired t-test. P values < 0.05 indicated a significant difference.

Results

Effect of INGAP-PP on insulin secretion

Normal neonatal islets cultured with INGAP-PP and then incubated with 2.8 and 16.7 mM glucose released significantly more insulin than those cultured in the control medium (Fig. 1).

Macroarray analysis

Of the 2,352 genes spotted in the membranes, 210 were up-regulated and only 4 were down-regulated by INGAP-PP-treatment; complete data are presented in a supplemental Table published on The Endocrine Society's Journals Online web site at (http://endo.endojournals.org). According to the macroarray manufacturer, the main categories of these genes were: Basic Transcription Factors; Facilitated Diffusion Proteins; Voltage-Gated Ion Channels; Targeting and Exocytosis Proteins; Carbohydrate and Amino Acid Metabolism; Chaperones, Heat Shock Proteins and Proteins Modification Enzymes; Post-translational Modifications Proteins and Ribossomal Proteins; Translation Factors; RNA Processing, Turnover and Transport Proteins; DNA-binding and DNA Damage Repair Proteins; Hormones and Neutrotransmitter Receptors; Growth Factors, Cytokines and Chemokines Receptors; Hormones; Intracellular Kinase Network Members; G-Proteins and Protein-Coupled Receptors; GTP/GDP Exchangers, GTPase Activity, Oncogenes and Tumor Supressors Proteins, and others.

A closer analysis of each one of the above categories indicated that, in neonatal islets, INGAP-PP modulated several genes related to general cellular processes and others specifically related to islet

metabolism (pyruvate dehydrogenase kinase and phosphatase, MAP3K1), the mechanism of insulin secretion (K^+- and Ca^{2+}-channels, calcium/calmodulin-dependent protein kinase), exocytosis (synapsin, SNAP25), insulin biosynthesis (insulin) and islet neogenesis (HNF-3β, USF-1).

RT-PCR and Western blotting

Based on the reported effect of INGAP-PP upon insulin secretion (14) and islet neogenesis (13, 15), we selected 9 of the genes affected by this peptide directly and/or indirectly related to these two processes to perform RT-PCR and further confirm such effect. Although not included in the macroarray membranes, we have also evaluated the transcription of PDX-1 due to its close relationship with islet neogenesis and its co-expression with INGAP-PP in islet cells during dietary-induced neogenesis (11). All the up-regulated genes analyzed by RT-PCR confirmed the results obtained in the macroarray (Fig. 2).

Otherwise, when we tested the effect of INGAP-PP on the expression (Western blot) of three transcripts involved in the regulation of PDX-1 expression (HNF3β), β-cell membrane potential (SUR-1), and insulin exocytosis (SNAP-25), we observed a significant increase in the concentration of the corresponding proteins (Fig. 3).

Discussion

Our results showed that INGAP-PP increased significantly the expression of 210 out of 2,352 genes, many of them involved in the regulation of function, growth and development of pancreatic β-cells.

The current data confirmed our own report that insulin secretion increased significantly in islets cultured with INGAP-PP (14). It also provides some alternative mechanistic explanation for this effect. Accordingly, the ATP-sensitive K^+ channels (K_{ATP} channels) in pancreatic β-cells couple insulin

secretion to the extracellular glucose concentration (16): the increase in ATP/ADP ratio induced by glucose metabolism triggers a cascade of events (closes these channels→ depolarizes the β-cell membrane→ opens the voltage-dependent Ca^{2+} channels→ promotes calcium influx→ increases intracellular calcium concentration) that leads to an increase of insulin secretion (17). The β-cell K_{ATP} channels comprise two components: a Kir6.2 subunit and a SUR1 subunit (18). The up regulation of SUR1 and Kir6.2 by INGAP-PP currently shown would play a role in its enhancing effect upon insulin secretion. Complementary, deletion of HNF3-β in pancreatic β-cells of mutant mice resulted in down-regulation of both K_{ATP} channel subunits (19, 20), showing that HNF3β is required for maintaining the expression of SUR1 and Kir6.2. Thus, the INGAP-PP induced increase in HNF3β could also contribute to its effect upon K_{ATP} channels and thereby upon insulin secretion.

The increment of SNAP-25 (synaptosomal-associated protein-25), a t-SNARE protein, in INGAP-PP-treated islets may also contribute to the secretagogue effect of this peptide. In fact, the insulin granule extrusion needs the assembly of a complex between proteins, named soluble N-ethylmaleimide-sensitive factors (NSF)-attachment protein receptors (SNAREs). Among these proteins, those associated with the plasma membrane (t-SNAREs) and those anchored on the membrane of secretory vesicles (v-SNAREs) are involved in the membrane fusion step of the b-cell exocytosis process (21).

It is well established that for most eukaryotic genes, initiation of transcription is a critical and rate-limiting step in gene expression. In our study, INGAP-PP induced an up-regulation of important transcription factors such as Maf2, CCAAT-binding transcription factor subunit B, Myocite-specific enhancer factor 2D (MEF2D), Hepatocyte nuclear factor 3β (HNF3β, FOXA2), Upstream stimulatory factor 1 (USF-1) and others.

HNF3β is a member of the fork head/winged helix transcription factor family and is essential for endoderm cell lineages (22, 23). It increases PDX-1 transcription by binding to specific sequences

in a nuclease hypersensitive site (24, 25), being essential for the control of embryonic development of the endocrine pancreas (26, 27). HNF3β and Neuro-D act synergistically to induce PDX-1 expression in pancreatic islet-cells. Further, overexpression of HNF3β overcomes the blocking effect of glucocorticoids upon its own expression and activity in cultured islet cells (28). Thus, the stimulatory effect of INGAP-PP upon HNF3β transcription and expression (Figs. 2 and 3) may explain, at least partly, the reported neogenic effect of this peptide (7, 10, 13).

INGAP-PP also enhanced USF-1 expression, a factor that also regulates PDX-1 transcription by binding to its promoter (29). Overexpression of a dominant-negative form of the USF2 protein (USF family proteins) reduces simultaneously PDX-1 transcription, its protein expression and insulin promoting activity and the insulin transcription level (29). Conversely, the levels of USF-1 and PDX-1 mRNA increased significantly in islets of rats reared artificially on a high carbohydrate formula during the suckling period (30, 31). Similarly, newborns of normal hamsters fed a sucrose diet during pregnancy showed an increase in the mass of β-cells, PDX-1 and INGAP-positive cells, together with the appearance of PDX-1/INGAP-positive cells (11). These results suggest that pancreas adaptation to a dietary-induced increased demand of insulin (insulin resistance state) includes up-regulation of this specific transcription factor gene in the islet-cells, thereby facilitating increased insulin gene transcription.

Although not included in table 2, we have also observed an up regulation of important transduction and translation factors [Translation initiation factor eIF-2B alpha-subunit (eIF-2α), Initiation factor eIF-2Be, Elongation factor 1 alpha, Eukaryotic initiation iactor 5 (eIF-5)] and of other proteins involved in translation [PHAS-I protein; Eukaryotic hemin-sensitive initiation factor 2a kinase (eIF-2a)]. These results together with the increased expression of several ribosomal proteins suggest that INGAP-PP would induce an overall increase in protein synthesis and metabolic activities in β-cells. INGAP-PP also increased the expression of several proto-oncogenes such as c-fos, c-myc, n-myc,

erbB3, FGR, and jun proto-oncogen transcription factor AP1. The participation of these immediate-early response genes, coding for transcription factors involved in the proliferation, growth and differentiation of different cell lines, including β-cells, is well documented (32, 33, 34). This effect of INGAP-PP on the expression of several immediate-early response genes resembles those obtained with glucose in different β-cell lines (33, 34). All these results indicate that INGAP-PP induces several modifications that favor β-cell growth and differentiation.

The plasticity of β-cell mass and function is important to maintain glucose homeostasis. In this regard, evidence in the literature has shown the importance of Insulin receptor substrate-2 (IRS-2) (35, 36, 37), as a mitogenic signal transduction pathway to pancreatic β-cell growth and survival. IRS-2 triggers at least two distinct signaling pathways in the β-cell, the Phosphatidylinositol 3-kinase (PI3K) pathway and Mitogen-activated protein kinase (MAPK) pathway (35, 38). The latter pathway involves the growth factor receptor-bound protein-2 (Grb2) and a cascade reaction that in its stream phosphorylates MAP/Erk kinase (MEK), leading to Erk-1/2 phosphorylation activation (39, 40, 41). Thus, the increase observed in the MEKK1 transcription induced by INGAP-PP might indicate that this pathway is involved in the neogenic effect of this peptide upon islets, previously described (13). A recent report provides evidence of the participation of the PI3K pathway in islet plastic reactivity (15). These authors showed that a short treatment of primitive duct-like structures, obtained from quiescent adult human islets, with INGAP-PP induced their reconversion to islet-like structures in a PI3-kinase-dependent manner. In their experimental conditions these neoislets resembled freshly isolated human islets regarding the presence and topological distribution of the four endocrine cell types, islet gene expression, hormone production, insulin content and glucose-induced secretion of insulin.

In brief, our results show that INGAP-PP enhances specifically the secretion of insulin and, for the first time, of the transcription of several islet genes, many of them directly or indirectly involved in

the control of islet metabolism, insulin synthesis/secretion, exocytosis and islet neogenesis. These results, together with other previously reported, strongly indicate the role of INGAP-PP, and possibly of INGAP, in the regulation of islet development and function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.regpep.2006.04.015.

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Figure legends

Figure 1. Effect of INGAP-PP-treatment on insulin secretion in isolated neonatal rat islets. After isolation, the islets were cultured for 4 days in RPMI-1640 medium with or without INGAP-PP (10 μg/ml). After culture, groups of 5 islets were incubated in HEPES-bicarbonate buffer containing 2.8 or 16.7 mM glucose/L. The columns represent the cumulative 1 h insulin secretion and are the means ± SEM of 3 independent experiments (n = 18). Means without a common letter differ ($P < 0.05$).

Figure 2. Confirmation by RT-PCR of 10 selected genes that were significantly up-regulated in neonatal pancreatic islets after INGAP-PP-treatment (10 μml). The bars represent the mean ± SEM of 3 experiments done with specific primer sets (Table 1) and normalized against the ribosomal protein S29 (RPS29). See Table 2 for definition of gene abbreviations. *$P < 0.05$, with P values calculated from t test on the average difference between CTL and INGAP-PP.

Figure 3. Protein expression of three transcripts to validate the macroarray analysis. Neonatal pancreatic islets were cultured in the absence or presence of INGAP-PP (10 μg/ml). After 4 days, the proteins were extracted (see Methods) and equal amounts of protein from control and INGAP-PP-treated islets were resolved by SDS–PAGE on 10% gels and transferred to a nitrocellulose membrane. The proteins were identified with anti-HNF3β (a), anti-SNAP-25 (b), and anti-SUR1 antibodies (c). The values are the mean ± SEM of 3 experiments. *$P < 0.05$ for CTL vs. INGAP-PP.

TABLE 1. RT-PCR primer sets with predicted product sizes

Gene	GeneBank	Forward primer	Reverse primer	TM (ºC)	Product (bp)
HNF3β	L09647	CTG AGT GGA AAC ATT GGG G	GAT TTG TGG AACTCT GGC CA	60	570
USF1	AF026476	GAG GGC TCA ACA TAA CGA AGT	AAT CAC ACT TGC CCA ACT CC	63	462
SUR1	AB052294	TTC CAC ATC CTG GTC ACA CCG	AGA AGG AGC GAG GAC TTG CCA C	60	425
Kir6.2	AB043638	TTA GCG CCA CCA TTC ATA TG	TCC GGA GAG ATG CTA AAC TTG	54	401
MKK1	U48596	AGT GAG GAG ACG GCA TTC ACC C	CTC GTT CGC TTT GGT ATG CCC	58	384
Insulin	V01242	ATT GTT CCA ACA TGG CCC TGT	TTG CAG TAG TTC TCC AGT T	57	340
SNAP25	NM030991	GAA TTC AAT GGC CGA GGA CGC AGA	ACT TAA CCA CTT CCC AGC ATC TTT GT	60	621
IAPP	J04544	ATT GCT GCC ACT GCC CAC TG	CCT CTG CCA CAT TCC TCT TCC C	58	280
PHAS1	U05014	GGA AAT TCC TGA TGG AGT G	CTG GAG TAG CAG CTC AGT ATC	58	401
RPS29	NM012876	AGG CAA GAT GGG TCA CCA GC	AGT CGA ATC ATC CAT TCA GGT CG	55	202
PDX-1	NM022852	AAC CGG AGG AGA ATA AGA GG	GTT GTC CCG CTA CTA CGT TT	56	225

SUR1 = Sulfonylurea receptor; Kir6.2 = Inwardly rectifying K^+ channel 6.2 family; RPS29 = Ribossomal protein S29; PDX-1 = Pancreatic duodenal homeobox-1. The remaining gene abbreviations are listed in Table 2.

TABLE 2. Pancreatic islet-related mRNA specificities modulated by INGAP-PP-treatment

mRNA	GeneBank accession	Fold modulation
1. Liver specific transcription factor (LF-B1; HNF1)	J03170	2.42
2. Upstream stimulatory factor 1 (USF1)	AF026476	2.61
3. Hepatocyte nuclear factor 3beta (HNF3β; FOXA2)	L09647	2.57
4. Calcium-activated potassium channel rSK3	U69884	2.30
5. Calcium channel, voltage-dependent, L type, alpha 1E subunit	L15453	2.50
6. Potassium inwardly-rectifying channel, subfamily J, member 2	AF021137	2.48
7. Potassium channel, voltage gated, KV3.4; RAW3; KCNC4	X62841	2.58
8. Calcium channel beta 1 subunit	X61394	2.52
9. Calcium-transporting ATPase	M93017	2.02
10. Synaptotagmin 4	L38247	2.05
11. Synapsin 1	M27812	2.10
12. Secretogranin 2	M93669	2.32
13. Syntaxin binding protein Munc18-2	U20283	2.01
14. Synaptosomal-associated protein, SNAP-25	AB003991	2.17
15. L-type pyruvate kinase	M11709	2.20
16. Islet Amyloid Polypeptide (IAPP)	J04544	2.13
17. Thiol-specific antioxidant protein (1-Cys peroxiredoxin)	Y17295	2.29
18. Eukaryotic initiation factor 4E-binding protein (4EBP1); PHAS-1	U05014	3.28
19. Insulin 1 gene	V01242	3.51
20. Insulin-like growth factor II, somatomedin A	M13969	2.16
21. Insulin-like growth factor I	M15480	2.07
22. Glucagon gene	K02813	3.55
23. Mitogen-activated protein kinase kinase 1(MAP3K1; MKK1)	U48596	2.20
24. Pyruvate dehydrogenase kinase 1	L22294	2.00
25. Cholinergic receptor, muscarinic 3	M18088	2.10

Atlas Rat 1.2 and 1.2 II Array: fold modulation as compared to expression in cultured neonatal normal islets

Figure 1

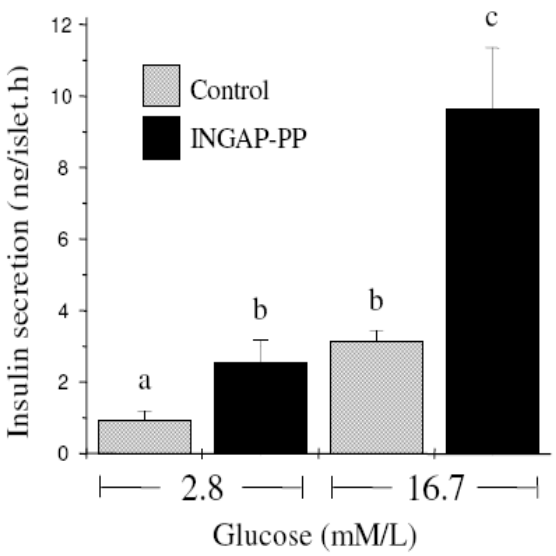

Insulin secretion (ng/islet.h)
12
10
8
6
4
2
0
Control
INGAP-PP
a
b
b
c
2.8
16.7
Glucose (mM/L)

Figure 2

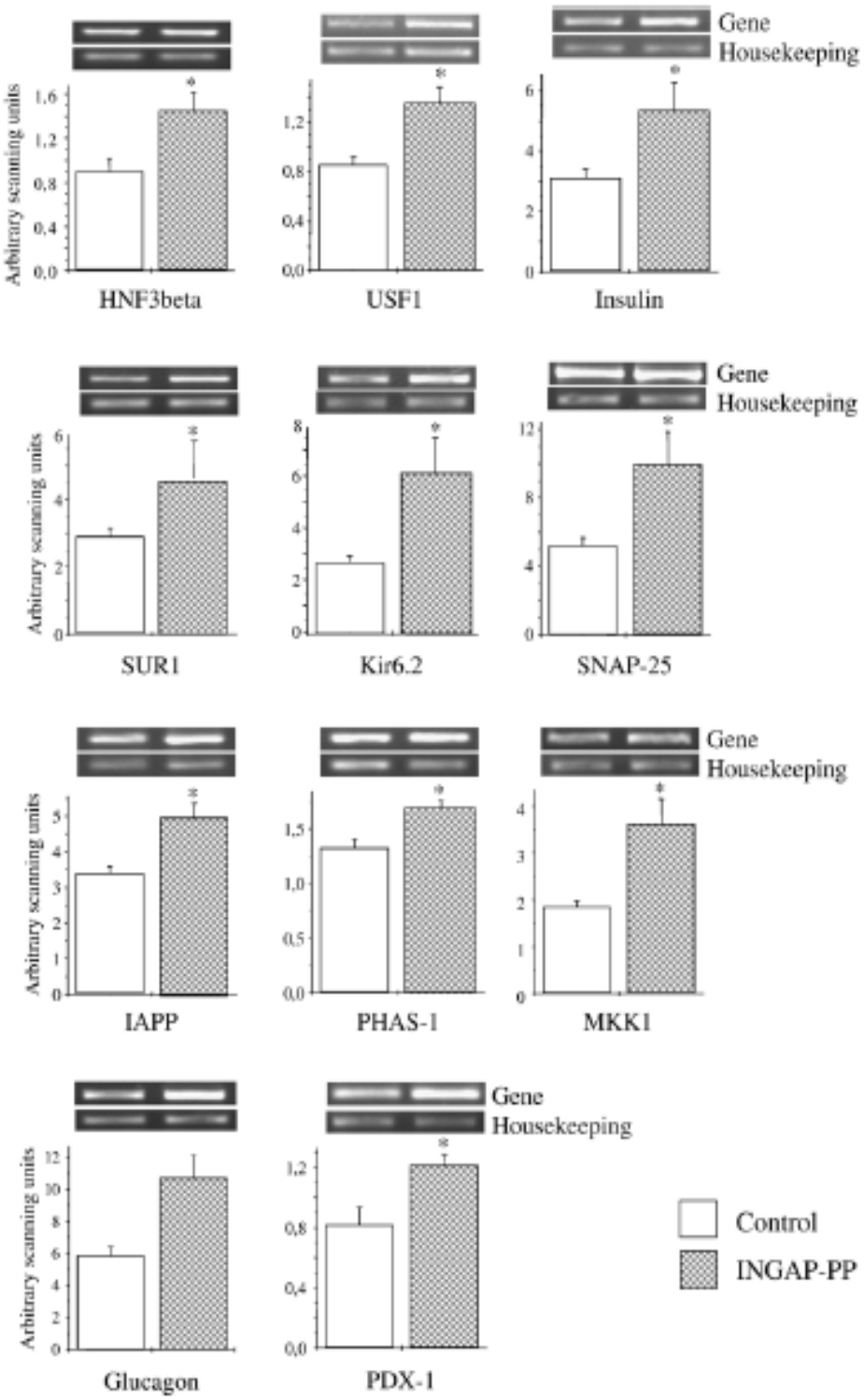
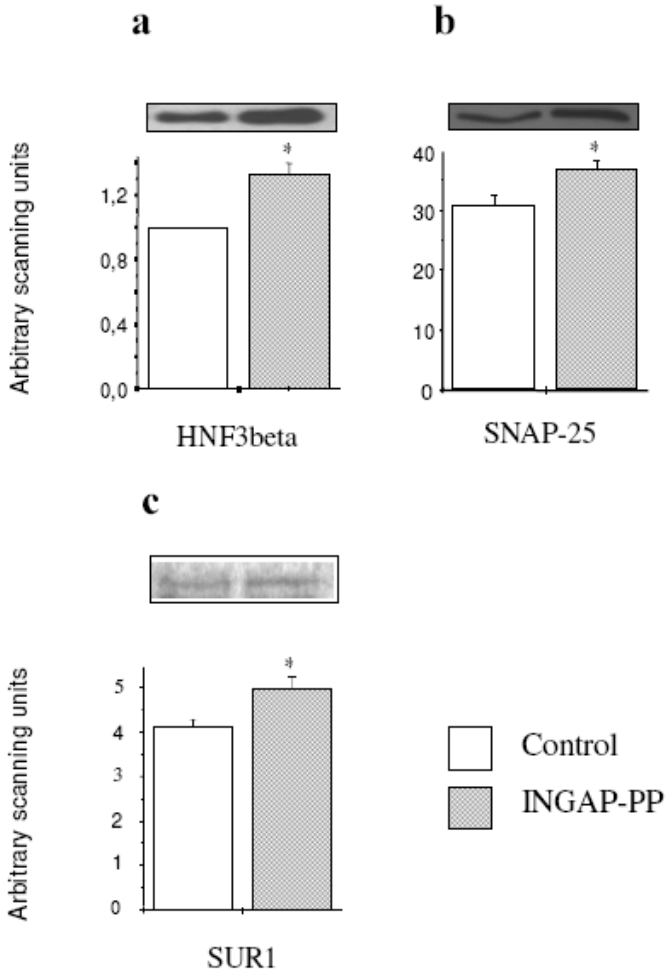

Gene
Housekeeping
Arbitrary scanning units
HNF3beta
USF1
Insulin
SUR1
Kir6.2
SNAP-25
IAPP
PHAS-1
MKK1
Glucagon
PDX-1
Control
INGAP-PP

Figure 3


a
b
c
Arbitrary scanning units
1,2
0,8
0,4
0,0
HNF3beta
40
30
20
10
0
SNAP-25
5
4
3
2
1
0
SUR1
Control
INGAP-PP

Artigo 2

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Islet Neogenesis Associated Protein signaling in neonatal pancreatic rat islets: involvement of the cholinergic pathway

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Keywords: Cholinergic stimulus, insulin secretion; INGAP-PP, MAPK and PI3K pathways, islets culture

Short title: INGAP signaling in pancreatic rat islets

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Abstract

Islet neogenesis associated protein (INGAP) increases islet mass and insulin secretion in neonatal and adult rat islets. In the present study, we measured the short- and long-term effects of INGAP-PP (a pentadecapeptide having the 104-118 amino acid sequence of INGAP) upon islet protein expression and phosphorylation of components of the PI3K, MAPK and cholinergic pathways, and on insulin secretion. Short-term exposure of neonatal islets to INGAP-PP (90 sec, 5, 15 and 30 min) significantly increased Akt$^{-Ser473}$ and ERK1/2$^{-Thr202/Tyr204}$ phosphorylation and INGAP-PP also acutely increased insulin secretion from islets perifused with 2 and 20 mM glucose. Islets cultured for four days in the presence of INGAP-PP showed an increased expression of Akt1, mTOR and ERK2 mRNAs as well as of the muscarinic M3 receptor subtype, and PLC-β2 proteins. These islets also showed increased Akt and ERK1/2 protein phosphorylation. Brief exposure of INGAP-PP-treated islets to carbachol (Cch) significantly increased P70S6K$^{-Thr389}$ and ERK1/2 phosphorylation and these islets released more insulin when challenged with Cch which was prevented by the M3 receptor antagonist 4-DAMP, in a concentration-dependent manner. In conclusion, these data indicate that short- and long-term exposure to INGAP-PP significantly affects the expression and phosphorylation of proteins involved in islet PI3K and MAPK signaling pathways. The observations of INGAPP-PP-stimulated up-regulation of cholinergic M3 receptors and PLC-β2 proteins, enhanced P70S6K and ERK1/2 phosphorylation and Cch-induced insulin secretion suggest a participation of the cholinergic pathway in INGAP-PP-mediated effects.

Introduction

Islet cell differentiation is controlled by several transcription factors including pancreatic duodenal homeobox-1 (PDX-1), neurogenin3 (ngn3), Nkx-1 and many others (Jensen *et al.* 2000). Hormonal, chemical and neural signals are also essential for β-cell mass adaptation during its intrauterine development (Nielsen *et al.* 2001). During the fetal and neonatal periods more new β-cells are formed by neogenesis (Bouwens *et al.* 1994). Neogenesis is also observed in adult rodents after pancreatic injury such as streptozotocin injection (Fernandes *et al.* 1997), partial pancreatectomy (Lee *et al.* 1989), pancreatic duct ligation (Wang *et al.* 1995, Xu *et al.* 2008), cellophane wrapping of the pancreas head (Rosenberg *et al.* 1993) and in sucrose-induced insulin resistance (Del Zotto *et al.* 1999, Del Zotto *et al.* 2000, Gagliardino *et al.* 2003).

INGAP was identified as the active part of a pancreatic protein complex isolated from normal hamsters, whose pancreas heads had been wrapped in cellophane (Pittenger *et al.* 1992). The INGAP gene is expressed both in normal hamster islets and exocrine cells (Gagliardino *et al.* 2003) and it has been shown that a pentadecapeptide having the 104-118 amino acid sequence of INGAP (INGAP-PP) reproduces the effect of the intact molecule upon thymidine incorporation into ductal cells and a ductal cell line (Rafaeloff *et al.* 1997). We have reported that offspring from normal hamsters fed a sucrose rich diet during pregnancy have an increase in β-cell mass, an increased number of PDX-1 and INGAP-positive cells together with the appearance of a small population of cells that co-express PDX-1/INGAP. Since these cells have a high replication rate and do not stain with insulin-, glucagon-, somatostatin-, or PP-antibodies, we postulated that they would be early precursors of islet-cells (Gagliardino *et al.* 2003). Otherwise, it was reported that administration of the pentadecapeptide to either normal adult mice and dogs or streptozotocin-induced diabetic mice induced an increase in β-cell mass and signs of neogenesis (Rosenberg *et al.* 2004, Pittenger *et al.* 2007). In addition, neonatal and adult normal rat islets cultured with INGAP-PP showed both increased β-cell size and insulin secretion in response to glucose (Borelli *et al.* 2005).

Since islet transplantation for therapy of Type 1 diabetes is hampered by the shortage of islet donors, the search for alternative sources of β-cells has been intensified. In this sense, endogenous β-cell mass expansion

and, consequently, the reversal of hyperglycemic states in animal models are being actively investigated (Rosenberg *et al.* 2004, Lipsett *et al.* 2006). Among the protocols used for this purpose INGAP-PP seems to be a suitable tool, but its mechanism of action is not yet completely elucidated.

It has been reported that the pancreas retains the ability to regenerate a functioning β-cell mass in the postnatal period (Rosenberg *et al.* 1995). This process of progenitor cell transformation that leads to islet neogenesis could be mediated, at least in part, by local pancreatic growth factors. It has been shown that INGAP-PP treatment of duct-like structures, obtained from quiescent adult human islets, induced their differentiation to islet-like structures with the four endocrine cell types showing a normal glucose-induced insulin secretion (Jamal *et al.* 2005). In clinical trials (Ratner *et al.* 2005a, Ratner *et al.* 2005b) INGAP-PP therapy has been found to reduce daily average blood glucose levels at 90 days in patients with type-2 diabetes and induce a significant increase in C-peptide secretion in patients with type-1 diabetes. Recently, we have further observed that in addition to its effects to stimulate insulin secretion, INGAP-PP treatment augmented the expression of several genes involved in the control of islet growth and development (Barbosa *et al.* 2006).

Different pathways are involved in the mechanism by which glucose and peptides such as incretin hormones stimulate β-cell growth and differentiation (Hui *et al.* 2003, Kluz *et al.* 2006). Incretin effects are apparently mediated through stimulation of cyclic AMP/PKA, p42 MAPK and PI3K pathways associated with the transcriptional activity of cyclin D1 (Friedrichsen *et al.* 2006), while glucose promotes human β-cell mitogenesis by stimulating p44/p42 MAPKs and mTOR/P70S6K phosphorylation (Guillen *et al.* 2006). These and other reports suggest that MAPK and PI3K cascades may be the major pathways involved in the stimulatory effect of different factors upon islet mass regulation.

In the present study, we show that the enhancing effect of short- and long-term exposure of neonatal rat islets to INGAP-PP upon dynamic insulin secretion is accompanied by an increase of both PI3K and MAPK protein phosphorylation, and of the expression of components of the cholinergic

pathway, such as the M3 receptor subtype and PLC-β2. Furthermore, Cch significantly increased the short-term induced phosphorylation of proteins from the PI3K and MAPK cascades as well as insulin secretion, suggesting a possible participation of the cholinergic pathway in this process.

Material and Methods

Reagents

Reagents used for RT-PCR were from Invitrogen (Invitrogen, USA) and Sigma (St Louis, MO, USA). The equipment for SDS-PAGE and immunoblotting was from Bio-Rad (Richmond, CA, USA). All the chemicals used in the experiments for immunoblotting and buffers were from Sigma (St Louis, MO, USA). Anti-muscarinic receptor M3 (rabbit polyclonal sc-9108), anti-PLC-β2 (rabbit polyclonal, sc-9018), anti-Akt (rabbit polyclonal, sc-8312), anti P70S6K (mouse monoclonal sc-8418) anti-phospho Akt$^{-Ser473}$ (rabbit polyclonal, sc-7985), anti-ERK1 (rabbit polyclonal, sc-94), anti-ERK2 (rabbit polyclonal, sc-154), and anti-phospho ERK1/2$^{-Thr202/Tyr204}$ (mouse monoclonal sc-7383) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and anti-phospho P70S6K$^{-Thr389}$ (mouse polyclonal, #9206S) was from Cell Signaling Technology, Inc. (Boston, MA, USA).

Islets isolation and culture

Islets of neonatal Wistar rats were obtained by collagenase digestion of pancreas in Hanks' balanced salt solution and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10 mM glucose, 100 IU of penicillin/mL, 100 μg of streptomycin/mL at 37ºC in a 5% CO_2/air atmosphere. In short-term experiments groups of 300 islets were cultured for two days to minimize contamination by exocrine tissue. In other studies the islets were cultured for 4 days in the absence (controls) or the presence of 10 μg/mL of INGAP-PP, as reported (Barbosa *et al.* 2006), renewing the medium every

day. All animal experiments were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas (CEEA/IB/UNICAMP).

Insulin secretion

To study dynamic secretion groups of 60 neonatal rat islets, previously cultured for two days in control conditions, were placed in individual chambers and perifused with KRB buffer, previously gassed with a mixture of CO_2/O_2 (5/95%), pH 7.4) and maintained at 37ºC in a temperature-controlled environment (Persaud *et al*. 1989). The islets were first pre-perifused for 1 h at 2 mM glucose, followed by a 70 min perifusion with the medium containing 2 or 20 mM glucose in the absence or presence of INGAP-PP (10 μg/mL). Medium samples were collected at 2 min intervals. To study static insulin secretion groups of 5 neonatal rat islets, previously cultured for 4 days in the absence or presence of INGAP-PP, were then pre-incubated in 0.6 mL of KRB containing 1.5% (w/v) BSA and 5.6 mM glucose at 37ºC for 45 min. After this period, the islets were further incubated in 1 mL KRB supplemented with 8.3 mM glucose for 1 h in the absence or presence of 200 μM Cch and 1-100 nM 4-DAMP (Boschero *et al.* 1995). Aliquots from the static incubation and perifusion experiments were stored frozen for insulin measurement by radioimmunoassay. Islets from all experiments were lysed in alcohol-acid solution for insulin extraction and subsequent insulin immunoassay (data not shown). All results were normalized against insulin content from each well or chamber.

RT-PCR

Semi-quantitative RT-PCR was performed using specific primers to analyze gene expression of Akt1 (NM 033230), mTOR (NM 019906), and ERK2 (M 64300). Reverse transcription was carried out with

3 μg of total RNA using Moloney murine leukemia virus-reverse transcriptase (Superscript II) and random hexamers, according to the manufacturer's instructions (Invitrogen, USA). RT-PCR assays were done in quadruplicate using recombinant Taq DNA polymerase (Invitrogen, USA) and 10 pM of each primer in a master mix of 50 μL. The primer sets used in the RT-PCR analyses are shown in Table 1. The number of cycles for each gene was defined after titration using 20 to 42 cycles and was within the logarithmic phase of amplification. PCR products were separated on 1.5% EtBr-agarose gels and the band intensities were determined by digital scanning (GelDoc 2000, Bio-Rad) followed by quantification using Scion Image analysis software (Scion Corp., Frederick, MD). The results were expressed as a ratio of target to RPS-29 signals. The RNAs used for RT-PCR analysis were obtained from three sets of experiments.

Tissue extracts and immunoblotting

Cultured islets were homogenized in 100 μL of solubilizing buffer (10% Triton-X 100, 100 mM Tris, pH 7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, and 2 mM PMSF) for 30 seconds using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY) and boiled for 5 min. The extracts were then centrifuged at 12,600 g at 4°C for 20 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method and the BioRad reagent. The proteins obtained were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min. 70 μg of protein from each sample was then applied to a 10% polyacrylamide gel and separated by SDS–PAGE in a BioRad miniature slab gel apparatus. The protein fractions were thereafter electrotransfered from the gel to nitrocellulose at 120 V for 90 min in a BioRad miniature transfer apparatus. Before incubation with the primary antibody, the nitrocellulose filters were treated with a blocking buffer (5% non-fat dried milk, 10 mM Trizma, 150 mM NaCl, and 0.02% Tween 20) for 2 h at 22°C. The membranes were then incubated for 4 h at 22°C

with antibodies against muscarinic receptor M3 (1:500), PLC-β2 (1:1,000), p-ERK1/2 (1:500), ERK1/2 (1:1,000), P70S6K (1:500), p-P70S6K (1:1,000), Akt (1:500) or p-Akt (1:500) diluted in blocking buffer with 3% non-fat dried milk, and then washed for 30 min in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated second antibody for 1 h. Specific protein bands were revealed using commercial enhanced chemiluminescence reagents with exposure to photographic film. The images were obtained by digital scanning on GelDoc 2000, BioRad (Richmond, CA, USA), followed by quantification using Scion Image analysis software (Scion Corp., Frederick, MD).

Statistical Analysis

Results are shown as means ± SEM; appropriate statistical comparisons between INGAP-PP-treated islets and the respective control groups were carried out using Student´s unpaired t-test. Differences were considered significant when P values were < 0.05.

Results

Short-term effect of INGAP-PP on islet Akt and ERK1/2 phosphorylation and on insulin secretion.

Akt phosphorylation increased 2 fold when neonatal rat islets were exposed to 10μg/mL INGAP-PP for either 90 sec or 5 min ($P < 0.05$), returning to control values after 15 min (Fig. 1A). INGAP-PP also significantly increased ERK1/2$^{-Thr202/Tyr204}$ phosphorylation that lasted for 30 min after the peptide exposure, being 4.1, 4.0, 2.2 and 2.8 fold higher than control at 90 sec, 5, 15 and 30 min, respectively ($P<0.05$) (Fig. 1B). To verify whether insulin secretion was also affected by exposure to INGAP-PP, islets were perifused with a buffer medium containing 2 mM or 20 mM glucose in the absence or presence of the peptide (10 μg/mL). At 2 mM glucose (Fig. 2A) addition of INGAP-PP provoked a rapid and sustained two fold increase in insulin secretion (min 11-30). This effect was rapidly reversed

by the removal of INGAP-PP from the perifusion medium. To determine whether the islets maintained their viability after INGAP-PP exposure, the glucose concentration was increased to 20 mM (min 51-70) and this resulted in a significant increase in the insulin secretion. Figure 2B shows that increasing the glucose concentration from 2 to 20 mM (min 11-30) stimulated insulin secretion from perifused islets and that this secretory response was further potentiated by INGAP-PP (min 31-50) to levels approximately two fold higher than those obtained with 20 mM glucose alone. However, the potentiation of insulin secretion induced by INGAP-PP was transient and insulin levels returned to values comparable to those elicited by 20 mM glucose before INGAP-PP withdrawal.

Long-term effect of INGAP-PP on islet Akt1, mTOR and ERK2 gene expression, and Akt and ERK1/2 phosphorylation.

RNA obtained from islets that had been maintained in the presence of INGAP-PP for 4 days was submitted to semi-quantitative RT-PCR analysis and normalized against the RPS-29 housekeeping gene. INGAP-PP induced in cultured islets a significant increase in Akt1, mTOR and ERK2 gene expression attaining 1.9, 1.6, and 1.5 fold higher values than control islets ($P<0.05$) (Fig. 3A). Islets that had been exposed to INGAP-PP for 4 days also showed increased basal phosphorylation of Akt and ERK1/2, with levels 1.8 and 1.5 fold respectively higher than control islets ($P<0.05$) (Fig. 3B).

Long-term effect of INGAP-PP on the expression of muscarinic M3 subtype receptor, PLC-β2 proteins, and on Cch-induced acute phosphorylation of P70S6K and ERK1/2.

Due to the role of cholinergic pathway in the mechanism of insulin secretion, induced either by acetylcholine or glucose, and considering that gene expression of the M3 receptor subtype was upregulated by INGAP-PP after 4 days culture, in the next series of experiments we analyzed M3 receptor and PLC-β2 expression as well as the effect of Cch on proteins phosphorylation of the PI3K

and MAPK pathways. Protein levels of the muscarinic M3 receptor subtype and PLC-β2 were significantly higher in islets cultured for 4 days in the presence of INGAP-PP than in controls (Fig. 4A, B). Basal P70S6K phosphorylation was similar in INGAP-PP-treated and control islets and exposure of these islets to 200 μM Cch for short periods of time (90 sec, 5 and 15 min) significantly increased phosphorylation in both groups. However, Cch-stimulated P70S6K phosphorylation was significantly higher in INGAP-PP-treated islets than in control islets (4.4, 6.9 and 4.3 fold for INGAP-PP-treated *vs.* 2.5, 1.5 and 2.0 fold for control islets at 90 sec, 5 and 15 min, respectively; $P<0.05$) (Fig. 5A). ERK1/2 phosphorylation was also similar in INGAP-PP-treated and control islets and exposure to Cch for 90 sec induced a similar and significant increase in ERK1/2 phosphorylation in treated and control islets (3.2 fold) compared to appropriate controls (no Cch). While the Cch-induced ERK1/2 phosphorylation increment returned to basal values after 5 min in control islets, it remained significantly higher in INGAP-PP-cultured islets at 5 and 15 min (3.7 and 2.3 vs. control, respectively; $P<0.05$) (Fig. 5B).

Effects of Cch on insulin release from INGAP-PP-cultured and control islets

Insulin secretion at 8.3 mM glucose was higher in INGAP-PP-cultured islets compared to control islets ($P < 0.05$) (Fig. 6). When 200 μM Cch was added to the incubation medium insulin secretion increased 5.7 times in the INGAP-PP-cultured and 1.5 times in the control islets. This stimulatory effect of Cch was dose-dependently inhibited by increasing concentrations of 4-DAMP, a selective antagonist of M3 receptors, with complete inhibition observed at 10 nM for INGAP-PP-cultured islets and at 1 nM for control islets.

Discussion

It is generally accepted INGAP-PP increases β-cell mass and insulin secretion in response to different stimuli (Borelli *et al.* 2005, Jamal *et al.* 2005, Lipsett *et al.* 2006, Barbosa *et al.* 2006). We have also

recently demonstrated that INGAP-PP simultaneously enhanced insulin secretion and up-regulated several genes related to protein synthesis and islet maturation in neonatal rat islets (Barbosa *et al.* 2006). However, the mechanism by which INGAP-PP produced these effects is not yet fully elucidated. It has been reported that culturing isolated adult human islets for ten days in a simple medium resulted in the disappearance of β-cells from the islet core and when these ghost islets were cultured with INGAP-PP for four days the islets were rebuilt and the newly formed β-cells released insulin in response to glucose at levels comparable to freshly isolated adult islets. Using a variety of blockers it was concluded that the effects of INGAP-PP were due to activation of the PI3K pathway (Jamal *et al.* 2005). It is still a matter of debate whether most of the new β-cells in adult animals are formed by the replication of resident β-cells (Dor *et al.* 2004) or if they are derived from ductal or other pancreatic precursor cells (Noguchi *et al.* 2006, Yatoh *et al.* 2007, Xu *et al.* 2008). Jamal and colleagues (2005) have suggested that the enhancing effect of INGAP-PP upon β-cell mass was obtained by a direct effect upon the remaining external islet cells that presumably conserves characteristics of ductal cells.

Neonatal islets exposed to INGAP-PP for short periods of time (90 sec, 5, 15 and 30 min) showed a significant increase in Akt1/2/3- and ERK1/2 phosphorylation (Fig. 1) supporting the early proposal that both PI3K and MAPK pathways may act as intracellular mediators of the beneficial effect of INGAP-PP upon β-cell mass and viability (Jamal *et al.*, 2005). These short-term effects of INGAP-PP upon the phosphorylation of proteins that belong to the PI3K and MAPK cascades were also observed when neonatal islets were cultured with INGAP-PP for four days (Akt, and ERK1/2) together with an increase of Akt1, mTOR, and ERK2 gene expression.

Long-term exposure of neonatal islets to INGAP-PP also increased expression of members of the cholinergic pathway, namely the M3 receptor subtype and PLC-β2. Furthermore, 4-DAMP (a selective M3 inhibitor) significantly inhibited Cch-stimulated insulin release. These results strongly

suggest that the enhanced insulin secretion induced by cholinergic stimulus in INGAP-PP-cultured islets was due, at least in part, to an increase in the expression of the M3 muscarinic receptor subtype. It has been reported that in MIN6 β–cells, PLC activation by Ca^{2+} is an essential step for the stimulatory effect of Cch upon insulin secretion (Thore *et al.* 2005); such a mechanism includes an intracellular Ca^{2+} mobilization phase and a sustained activation phase dependent on Ca^{2+} entry through a non voltage-dependent channels present in the plasma membrane. Since the expression of PLCβ2 was increased in INGAP-PP-cultured islets it is conceivable that higher levels of intracellular [Ca^{2+}] in these islets (Silva *et al.* 2008) could account for the increased secretion of insulin *via* a higher PLC-β2 activity. The increased expression of the two K^{+}-$_{ATP}$ dependent channels component genes (Barbosa *et al.* 2006) and the increase in intracellular [Ca^{2+}], would also contribute to the enhanced release of insulin observed in INGAP-PP cultured islets. Furthermore, Cch significantly augmented P70S6K and ERK1/2 protein phosphorylation in these islets. Altogether, these results suggest participation of the cholinergic pathway in the mechanism by which INGAP-PP increases insulin secretion.

Activation of PI3K and MAPK pathway proteins occurs following both short- and long-term exposure to INGAP-PP. It has been shown that activation of those pathways is a common mechanism shared by several hormones, growth factors and substances that affect tissue growth and differentiation in many cell types. For example, ERK1/2 phosphorylation is potentiated by glucose, incretins, IGF-1 and glyburide in INS-1 cells (Briaud *et al.* 2003). Prolactin also activates PI3K and MAPK cascades in cultured neonatal rat islets and in islets from pregnant rats, underlining the importance of these pathways in growth and development of islets in both periods of life (Amaral *et al.* 2003, Amaral *et al.* 2004). In addition, ERK3, a member of the MAPK cascade, is involved in the process of insulin granule exocytosis possibly through the complex ERK3/MAP2, and via conventional PKCs activation (Anhe *et al.* 2006; Cunha *et al.* 2007). Moreover, it has been postulated that phosphorylation of synapsin I, a protein involved in glucose-induced insulin secretion, is dependent on ERK1/2 activation

(Longuet *et al.* 2005). Thus, our findings concerning INGAP-PP-induced phosphorylation of proteins of the MAPK and PI3K pathways are in good agreement with the literature mentioned above.

There is also evidence of participation of the cholinergic pathway in cell growth in other tissues. Thus, a) Cch increases $[Ca^{2+}]i$, MAPK/ERK phosphorylation, protein synthesis, and cell proliferation in a human breast cancer cell line (Jimenez *et al.* 2005); b) M3 muscarinic cholinergic receptors activate p70S6K1 *via* PI3K signaling pathway in astrocytoma cells (Batty *et al.* 2004, Tang *et al.* 2003); c) a novel pathway of acetylcholine-stimulated activation of eNOS, involving the JAK2/IRS-1/PI3K/Akt pathway, has recently been reported in aorta of obese rats that are simultaneously resistant to insulin and Cch (Zecchin *et al.* 2007); and d) islet M1 and M3 muscarinic receptor expression is increased during islet cell regeneration (Renuka *et al.* 2005). The increased expression of such receptors is associated with an increase in insulin secretion that, in turn, might modulate β-cell growth and differentiation *via* IRS2 activation (Velloso *et al.* 1995).

In conclusion, our data show that both short- and long-term exposure of neonatal normal rat islets to INGAP-PP activates islet PI3K and MAPK pathways and increases the expression of muscarinic M3 receptors and PLC-β2 proteins. In INGAP-PP-cultured islets, a brief exposure to Cch enhanced the phosphorylation of several components of both signaling cascades leading to an increase in insulin secretion. This latter phenomenon suggests that the effect of INGAP-PP upon pancreatic islets is due, at least in part, to activation of the cholinergic pathway. In light of the beneficial effects of INGAP-PP on β-cell function INGAP-PP supplementation of human islets, maintained in culture prior to transplantation therapy, could be a suitable strategy to optimize their viability and secretory function.

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Figure legends

Figure 1 *Acute effects of INGAP-PP on Akt and ERK1/2 phosphorylation in neonatal rat islets*. Two days cultured control islets were acutely exposed to 10 μg/mL of INGAP-PP for 90 sec, 5, 15 and 30 min and islet extracts were immunoblotted with anti-phospho-Akt (**A**) and anti-phospho-ERK1/2 (**B**) antibodies. The bars represent the relative phosphorylation of the proteins (normalized against the total protein) measured by optical densitometry, and are the means ± SEM of four experiments. The same letters indicate the pairs comparisons that are significantly different ($P<0.05$).

Figure 2 *Kinetics of INGAP-PP-evoked stimulation of insulin secretion.* Two days cultured control islets were continuously perifused with KRB buffer at 37ºC. After a pre-incubation period of 1 h (2mM glucose) the effluent was collected each 2 min for 70 min. INGAP-PP (10 μg/mL) was applied as indicated either in the presence of 2 mM (**A**) or 20 mM glucose (**B**). Results are expressed as % of mean values calculated for min 0 to 10 (2 mM glucose), and normalized against total insulin content. Insulin secretion rates are means ± SEM from three different experiments, * $P<0.05$ compared to basal insulin secretion (2 mM glucose) and ** $P<0.05$ compared to 20 mM glucose.

Figure 3 *Effects of culture with 10 μg/ml of INGAP-PP for 4 days on mRNA levels of Akt1, mTOR, and ERK2 (**A**), and on Akt and ERK1/2 (**B**) phosphorylation.* A: RT-PCRs annealing temperatures and cycle numbers used were as follows: 58ºC and 26 cycles for Akt; 58ºC and 29 cycles for mTOR; 56ºC and 24 cycles for ERK2. Results were normalized to the RPS-29 housekeeping gene. B: Islet extracts were immunoblotted with anti-phospho-Akt and anti-phospho-ERK1/2 antibodies (normalized against the total protein). Plotted columns are means ± SEM of 12 experiments for mRNA and 4 experiments for protein phosphorylation. * $P<0.05$ *vs* control.

Figure 4 *Effect of culture with 10 μg/mL of INGAP-PP for 4 days on muscarinic receptor M3 sybtype (**A**) and PLC-β2 (**B**) expression.* Islet extracts were immunoblotted with anti-muscarinic receptor and anti-PLC-β2 antibodies. The bars represent the relative protein expression as determined by optical densitometry, and are the means ± SEM of three experiments, normalized to β-actin protein expression, * P<0.05 *vs* controls. Open and closed bars represent islets from control and INGAP-PP-cultured rat islets, respectively.

Figure 5 *Acute effects of 200 μM Cch on P70S6K and ERK1/2 phosphorylation in islets for 4 days in the absence or presence of 10 μg/mL INGAP-PP*. After culture, control and INGAP-PP islets were exposed to Cch for 90 sec, 5 and 15 min. Islet extracts were immunoblotted with anti-phospho-P70S6K (**A**) and anti-phospho-ERK1/2 (**B**) antibodies. The bars represent the relative phosphorylation of the proteins (normalized against the total protein) determined by optical densitometry, and are the means ± SEM of four experiments. The same letters indicate the pairs comparisons that are significantly different (P<0.05).

Figure 6 *Effect of 4-DAMP on 200 μM Cch-induced insulin secretion in control and INGAP-PP-cultured (10 μg/mL) neonatal rat islets for 4 days.* After culture, groups of 5 islets were incubated in KRB buffer containing 8.3 mM glucose in the absence or presence of 200 μM Cch and increasing concentrations of the M3 receptor antagonist 4-DAMP (1 nM, 10 nM and 100 nM). The bars represent cumulative 1 h insulin secretion and are means ± SEM of 14-20 groups of islets from three different experiments. The same letters indicate the pairs comparisons that are significantly different (P<0.05) between groups. Light and dark bars represent islets from control and INGAP-PP-cultured islets, respectively.

Figure 1

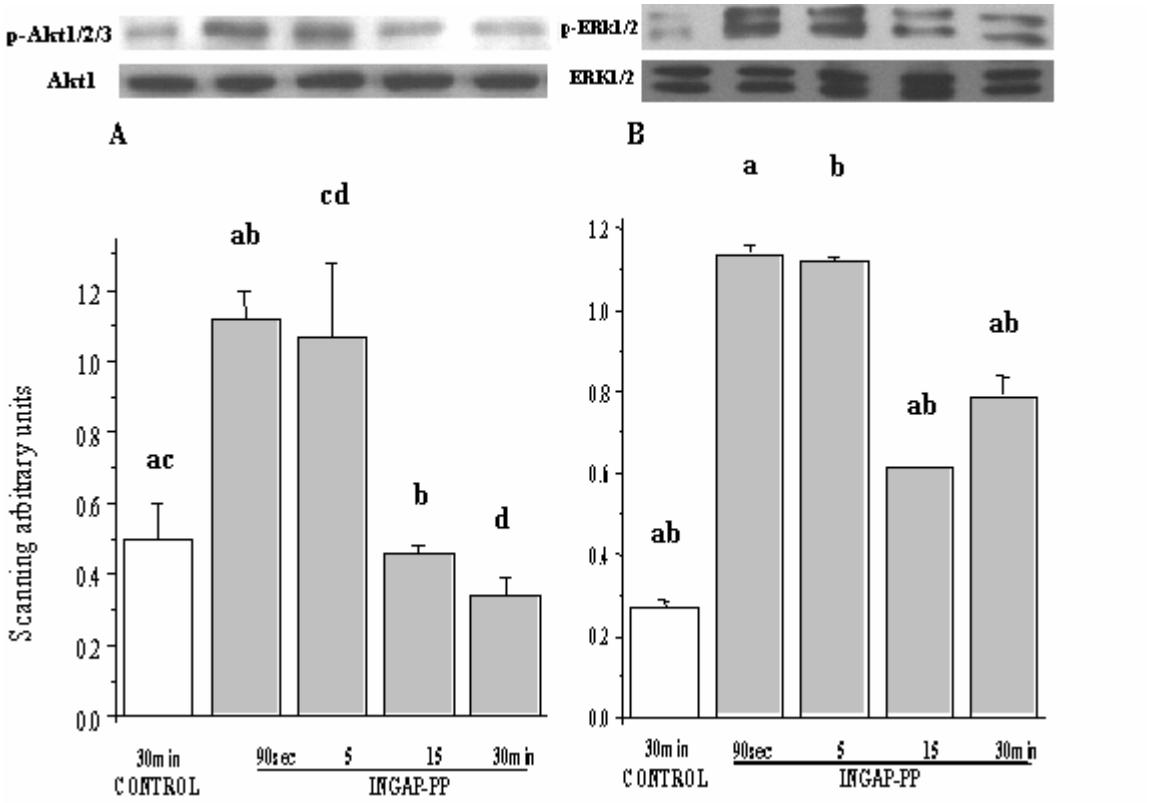
p-Akt1/2/3
Akt1
p-ERK1/2
ERK1/2
A
B
Scanning arbitrary units
ac
ab
cd
b
d
a
b
ab
ab
ab
30m in
CONTROL
90sec
5
15
30m in
INGAP-PP

Figure 2

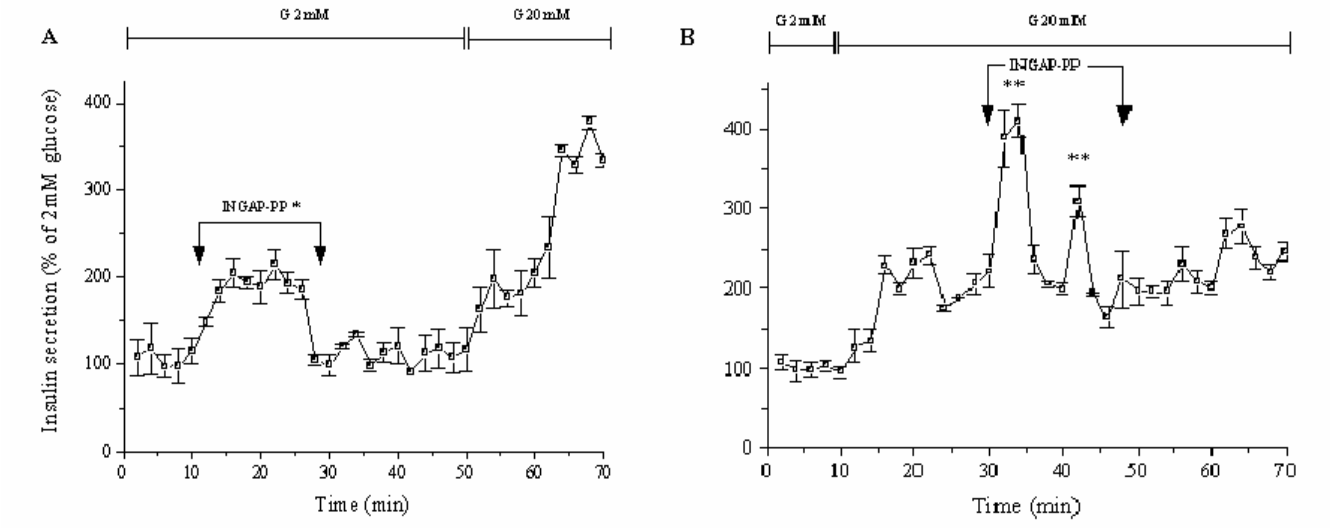
A
G 2mM
G 20mM
INGAP-PP *
Insulin secretion (% of 2mM glucose)
Time (min)
B
G 2mM
G 20mM
INGAP-PP
**
**
Time (min)

Figure 3

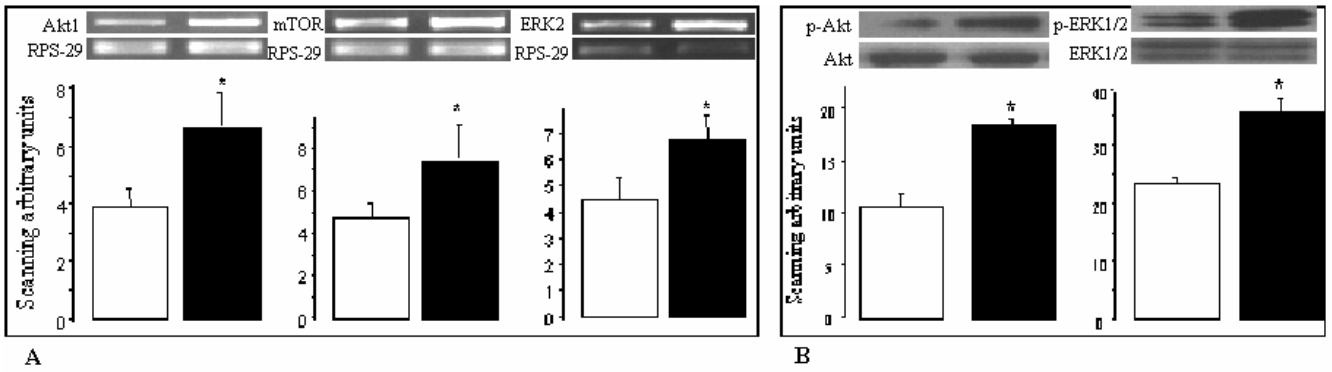
Akt1
RPS-29
mTOR
RPS-29
ERK2
RPS-29
Scanning arbitrary units
p-Akt
Akt
p-ERK1/2
ERK1/2
Scanning arbitrary units
A
B

Figure 4

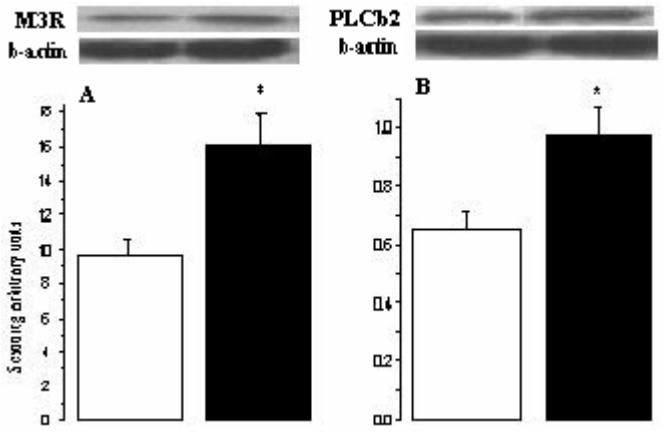

Figure 5

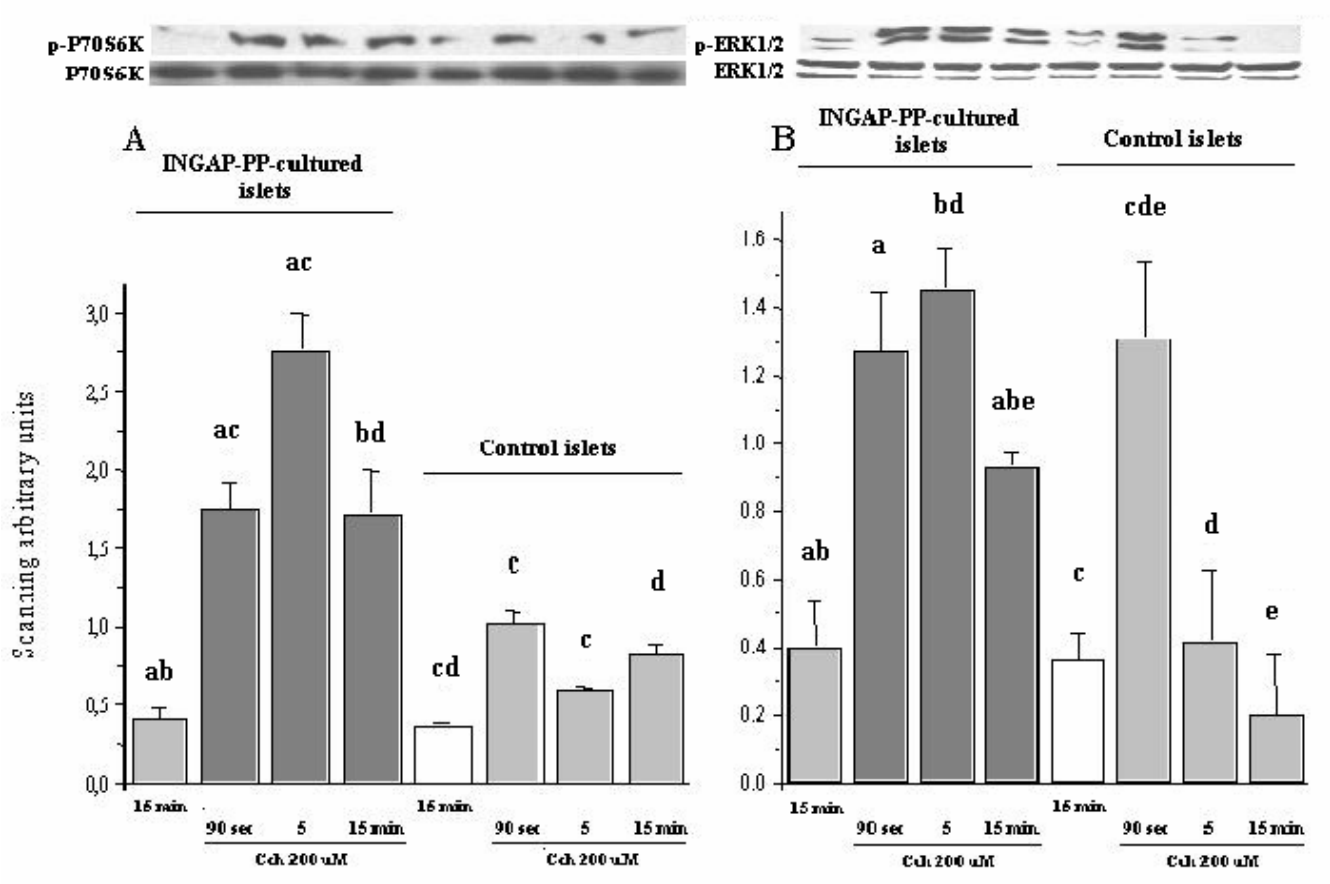
p-P70S6K
P70S6K
p-ERK1/2
ERK1/2
A
INGAP-PP-cultured islets
Control islets
Scanning arbitrary units
ab
ac
ac
bd
cd
c
c
d
16 min.
90 sec
5
15 min.
Cch 200 uM
B
INGAP-PP-cultured islets
Control islets
ab
a
bd
abe
c
cde
d
e
15 min.
16 min.
90 sec
5
15 min.
Cch 200 uM

Figure 6

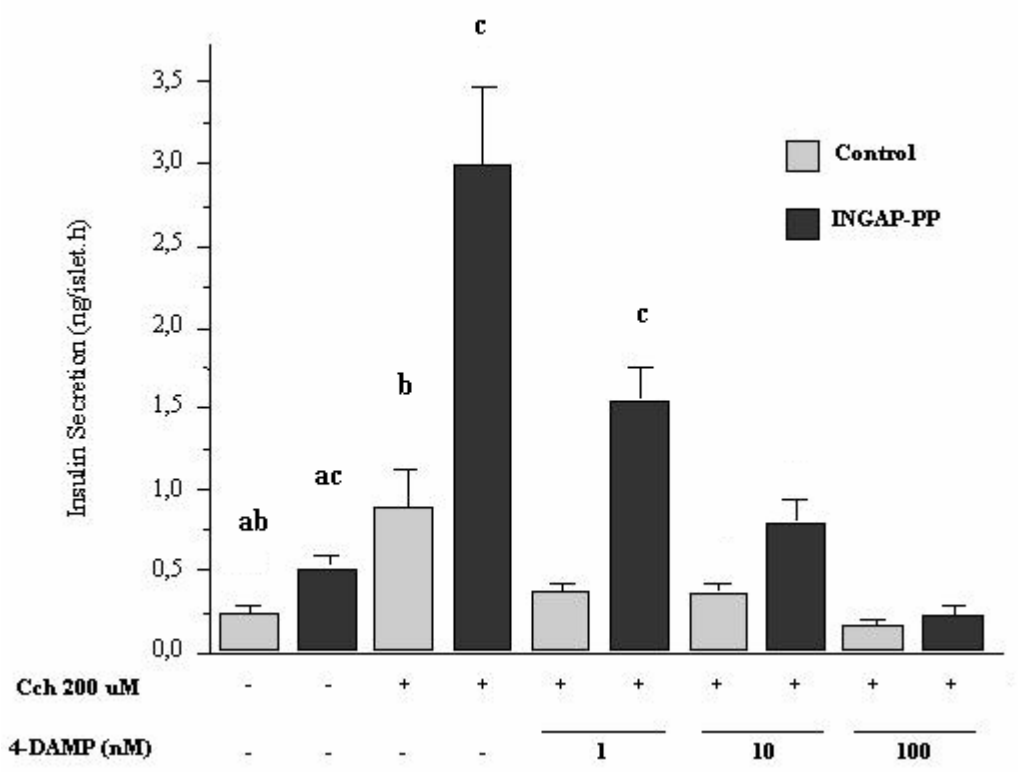
Insulin Secretion (ng/islet.h)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
ab
ac
b
c
c
Control
INGAP-PP
Cch 200 uM
4-DAMP (nM)
1
10
100

Table 1

RT-PCR primer sets with predicted product sizes

Gene	GeneBank	Forward primer	Reverse primer	TM (°C)	Cycle number	Product (bp)
Akt1	NM 033230	CCTCAAGTACTCATTCCAGAC	CTCATACACATCTTGCCACAC	58	26	619
mTOR	NM 019906	AAGGAGATGCAGAAGCCTCAGT	TGTCCCAAAGCCCATTAGGTCT	58	29	249
ERK2	M 64300	GACCCAAGTGATGAGCCCATTG	AAGCCACTACGACCAGACTGCC	56	24	253

Discussão

O INGAP é capaz de aumentar a massa de célula β e secreção de insulina em resposta a diferentes estímulos (Borelli *et al*. 2005, Jamal *et al*. 2005, Lipsett *et al*. 2006, Barbosa *et al*. 2006), porém o mecanismo de ação desse peptídeo ainda não é completamente compreendido. Como já relatado, o INGAP foi inicialmente ligado à neogênese de ilhotas em situações de estresse do pâncreas. A origem de novas células β no pâncreas ainda é motivo de debate: se são formadas via replicação de células pré-existentes (Dor *et al*. 2004) ou derivadas de células precursoras ductais ou outras células pancreáticas (Noguchi *et al*. 2006, Yatoh *et al*. 2007, Xu *et al*. 2008). Foi demonstrado que células semelhantes a células ductais, obtidas através de cultura de ilhotas humanas por 10 dias, foram capazes de se diferenciarem novamente em estruturas semelhantes a ilhotas, após 4 dias em cultura com INGAP (Jamal *et al*. 2005). Isso sugere que o efeito do INGAP sobre aumento de massa das células β seria sobre remanescentes de células de ilhotas, que conservariam características de células ductais.

Neste trabalho, avaliamos os efeitos do INGAP-PP sobre ilhotas de ratos neonatos uma vez que, durante a primeira semana de vida, ocorrem altas taxas de proliferação tanto via neogênese quanto replicação celular, sendo provável que a ilhota responda melhor à ação desse peptídeo, a exemplo do que ocorre com fatores de crescimento.

Inicialmente, cultivamos ilhotas por 4 dias na ausência ou presença de INGAP-PP. Após extração de RNA e obtenção de cDNA marcado com (α^{33}P) dATP este foi hibridizado em membranas comercias, contendo 2.352 genes previamente fixados. Observamos que o tratamento com INGAP-PP aumentou a expressão de 210 genes e reduziu a expressão de apenas 4 genes (Tabela Suplementar, Anexo 1). Dentre esse genes incluem-se genes que codificam fatores de transcrição, hormônios e outras proteínas específicas da ilhota e das células β, além de proteínas ribossomais e outras envolvidas na transcrição e tradução protéica. Dos fatores de transcrição que tiveram sua expressão aumentada, destacam-se Maf2, *CCAAT-binding transcription factor subunit B*, MEF2D, HNF3β (FOXA2), USF-1 e Pdx-1. A modulação de fatores como FOXA2, USF1 e Pdx-1 evidencia a atuação do INGAP-PP em

processos relacionados à manutenção da funcionalidade do pâncreas endócrino, uma vez que esses fatores podem regular direta ou indiretamente a maturação da célula β, especialmente a síntese de insulina (Ahlgren *et al*. 1997, Gualdi *et al*. 1996, Sharma *et al*. 1997, Wu *et al*. 1997, Ahlgren *et al*. 1998, Qian *et al*. 1999, Gerrish *et al*. 2000, Marshak *et al*. 2000). Por outro lado, a análise do promotor do INGAP mostrou que Pdx-1 está associado à inibição da expressão do polipeptídeo (Taylor-Fishwick *et al*. 2003), o que indica um possível controle da expansão da ilhota por *feedback* via ação do Pdx-1. A expressão de FOXA2 está relacionada à regulação da expressão das subunidades SUR1 e Kir6.2 do canal de K_{ATP} (Sund *et al*. 2001, Lantz *et al*. 2004), estes aumentados pelo tratamento com INGAP-PP (Silva *et al*. 2008). Observamos também *up-regulation* de proteínas associadas à ancoragem de vesículas na membrana celular e extrusão dos grânulos de insulina (SNAP-25, Munc18, sinapsina, entre outras). A modulação destas pelo INGAP-PP sugere ação desse peptídeo na regulação de proteínas participantes do processo de secreção de insulina, seja na despolarização da membrana (Kir6.2 e SUR1), ou durante a extrusão do grânulo. A expressão aumentada de fatores relacionados à transcrição e tradução protéica, como *elongation factor 1 alpha*, eIF-2α, eIF-2Be, eIF-5, eIF-2a, PHAS-I, além de uma série de proteínas ribossomais, evidencia a participação do INGAP-PP na modulação de proteínas ligadas às funções gerais das células. Por outro lado, o aumento da expressão de proto-oncogenes (c-fos, c-myc, n-myc, erbB3, FGR e *jun proto-oncogen transcription factor* AP1) sugere participação do INGAP-PP na modulação de genes relacionados à proliferação, crescimento e diferenciação. A relação entre a expressão desses genes e processos de proliferação e diferenciação já é bem documentada na literatura (Susini *et al*. 1998, Jona *et al*. 1999, Josefsen *et al*. 1999).

Ilhotas cultivadas em presença de INGAP-PP por 4 dias tiveram expressão aumentada de Akt1, mTOR e ERK2. Enquanto que, ilhotas controle expostas agudamente ao INGAP-PP mostraram aumento da fosforilação basal de P70S6K e ERK1/2. Ativação de proteínas das vias PI3K e MAPK está relacionada ao mecanismo de ação de vários hormônios, fatores de crescimento e substâncias que

estimulam crescimento e diferenciação em muitos tecidos (Briaud 2003, Briaud *et al*. 2003), por exemplo a prolactina, que ativa estas vias tanto em animais prenhes quanto neonatais (Amaral *et al*. 2003, 2004). A formação do complexo ERK3/MAP2 para exocitose do grânulo de insulina, além da ativação de PKC´s convencionais (Anhe *et al*. 2006, Cunha *et al*. 2007) evidenciam a participação da via da MAPK na secreção de insulina. Além disso, foi demonstrado que a fosforilação da sinapsina parece ser dependente da ativação de ERK (Longuet *et al*. 2005). ERK ativada também pode se translocar para o núcleo, onde catalisa a fosforilação de fatores de transcrição como p62 (Lackner *et al*. 2005), iniciando uma série de eventos que culminam com a proliferação ou diferenciação celulares (Boulton *et al*. 1991).

Cinco subtipos de receptor muscarínico foram definidos em escala molecular (M1-M5), todos associados a membros da família de proteína G acoplada ao receptor (Bonner 1989). Análises farmacológicas sugerem que o subtipo M3 é o principal desses receptores presente na célula β (Boschero *et al*. 1995, Henquin 1998). Após cultura de 4 dias em presença de INGAP-PP observamos aumento de expressão gênica e protéica do receptor muscarínico M3, e de expressão protéica da PLC-β2, uma proteína *downstream* ao respectivo receptor. Além disso, ilhotas tratadas com o peptídeo secretaram significativamente mais insulina em presença de Cch. A secreção de insulina foi reduzida quando da adição do inibidor específico para o M3, 4-DAMP, ao meio de incubação. A possível ativação de PLC-β2 pela elevação de Ca^{2+} (Thore *et al*. 2005, Silva *et al*. 2008), associada à maior expressão do receptor M3 e da PLC-β2, sugere uma maior sensibilidade ao estímulo colinérgico, justificando o aumento da secreção de insulina em ilhotas tratadas com INGAP-PP. Associado ao aumento da secreção de insulina, Cch também ativou as vias PI3K e MAPK, representadas respectivamente pelo aumento da fosforilação de P70S6K e ERK1/2, em maior proporção nas ilhotas tratadas com INGAP-PP. A ativação de proteínas dessas vias pelo Cch foi demonstrado em experimentos com linhagem humana de câncer de mama (Jimenez *et al*. 2005) e em astrocitomas (Tang

et al. 2003, Batty *et al*. 2004), indicando a participação colinérgica em processos de proliferação celular. Aumento da expressão de M1 e M3 também foi observado em ilhotas em regeneração (Renuka *et al*. 2005).

Concluindo, nossos resultados indicam claramente que o INGAP-PP melhora as condições das ilhotas pancreáticas através da ativação das vias PI3K e MAPK com possível participação da via colinérgica no processo. Esses resultados, juntamente com outros da literatura, mostram ainda que o INGAP-PP representa uma ferramenta útil na manutenção da viabilidade de ilhotas humanas mantidas em cultura para fins de transplante.

Conclusões

Os resultados obtidos nesse trabalho nos levam a concluir que:

- O INGAP-PP modula a expressão de inúmeros genes muitos dos quais participam da síntese e secreção de insulina em ilhotas de ratos neonatos cultivadas por 4 dias . Tais efeitos se refletem no aumento da secreção de insulina glicose-induzida;
- A expressão aumentada da insulina e de fatores de transcrição como UFS1, Pdx1 e HNF3β, mostra que o INGAP-PP exerce função importante sobre a manutenção da massa e da funcionalidade das células β pancreáticas;
- A maior expressão das subunidades SUR1 e Kir6.2 do canal de K_{ATP} em ilhotas tratadas com INGAP-PP, bem como do fator de transcrição HNF3β que regula a expressão dessas subunidades, sugerem a participação do INGAP-PP sobre estruturas envolvidas no controle do potencial de membrana das células β, e reguladas pelo metabolismo da glicose durante o processo secretório;
- A modulação da expressão do receptor muscarínico M3, da PLC-β2, bem como o aumento da secreção de insulina induzida por Cch sugere participação da via colinérgica no mecanismo de ação do INGAP-PP, provavelmente através da fosforilação de proteínas das vias MAPK e PI3K, essenciais para proliferação e maturação celulares;
- A habilidade do INGAP-PP em aumentar a secreção de insulina crônica ou agudamente parece ser processada de maneira distinta. Cronicamente através do aumento da expressão de diferentes genes e proteínas. Agudamente via fosforilação de proteínas pertencentes às cascatas MAPK e PI3K com aumento da $[Ca^{2+}]i$ (resultados não publicados);
- A melhora das funções da ilhota pancreática estimulada pelo INGAP torna o peptídeo uma ferramenta importante para o tratamento prévio de ilhotas isoladas de doadores para transplante.

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Anexo 1

TABLE 2. List of regulated genes in neonatal pancreatic islets following treatment with INGAP-PP.

GENBANK ID	GENE	RATIO*
Cell Surface Antigens		
AF009133	Antigen CD37	0.50
X53430	CD3d mRNA for T3 delta protein	2.32
Basic Transcription Factors		
J03170	Liver specific transcription factor (LF-B1)	2.42
L36388	HFH-4	2.11
MM24393	Myogenin	2.24
M34238	CCAAT-binding transcription factor B (CBFB)	2.52
X56546	Variant hepatic nuclear factor 1 (vHNF1)	2.35
X94246	Paired box protein 9 (PAX8)	2.26
AF026476	Upstream stimulatory factor 1 (USF1)	2.61
AJ005425	Myocyte-specific enhancer factor 2D (MEF2D)	2.14
L09647	Hepatocyte nuclear factor 3a (HNF3-beta)	2.57
U56242	Transcription factor Maf2	2.50
Others Transcription Proteins		
AF090306	Retinoblastoma-binding protein	0.46
AF013967	Zinc finger splicing protein (ZIS)	2.13
Cell Adhesion Receptors & Proteins		
D87248	NB3 neural adhesion molecule	2.21
Cell-Cell Adhesion Receptors		
L43592	Protocadherin 3 (PCDH3)	2.42
U41663	Neuroligin	2.11
Extracellular Transporters & Carrier Proteins		
M63574	Selenoprotein P	2.18
X73911	Amiloride binding protein (long form)	2.12
Facilitated Diffusion Proteins		
AB013890	ATP-sensitive inwardly rectifying K channel Kir7.1	2.54
AF059258	Monocarboxylate transporter 3 (MCT3)	2.37
D82883	Sulfate transporter	2.17
M00001	apolipoprotein A-I	2.06
M00002	apolipoprotein A-IV	2.04
Voltage-Gated Ion Channels, Symporter & Antiporter		
M84210	Potassium channel protein	2.10
U69884	Calcium-activated potassium channel rSK3	2.30
L15453	Calcium channel, voltage-dependent, L type, alpha 1E subunit	2.50
M59980	Potassium voltage gated channel, Shal-related family, member 2	2.18

AF021137	Potassium inwardly-rectifying channel, subfamily J, member 2	2.48
M92905	Calcium channel, voltage-dependent, N type, alpha 1B subunit	2.17
X16476	Potassium voltage gated channel, Shab-related subfamily, member 1	2.03
X17621	Potassium voltage gated channel, shaker related subfamily, member 6	2.60
X62841	Potassium channel, voltage gated, KV3.4; RAW3; KCNC4	2.58
X16002	Potassium voltage gated channel, shaker related subfamily, member 4	2.02
X61394	Calcium channel beta 1 subunit	2.52
U28927	GABA transporter	2.04
D63772	Solute carrier family 1, member 1	2.06
Other Facilitated Diffusion Proteins		
AJ000515	Cyclic nucleotide-gated cation channel beta subunit (CNCbeta)	2.61
L21672	Glycine transporter 2 (GLYT2)	2.63
ATPase Transporter		
AF035387	C7-1 protein	2.54
M93017	Calcium-transporting ATPase	2.02
Other Membrane Channels & Transporters		
X66494	CHOT1 mRNA	3.02
L05435	Synaptic vesicle glycoprotein 2 a	2.03
M97381	Solute carrier family 18, member 2	2.00
Extracellular Matrix Proteins		
U57362	Collagen XII alpha 1 subunit (COL12A1)	2.46
U82626	Basement membrane-associated chondroitin proteoglycan	2.13
Targeting & Exocytosis Proteins		
AF019974	Chromogranin B (CHGB)	2.22
U49099	28-kDa Golgi SNARE (GS28)	2.00
J04597	Protein P150 RII-B-binding domain	2.00
AF030253	Vesicular GABA transporter (VGAT)	2.04
AF056704	Synapsin III (SYN3)	2.00
U75361	Mammalian unc-13-3 homology (MUNC13-3)	2.21
G Proteins, Others Trafficking & Targeting Proteins		
X12535	Ras-related protein RAB7	2.31
L38247	Synaptotagmin 4	2.05
M27812	Synapsin 1	2.10
M93669	Secretogranin 2	2.32
U20283	Syntaxin binding protein Munc18-2	2.01
AB003991	Synaptosomal-associated protein, SNAP-25	2.17
Simple Carbohydrate & Energy Metabolism		
M11709	L-type pyruvate kinase	2.20
J04544	Islet Amyloid Polypeptide	2.13
M73553	Intestinal lactose-binding lectin (L-36)	2.47
X74593	Sorbitol dehydrogenase	2.09

AF062741	Pyruvate dehydrogenase phosphatase isoenzyme 2 (PDP2)	2.22
K02813	Glucagon gene	3.55
AB013732	UDP-glucose dehydrogenase (UDPGDH, UGDH)	2.29
U07177	Lactate dehydrogenase C (LDH-C)	2.24
J04473	Mitochondrial fumarase	2.31
U67995	Stearyl-CoA desaturase 2	2.06
Amino Acid Metabolism		
D83481	Cysteine dioxygenase	3.51
M12337	Phenylalanine hydroxylase	0.46
J05499	L-glutamine amidohydrolase	2.75
M58308	Histidase	2.09
Other Metabolism Enzymes		
U18729	Cytochrome b-558 alpha polypeptide (CYBA)	2.22
Y17295	Thiol-specific antioxidant protein (1-Cys peroxiredoxin)	2.29
U53706	Mevalonate pyrophosphate decarboxylase	2.24
M34464	S-adenosylmethionine decarboxylase	2.22
Chaperones, Heat Shock Proteins & Proteins Modification Enzymes		
AF006617	Microsome-associated 60-kDa stress 70 protein chaperone (STCH)	2.40
U62940	Stress-inducible chaperone mt-GrpE 1 nuclear gene encoding mitochondrial protein	2.12
L10416	Rab geranylgeranyl transferase beta-subunit	2.04
L25331	Lysyl hydroxylase	2.37
M81225	Farmesyltransferase alpha subunit	2.24
AB010999	Peptidylarginine deaminase type IV (PDI4)	3.02
AF016048	Platelet-activating factor acetylhydrolase alpha 2 subunit (PAF-AH alpha 2) gene	2.70
D88035	Glycoprotein specific UDP-glucuronyltransferase, GL-CAT-P	2.47
U35890	Polypeptide GalNAc transferase T1	2,21
D88034	Peptidylarginine deiminase type III (PDI3)	2.91
M12516	Cytochrome P450 oxidoreductase	2.27
Post-translational Modifications Proteins		
M27466	Cytochrome c oxidase polypeptide Vlc2 (COX6C2)	2.70
M32725	Dorsal protein 1	2.45
M57263	Protein-glutamine gamma-glutamyltransferase	2.75
Ribossomal Proteins		
D11388	Ribossomal protein S15 (RPS15)	2,04
M20156	Ribossomal protein L18	2.08
X06148	Ribossomal protein L15	2.19
X06483	60S Ribossomal protein L32 (RPL32)	2.56
X78167	Ribossomal protein L15	2.16
X94242	Ribossomal protein L14	3,91
X58465	Ribossomal protein S5 (RPS5)	2.47
X53377	Ribossomal protein S7 (RPS7)	2.21
X68283	Ribossomal protein L29	2.08

X82180	Ribossomal protein L4	2.16
X82551	60S ribosomal protein (RPL39)	2.25
X93352	Ribossomal protein L10a	2.17
Translation Factors		
U05821	Translation initiation factor eIF-2B alpha-subunit (eIF-2a)	2.18
U19516	Initiation factor eIF-2Be	2.11
X61043	Elongation factor 1 alpha	2.01
L11651	Eukaryotic initiation factor 5 (eIF-5)	2.05
Other Proteins Involved in Translation		
X05300	Ribophorin I	2.42
U05014	Eukaryotic initiation factor 4E-binding protein (4EBP1); PHAS-1	3.28
L27707	Eukaryotic hemin-sensitive initiation factor 2a kinase (eIF-2a)	3.70
RNA Processing, Turnover & Transport Proteins		
X52311	Unr mRNA for unr protein with unknown function	2.30
X60790	PYBP2 mRNA for pyrimidine binding protein 2	2.71
AF041066	Ribonuclease 4	2.29
AF068268	2'-5' oligoadenylate synthetase 2 (OAS2)	2.72
U18942	Double-stranded RNA-specific adenosine deaminse	2.13
U20181	Iron-regulatory protein 2 (IRP2)	2.31
U74586	Double-stranded RNA-specific adenosine deaminase, RED2	2.80
M12156	Helix-destabilizing protein	2.06
DNA-binding, Chromatin Proteins & DNA Damage Repair Proteins		
M55022	Nucleolin (NCL)	2.07
M17096	Transition protein	2.05
U89695	High mobility group protein I isoform C (HMGIC)	2.26
U22893	DNA-binding protein A (DBPA)	2.27
U83112	INS-1 winged helix protein (INSWH)	2.48
X14776	Transition protein 2 (TP2)	2.37
X93591	MSH2 DNA mismatch repair protein	2.16
Hormone Receptors		
X70658	Calcitonin-like receptor	2.69
AF061443	Leucine-rich repeat containing G protein-coupled receptor 4	2.26
U31968	Caveolin-3	2.56
M35077	Dopamine receptor D1A	2.19
U09631	Vasopressive intestinal peptide receptor 2	
Other Receptors (by Ligands) & Endocytosis Proteins		
AB009463	Low-density lipoprotein receptor protein 105 (LRP105)	2.20
AF022247	Intrinsic factor-vitamin B12 receptor; cubulin (CUBN)	2.46
X61159	Glycine receptor alpha 2 subunit (glycine receptor, neonatal)	2.15
AF005099	Neuronal pentraxin receptor	2.12
M96377	Neurexin 2	2.22
L06096	Inositol 1, 4, 5-triphosphate receptor 3	2.34

X61677	Inositol 1,4,5-triphosphate receptor 2	2.06
Neuropeptides		
L25633	Neuroendocrine-specific protein (RESP18)	2.77
L15011	Neuron-specific cortexin	2.67
X97375	Prepronociceptin (neuropeptide nociceptin) (N23K)	2.11
Growth Factors, Cytokines & Chemokines Receptors		
L40030	Placenta growth factor (PIGF)	2.46
X67108	Brain-derived neurotrophic factor (BDNF)	2.41
X95096	Macrophage stimulating protein (MSP); hepatocyte growth factor-like Protein (HGFL)	2.43
U68725	Netrin receptor	2.34
U68726	Neogenin	2.06
M77809	Transforming growth factor, beta receptor 3	2.05
M91599	Fibroblast growth factor receptor 4	2.06
U97143	Glial cell line derived neurotrophic factor family receptor alpha 2	2.09
U03491	Transforming growth factor, beta 3	2.08
M32748	Leukemia inhibitory factor, cholinergic neuronal differentiation factor	2.11
L15305	Glial cell line derived neutrophic factor	2.51
U37101	Colony stimulating factor 3	2.21
U00620	Colony stimulating factor 2, granulocyte-macrophage	2.04
M13969	Insulin-like growth factor II, somatomedin A	2.16
M15480	Insulin-like growth factor I	2.07
S69323	Neurotrophin 5, NT4	2.27
S77492	Bone morphogenetic protein 3	2.29
X55183	Growth factor Schwannoma-derived, amphiregulin	2.11
Hormones & Interleukins		
V01242	Insulin 1 gene	3.51
X01032	Brain cholecystokinin (CCK)	2.95
X02318	Fragment for thyroglobulin	2.40
M32754	Inhibin alpha chain	2.16
X01118	Natriuretic peptide precursor type A	2.15
X01454	Thyroid stimulating hormone beta subunit	2.18
M22899	Interleukin 2, IL2	2.22
Other Extracellular Communication Proteins & Immune System Proteins		
D50568	Eosinophil granule major basic protein (MBP); proteoglycan 2 (PRG2)	2.74
X58023	Corticotropin releasing hormone binding protein	2.35
L08831	Glucose-dependent insulinotropic peptide	2.02
Intracellular Kinase Network Members		
D86556	Calcium/calmodulin-dependent protein kinase 1 beta (CAMK1B)	2.46
U22297	Casein kinase 1 gamma 2 isoform	2.57
U38481	rhoA-binding serine/threonine kinase alpha (ROK-alpha)	2.61
U48596	Mitogen-activated protein kinase kinase 1(MAP3K1)	2.20
U78857	Calcium calmodulin dependent kinase CPG16 (cpg16)	2.43

U63971	Rhodopsin kinase	2.04
L22294	Pyruvate dehydrogenase kinase 1	2.00
Intracellular Protein Phosphatases		
D90163	Serine/threonine protein phosphatase alpha 1 catalytic subunit (PP1CA)	2.58
U69673	Protein tyrosine phosphatase 20 (PTP20)	2.37
G Proteins G & Protein-Coupled Receptors		
X65747	Gnat-3 mRNA for gustducin	2.35
X76921	ARF-like mRNA 3	3.53
AF097887	CDC42 homology protein (CHP)	2.75
D12820	G protein-coupled receptor 27; gustatory receptor 27 (GUST27)	2.01
U22830	Purinergic receptor P2Y, G-protein coupled 1	2.30
D63665	Pyrimidinergic receptor P2Y, G-protein coupled 6	2.58
L35767	Very low density lipoprotein receptor, LDL receptor	2.02
L19699	GTP-binding protein, Ral B	2.19
J03773	Guanine nucleotide binding protein, alpha	2.02
M17525	GNAS complex locus	2.02
X06889	RAB3A, member RAS oncogene family	2.09
GTP/GDP Exchangers & GTPase Activity, Oncogenes & Tumor Supressors		
L10336	Guanine nucleotide-releasing protein (mss4)	2.94
U72995	RAB3 GDP/GTP exchange protein	3.40
U92279	Regulator of G-protein signaling 14 (RGS14)	2.75
X67250	N-chimaerin	3.44
Z35654	Ost oncogene	2.55
X06769	c-fos proto-oncogene	2.22
Y00396	c-myc proto-oncogene	2.00
X63281	n-myc proto-oncogene	2.17
X17163	jun proto-oncogene transcription factor AP1	2.00
U29339	erbB3 proto- oncogene	2.23
X57018	FGR proto-oncogene	2.36
D38629	Adenomatous polyposis coli protein	2.13
Phospholipases & Phosphoinositol Kinases		
U69550	Phospholipase D1 (rPLD1)	2.23
Adenylate/Guanylate Cyclase & Diesterases		
U60835	Soluble guanylyl cyclase alpha 1 subunit	2.33
L41045	Cyclic nucleotide phosphodiesterase (PDE1C2)	2.86
M57507	Guanyl cyclase (GC-S-beta-2)	2.27
M22562	Soluble guanylate cyclase 70 kd subunit	2.29
Other Intracellular Transducers, Effectors & Modulators		
L09119	C kinase substrate calmodulin-binding protein (RC3)	2.71
U61772	Neurofibromatosis 2	2.00
Amino- & Carboxypeptidases		

J00713	Carboxypeptidase A (CPA)	2.23
Cytoskeleton & Motility Proteins		
X79321	Tau microtubule associated protein	2.52
X60370	Microtubule associated protein 1B (MAP1B)	2.16
Xenobiotic Metabolism		
U09870	Major vault protein, MVP	2.01
X67654	Glutathione S-transferase 1, theta	2.30
Apoptosis-Associated Proteins		
X80477	Purinergic receptor P2X, ATP receptor	2.02
Neutransmitter Receptors & Ligand-Gated Íon Channels		
X74833	Cholinergic receptor, nicotinic, beta polypeptide 1	2.30
M18088	Cholinergic receptor, muscarinic 3	2.10
Functionally Unclassified Proteins		
Z54212	Epithelial membrane protein-1	0.46

*****Two membranes were averaged, corrected for the background and normalized against housekeeping genes present in the membranes.

Anexo 2

1: Regul Pept. 2006 Sep 11;136(1-3):78-84. Epub 2006 Jun 9.

Islet Neogenesis Associated Protein (INGAP) modulates gene expression in cultured neonatal rat islets.

Barbosa H, Bordin S, Stoppiglia L, Silva K, Borelli M, Del Zotto H, Gagliardino J, Boschero A.

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The Islet Neogenesis Associated Protein (INGAP) increases pancreatic beta-cell mass and potentiates glucose-induced insulin secretion. We currently studied the effects of a pentadecapeptide having the 104-118 amino acid sequence of INGAP (INGAP-PP) on insulin secretion and on transcript profile expression in 4-day-cultured normal pancreatic neonatal rat islets. Islets cultured with INGAP-PP released significantly more insulin in response to 2.8 and 16.7 mM glucose than those cultured without the peptide. The macroarray analysis showed that 210 out of 2352 genes spotted in the nylon membranes were up-regulated while only 4 were down-regulated by INGAP-PP-treatment. The main categories of genes modified by INGAP-PP included several related with islet metabolism, insulin secretion mechanism, beta-cell mass and islet neogenesis. RT-PCR confirmed the macroarray results for ten selected genes involved in growing, maturation, maintenance of pancreatic islet-cells, and exocytosis, i.e., Hepatocyte nuclear factor 3beta (HNF3beta), Upstream stimulatory factor 1 (USF1), K(+)-channel proteins (SUR1 and Kir6.2), PHAS-I protein, Insulin 1 gene, Glucagon gene, Mitogen-activated protein kinase 1 (MAP3K1), Amylin (IAPP), and SNAP-25. INGAP-PP also stimulated PDX-1 expression. The expression of three transcripts (HNF3beta, SUR1, and SNAP-25) was confirmed by Western blotting for the corresponding proteins. In conclusion, our results show that INGAP-PP enhances specifically the secretion of insulin and the transcription of several islet genes, many of them directly or indirectly involved in the control of islet metabolism, beta-cell mass and islet neogenesis. These results, together with other previously reported, strongly indicate an important role of INGAP-PP, and possibly of INGAP, in the regulation of islet function and development.

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Anexo 3

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RESEARCH

Islet Neogenesis Associated Protein signaling in neonatal pancreatic rat islets: involvement of the cholinergic pathway

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Abstract

Islet neogenesis associated protein (INGAP) increases islet mass and insulin secretion in neonatal and adult rat islets. In the present study, we measured the short- and long-term effects of INGAP-PP (a pentadecapeptide having the 104-118 amino acid sequence of INGAP) upon islet protein expression and phosphorylation of components of the PI3K, MAPK and cholinergic pathways, and on insulin secretion. Short-term exposure of neonatal islets to INGAP-PP (90 sec, 5, 15 and 30 min) significantly increased Akt-Ser473 and ERK1/2-Thr202/Tyr204 phosphorylation and INGAP-PP also acutely increased insulin secretion from islets perifused with 2 and 20 mM glucose. Islets cultured for four days in the presence of INGAP-PP showed an increased expression of Akt1, mTOR and ERK2 mRNAs as well as of the muscarinic M3 receptor subtype, and PLC-β2 proteins. These islets also showed increased Akt and ERK1/2 protein phosphorylation. Brief exposure of INGAP-PP-treated islets to carbachol (Cch) significantly increased P70S6K-Thr389 and ERK1/2 phosphorylation and these islets released more insulin when challenged with Cch which was prevented by the M3 receptor antagonist 4-DAMP, in a concentration-dependent manner. In conclusion, these data indicate that short- and long-term exposure to INGAP-PP significantly affects the expression and phosphorylation of proteins involved in islet PI3K and MAPK signaling pathways. The observations of INGAPP-PP-stimulated up-regulation of cholinergic M3 receptors and PLC-β2 proteins, enhanced P70S6K and ERK1/2 phosphorylation and Cch-induced insulin secretion suggest a participation of the cholinergic pathway in INGAP-PP-mediated effects.

Anexo 4

UNICAMP

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Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1199-1**, sobre **"Maturação da resposta secretória à glicose pelo INGAP (islet neogenesis associated protein) em ilhotas de Langerhans de ratos recém nascidos. Possível cross-talk com o sinal da prolactina"**, sob a responsabilidade de **Prof. Dr. Antonio Carlos Boschero / Helena Cristina de Lima Barbosa**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 27 de fevereiro de 2007.

CERTIFICATE

We certify that the protocol nº **1199-1**, entitled **"Maturation of the glucose-responses insulin secretion ny INGAP (islet neogenesis associated protein). Possible cross-talk with the prolactin signal"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on February 27, 2007.

Campinas, 27 de fevereiro de 2007.

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