UNIVERSIDADE ESTADUAL DE CAMPINAS

VIVIANE DELGHINGARO AUGUSTO

Modulação da expressão gênica e de proteínas envolvidas no mecanismo de secreção de insulina em ilhotas pancreáticas de ratos submetidos à restrição protéica

Tese apresentada ao Instituto de Biologia para obtenção do Titulo de Doutor em Biologia Funcional e Molecular, na área de Fisiologia

Orientador: Prof. Dr. Everardo Magalhães Carneiro

Co-orientador: Prof. Dr. Antonio Carlos Boschero

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Prof. Dr. José Roberto Bosqueiro _____

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SUMÁRIO

LISTA DE ABREVIATURAS

ATP	trifosfato de adenosina
ANOVA	análise de variância
$[Ca^{2+}]_i$	concentração de cálcio intracelular
cAMP	adenosina monofosfato na forma cíclica
cDNA	ácido desoxiribonucléico complementar
DAG	diacilglicerol
EDTA	ácido etilenodiaminotetracético
FFA	ácido graxo livre
GLUT-2	transportador de glicose - 2
IP_3	1, 4, 3 inositol trifosfato
IR	receptor de insulina
IRS-1	substrato 1 do receptor da insulina
IRS-2	substrato 2 do receptor da insulina
K^+	potássio
LP	hipoprotéico
mRNA	ácido ribonucléico mensageiro
NP	normoprotéico
PCR	reação em cadeia da polimerase
PI3-K	fosfatidilinositol 3-quinase
PKAα	proteína quinase A - subunidade alfa
PKB	proteína quinase B
PKCα	proteína quinase C - subunidade alfa
PVX	vírus X da batata
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	duodecil sulfato de sódio
TCA	ácido tricloroacético

RESUMO

RESUMO

A biossíntese e secreção de insulina pelas células beta pancreáticas são reguladas por fatores humorais, estímulos nervosos e interações celulares. Alterações nutricionais durante os períodos fetais e/ou infância, induzidos por restrição protéica ou calórica, têm sido relacionadas a mudanças estruturais e funcionais do pâncreas. Neste estudo, utilizando a técnica de "cDNA array", avaliamos a expressão gênica de ilhotas pancreáticas de ratos alimentados, durante dois meses, pós-desmame, com dieta isocalórica de baixo teor protéico (6%; LP) ou normoprotéica (17%; NP). Nossos resultados mostraram alterações na expressão de 32 genes, sendo que a maior parte das proteínas codificadas por esses genes eram relacionadas à biossíntese/secreção de insulina e remodelamento celular. Numa segunda etapa, através de RT-PCR e Western blot, analisamos a expressão gênica de proteínas ligadas ao processo de secreção de insulina, onde também observamos redução de GLUT-2, PKAα e PKCα, em ilhotas de ratos LP quando comparadas à NP. Essas mudanças foram acompanhadas por reduções significativas na secreção de insulina em resposta à diferentes nutrientes. Diminuições no conteúdo total de insulina (ilhota e pâncreas) e na quantidade de RNAm da insulina, foram verificadas em ilhotas LP. Além disso, variações na expressão de IR e IRS-1, componentes envolvidos na cascata de sinalização da insulina, foram observadas em ilhotas pancreáticas LP. Diante disso, o período de restrição alimentar a que foram submetidos esses animais induziu alterações na expressão gênica, incluindo proteínas envolvidas no processo de secreção de insulina em ilhotas de Langerhans, podendo explicar, em parte, a redução da secreção desse hormônio, frente a diferentes agentes insulinotrópicos, observada nesse modelo experimental.

ABSTRACT

The biosynthesis and insulin secretion by pancreatic B cells are regulated by humoral factors, nervous stimulus and intercellular interactions. Nutritional alterations during fetal and early postnatal periods, induced by either low protein or caloric restriction diets, have been related to changes in the structure and function of pancreas. In this study, using cDNA array technique, we evaluated the pattern of gene expression in pancreatic islets from rats fed an isocaloric low (6%, LP) and normal (17%, NP) protein diet, after weaning. Our results show 32 genes with altered expression, and the majority of them encode for proteins related to biosynthesis/insulin secretion and cellular remodeling. By RT-PCR and Western blot, we evaluated the gene expression of proteins related to insulin secretion, and we also observed reduction in GLUT-2, PKAα and PKC expression in LP compared to NP islets. These alterations were accompanied by a significant reduction in insulin secretion in response to different nutrients. The islets from LP rats showed a reduction in the total insulin content associated with decreased insulin mRNA. In addition, components involved in insulin signaling systems such as IR and IRS-1 were also altered in LP islets. Taken together, these results indicate that low protein diet, after weaning, induce alteration in gene expression, including protein belonging to insulin secretion process in pancreatic islets, which could explain partially the impaired insulin secretion in response to different insulinotropic agents, observed in this experimental model.

INTRODUÇÃO

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Desnutrição e Diabetes

Mudanças pronunciadas no estilo de vida humano têm acompanhado o processo de globalização no último século. O padrão de vida sedentário, associado à alimentação hipercalórica, pobre em fibras e rica em proteínas, gorduras saturadas e carboidratos simples, tem contribuído para o rápido aumento da incidência de Diabetes Mellitus do tipo 2 (Rao, 1988).

O diabetes tipo 2 é uma doença multifatorial, geralmente associada à Síndrome X ou Síndrome Metabólica, a qual é caracterizada por obesidade, hipertensão arterial sistêmica, dislipidemia e intolerância à glicose (Reaven, 1988; Barker et al 1993). Dessa forma, quando a resistência à insulina é agravada pela obesidade, inatividade física ou idade, o pâncreas não consegue suprir os requerimentos aumentados de insulina e o diabetes se instala.

Hales & Barker (1992) formularam a hipótese do "*thrifty phenotype*" para explicar a etiopatogenia do Diabetes Mellitus tipo 2 e da "Síndrome X". De acordo com essa hipótese, o desenvolvimento do diabetes e da Síndrome X são determinados por fatores ambientais, inclusive nutricionais, e são originadas *in útero* ou durante a infância.

Dessa forma, quadros de desnutrição materna ou disfunção placentária seriam responsáveis por mudanças funcionais e estruturais permanentes no organismo, decorrentes de uma distribuição seletiva de nutrientes, levando ao crescimento diferencial dos órgãos, tais como fígado e pâncreas, além dos tecidos muscular e adiposo.

Todas essas alterações provocadas pela desnutrição são decorrentes de processos adaptativos, proporcionando, dessa forma, maiores chances de sobrevivência. Porém, quando esses organismos encontram-se em condições nutricionais adequadas na vida pós-natal, podem tornar-se obesos e, consequentemente, desenvolver diabetes, doenças cardíacas e hipertensão.

Recentemente, o "Expert Committee on the Diagnosis and Classification of Diabetes Mellitus" (2003), divulgou uma nova classificação do Diabetes Mellitus, no qual a desnutrição não está mais incluída como fator diabetogênico. Entretanto, diversas evidências têm demonstrando que a desnutrição exerce um papel primordial no desenvolvimento da síndrome metabólica, uma vez que causa intolerância à glicose, insulinopenia e resistência à insulina em

modelos animais e em seres humanos (Cook, 1967, Heard & Turner, 1967; Becker et al., 1971; Smith et al., 1975; Swenne et al., 1987).

Dados epidemiológicos humanos e estudos realizados em animais tem relacionado o baixo peso ao nascimento a uma redução no número de ilhotas pancreáticas. Essas observações sugerem que condições nutricionais adversas causam diminuição no desenvolvimento fetal do pâncreas endócrino, visto que o período gestacional, lactação e início da infância são considerados como fases críticas para a ontogenia e desenvolvimento deste órgão (Van Assche & Aerts, 1979; Dahri et al. 1991; Fowden, 1995).

Dessa forma, alterações morfológicas e funcionais das ilhotas pancreáticas têm sido observadas em modelos de desnutrição pré e pós-natal, além de uma redução do peso absoluto do pâncreas (Snoeck et al., 1990; Dahri et al., 1995; Berney et al., 1997). Com relação à morfologia, as células beta apresentam um volume reduzido, com menor quantidade de grânulos secretores, tendo muitos deles aspecto de imaturos. As células beta apresentam também degenerações mitocondriais e indícios de permeabilidade celular diminuída (Dahri et al., 1991; Hoet et al., 1992; Latorraca et al., 1998).

Quando se avalia a capacidade funcional das ilhotas de ratos desnutridos, nota-se um comprometimento da secreção de insulina estimulada por aminoácidos; perda do padrão bifásico de secreção; sensibilidade à glicose diminuída, esta representada por uma curva dose-resposta glicose-insulina deslocada para a direita em relação à curva dos ratos controles (Snoeck et al., 1990; Dahri et al.,1991; Wilson 1997). Mediante estímulos com diferentes concentrações de glicose, essas ilhotas apresentaram menor captação de $^{45}Ca^{2+}$ do que as ilhotas controle. Portanto, defeito na mobilização do íon Ca^{2+} parece contribuir para as alterações secretórias verificadas em ilhotas de ratos desnutridos (Carneiro et al. 1995, Latorraca et al. 1999).

Curiosamente, algumas anormalidades observadas no animal desnutrido, tais como redução do volume das ilhotas, atrofia e perda de granulação das células beta, insensibilidade das ilhotas à glicose e alteração da cinética secretória são similares àquelas observadas no diabético do tipo 2 (Gepts & Lecompt, 1981; Grodsky, 1996; Leahy, 1996). Alterações no balanço entre neogênese, proliferação e apoptose também têm sido observadas na prole de ratas submetidas à desnutrição protéica durante a prenhez (Snoeck et al., 1990; Dahri 1991, Hoet 1992, Petrik et al., 1999).

Secreção de Insulina

As células beta, presentes nas ilhotas pancreáticas, são a única fonte do hormônio insulina e, portanto, são de extrema importância para a manutenção da homeostase glicêmica. Disfunções nas células beta são as maiores causas do desenvolvimento do diabetes mellitus.

A síntese e secreção de insulina pelas células beta pancreáticas são estimuladas pela glicose de maneira dose-dependente. Porém, outros nutrientes, tais como, os aminoácidos leucina e arginina, além de hormônios peptídicos, ácidos graxos, nucleotídeos cíclicos e outros, presentes na circulação sanguínea, potencializam a secreção de insulina induzida por glicose e aumentam a sua expressão gênica e biossíntese (Vuguin et al., 2001).

A glicose é transportada para o interior da célula beta através do GLUT-2, um transportador de membrana. Uma vez internalizada, a glicose é fosforilada pela glicoquinase, seguindo a via glicolítica. Os produtos originados por essa via são então oxidados para a geração de ATP através do ciclo de Krebs (Meglasson, 1986). Este processo leva à um aumento na razão entre ATP:ADP, que promove o fechamento dos canais de K^{-}_{ATP}-dependentes, induzindo despolarização da membrana plasmática, abertura dos canais de Ca^{2-} voltagem-dependentes culminando com influxo de Ca^{2-} na célula beta (Cook & Hales, 1984, Aschroft et al., 1984, Satin & Cook, 1985, Prentki & Matschinsky, 1987). Este aumento de cálcio intracelular induz extrusão imediata dos grânulos posicionados próximos à membrana plasmática, além de mobilizar outros que se encontram mais distantes desta região (Rorsman et al., 2000). Todo este processo ocorre em ordem de segundos a minutos. A resposta da célula beta ao estímulo com nutrientes secretagogos também pode ser observada após um período maior de tempo (minutos/horas), sendo caracterizada por um aumento na biossíntese da pré-proinsulina (Bratanova-Tockhova et al., 2002).

A secreção de insulina também pode ser amplificada através do aumento das concentrações de inositol trifosfato (IP_3), resultando na mobilização de cálcio armazenado no retículo endoplasmático. Aumentos nos níveis de cálcio intracelular em associação com elevações citoplasmáticas de diacilglicerol (DAG) causam ativação de isoformas de proteínas kinases C (PKC) que, por sua vez, são capazes de fosforilar proteínas chave no processo secretório. Além disso, o metabolismo da glicose está ligado ao aumento da atividade da adenilato ciclase, enzima responsável pela clivagem de ATP e geração de adenosina monofosfato

cíclico (AMPc). Elevações nos níveis de AMPc causam a ativação da proteína kinase A (PKA), a qual tem sido relacionada ao processo de secreção de insulina por promover aumentos na movimentação dos grânulos secretórios (Wei et al., 2000; Yajima et al., 1999).

Além dos estímulos descritos acima, diversos estudos têm demonstrado que a célula beta expressa componentes do sistema de sinalização da insulina, tais como: IR (Verspohl & Ammon, 1980; Gazzano et al., 1985, Rothenberg et al., 1995), IRS-1 e IRS-2 (Velloso et al., 1995; Harbeck et al., 1996; Sun et al., 1997, Araújo et al., 2002), PI3-K (Alter & Wolf, 1995; Gao et al., 1996) e PKB (Holst et al., 1998).

Vários trabalhos têm evidenciado que a associação da insulina com seu receptor na membrana da célula beta, e posterior fosforilação de IRS-1 e demais proteínas envolvidas na cascata de sinalização da insulina, contribuem para a regulação de sua própria síntese e secreção (Xu & Rothenberg, 1998; Withers et al., 1998; Aspinwall et al., 1999; Kulkarni et al., 1999; Leibinger et al., 2000). Dessa forma, utilizando camundongos knockout para IRS-1, em células beta pancreática, Kulkarni (1999) demonstrou que o bloqueio desta via pode estar associado à hiperplasia das ilhotas pancreáticas, defeitos na resposta secretória frente a diversos estímulos, tanto in vivo quanto in vitro, além de reduzido conteúdo de insulina.

Nutrição e modulação da expressão gênica

Em organismos multicelulares, o controle da expressão gênica difere em muitos aspectos do operado em organismos unicelulares, envolvendo interações hormonais, neuronais e nutricionais (Farfournoux et al., 2000). Esses estímulos ocorrem tanto na superfície das células quanto no seu interior e, freqüentemente, desencadeiam múltiplas vias de amplificação de sinal que convergem em resposta celular e/ou tecidual.

É sabido que as inúmeras proteínas responsáveis pela manutenção da homeostase celular estão igualmente codificadas em todas as células de um organismo. As respostas especializadas de cada célula ou tecido é, em princípio, decorrente da expressão diferencial dessas proteínas, que podem ser moduladas ou alteradas por sinais presentes no ambiente extracelular.

Alterações bioquímicas e fisiológicas têm sido observadas em ilhotas de ratos neonatos e músculo esquelético de camundongos submetidos a diferentes intervenções nutricionais (Girardi et al., 1994; Gurney et al., 1994, Vaulont & Kahn, 1994; Towle, 1995, Foufelle et al., 1998; Lee

et al., 1999; Song et al., 2001). Além disso, mudanças no perfil de aminoácidos têm sido registradas em situações onde a captação de proteínas é diminuída, ou em dietas não balanceadas, ou ainda, em quadros de deficiência de algum aminoácido essencial. Em tais situações, a concentração no plasma de certos aminoácidos pode ser dramaticamente reduzida (Cherif et al., 2001).

Aminoácidos essenciais são fornecidos através da dieta e uma deficiência em qualquer um desses pode levar a um balanço negativo de nitrogênio causando mudanças no perfil de proteínas plasmáticas e estruturais. Essas alterações levam o organismo a ajustar suas funções fisiológicas através da regulação da expressão gênica de proteínas envolvidas no controle da homeostase celular e orgânica. Dessa forma, análises paralelas da atividade transcricional de um grande número de genes podem refletir os padrões que são necessários para suportar tais mudanças adaptativas (Farfournoux et al., 2000).

Assim sendo, através do grande esforço destinado ao sequenciamento e identificação de todos os genes codificados nos genomas de vários organismos, tecnologias novas foram desenvolvidas. Em paralelo aos projetos de sequenciamento de genomas, destaca-se a análise em larga escala da expressão simultânea de genes, através de "chips"ou arranjos ("arrays") de DNA. O racional deste método é imobilizar em membrana de nylon ou lâmina de vidro fragmentos de genes caracterizados ou de candidatos a genes ("*ESTs, expressed sequence tags*") para posterior hibridação com cDNAs marcados por isótopos radioativos ou fluorescência. Esses cDNAs são obtidos a partir da transcrição reversa de RNAs extraídos de diferentes tecidos de um mesmo organismo, ou do mesmo tecido exposto a estímulos distintos. O resultado observado são os perfis de expressão diferencial dos genes, ativados ou inativados, nas diferentes condições. Assim, esta nova tecnologia permite que as pesquisas no campo da fisiologia endócrina sejam postuladas sob a perspectiva molecular (Weeb et al. 2000; Cardozo et al., 2001; Song et al.,2001). Diante disso, modelos experimentais, que utilizem restrição protéica, podem constituir ferramentas importantes para o estudo da expressão gênica, podendo ser correlacionados ou não ao Diabetes Mellitus e/ou Síndrome Metabólica.

Baseados nessas informações, objetivamos neste trabalho avaliar o padrão da expressão gênica e protéica de moléculas, envolvidas no processo de secreção da insulina, provenientes de ilhotas pancreáticas de ratos submetidos à restrição protéica, após o período de amamentação. Alterações significativas em vários genes e proteínas, pertencentes a diferentes vias de transdução

de sinal na ilhota pancreática, foram observadas. Esses resultados sugerem que as alterações na expressão gênica, observadas nesse modelo, podem contribuir para as mudanças celulares, explicando em parte a deficiente secreção de insulina apresentada por essas ilhotas.

Desta forma, para uma maior compreensão deste estudo, apresentamos nossos resultados experimentais divididos em dois manuscritos. O primeiro trabalho consiste na avaliação da expressão gênica causada pela restrição protéica e o segundo, envolve a análise da expressão gênica e de proteínas envolvidas diretamente com o mecanismo de secreção de insulina. Portanto, a associação entre esses dois manuscritos nos permitiu uma visão global da influência da redução de nutrientes no processo de secreção da insulina, bem como sua associação com a Síndrome Metabólica e Diabetes.

ARTIGO 1

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A low protein diet alters gene expression in rat pancreatic islets

Viviane Delghingaro-Augusto[1], Silvana Bordin[2], Fabiano Ferreira[1], Maria Esméria Corezola do Amaral[1], Marcos H. Toyama[1], Antonio Carlos Boschero[1*], Everardo Magalhães Carneiro[1]

[1]Departamento de Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), SP, Brazil.
[2]Departamento de Fisiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

* To whom correspondence should be addressed: Departamento de Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brazil.
Phone: (55) (19) 3788 6202
FAX: (55) (19) 3788 6185
Email: boschero@unicamp.br

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Running title: Low protein and gene expression.

Key words: protein restriction, gene expression, cDNA array, pancreatic islet

Abbreviations: cDNA – Complementary Deoxinucleic Acid; EDTA – Ethylenediamine Tetraacetic Acid; FFA – Free Fatty Acid; GLUT2 – Glucose Transporter 2; LP – Low Protein Group; mRNA – Messenger Ribonucleic Acid; NP – Normal Protein Group; PCR – Polymerase Chain Reaction; PVX – Potato Virus X; RT-PCR – Reverse Transcriptase-Polymerise Chain Reaction; TCA – Trichloroacetic Acid; TTBS - Tris-Tween 20 Buffered Saline.

ABSTRACT

Insulin secretion is regulated mainly by circulating nutrients, particularly glucose, and is also modulated by hormonal and neuronal inputs. Nutritional alterations during fetal and early postnatal periods, induced by either low protein or caloric restriction diets, produce beta cell dysfunction. As a consequence, insulin secretion in response to different secretagogues is reduced as well as the number of β-cells and size and vascularization of islets. In this study, we evaluated the pattern of gene expression in pancreatic islets from rats fed an isocaloric low (6%, LP) and normal (17%, NP) protein diet, after weaning, using a cDNA array technique and reverse transcriptase-polymerase chain reaction (RT-PCR). We identified 32 genes with altered expression (up or down-regulated), related to metabolism, neurotransmitter receptors, trafficking and targeting proteins, intracellular kinase network members and hormones. The levels of selected gene transcripts, such as clusterin, secretogranin II precursor, eukaryotic translation initiation factor 2, phospholipase A2, and glucose transporter were evaluated by RT-PCR to confirm results from cDNA array. In conclusion, cDNA array analysis indicated significant changes in the gene expression pattern in rats fed a low protein diet after suckling, suggesting that a wide range of molecular alterations contribute to several adaptations in cellular, biochemical and molecular levels in the endocrine pancreas, including impaired glucose-induced insulin secretion.

INTRODUCTION

Adequate nutrition during prenatal and early postnatal periods is very important for the development of endocrine pancreas. Human and animal epidemiologic data from different populations indicate that poor nutrition during these periods of life is associated with an increased incidence of glucose intolerance and type 2 diabetes in adulthood (1).

Several studies have associated the control of gene expression with nutritional signals (2-6). The availability of free amino acids to the tissues is important for the maintenance of organ and body protein homeostasis, therefore amino acids must be supplied in the diet, and a deficiency of any one of the essential amino acids can lead to a negative nitrogen balance. Alterations in nitrogen metabolism cause a change in the plasma amino acid profile (7-11). As a consequence, the organism has to adjust several physiological functions involved in defense/adaptation to amino acid limitation by regulating the expression of several genes (12-14).

Intrauterine malnutrition models induced by low protein diet during pregnancy and lactation also produce changes in the structure and function of several organs in the offspring. Reduced insulin secretion in response to glucose and different secretagogues, reduced rate of islet-cell proliferation, reduced islet size and a decrease in islet vascularization have been observed in endocrine pancreas from malnourished rats. (15-18). Alterations in the plasma amino acid profiles from dams and offspring have been observed when a low protein diet is introduced during gestation.

In order to analyze the influence of protein restriction on gene expression, pancreatic islets RNA from rats subjected to low and normal protein diets were reverse-transcribed and hybridized to Atlas cDNA array (Clontech), a commercial nylon membrane containing 1176 genes. To further validate the results of the macroarray analysis, we selected 5 genes for confirmation by RT-PCR. Our results showed 32 genes with altered expression, and the majority of them encode for proteins related to biosynthesis/insulin secretion and cellular remodeling. These results indicate that low protein diet, after weaning, induce also alterations in gene expression which could explain partially the impaired insulin secretion, in response to different insulinotropic agents, observed in this experimental model.

MATERIALS AND METHODS

Animals and diet

The Institutional (UNICAMP) Committee for Ethics in Animal Experimentation (São Paulo-Brazil) approved all the experiments described here. Male Wistar rats (28 days old) from the breeding colony at UNICAMP were maintained at 24°C with 12 h light dark cycle and had free access to food and water. The rats were distributed randomly and were fed with 17% (normal protein diet, NP) or 6% (low protein diet, LP) protein, for eight weeks, as described elsewhere (19) (Table 1). The calorie difference between the two diets was balanced with additional carbohydrate instead of protein in the low protein diet. At the end of the experimental period, the nutritional status of the animals was evaluated by determination of body weight. After decapitation, blood samples were colleted and the sera were stored at -20°C for the subsequent measurement of total serum protein, serum albumin, serum glucose and free fatty acids content.

Amino acid profile

Plasma samples were collected from fed and fasted (13 h) animals (NP and LP groups). Samples were deproteinized by the addition of 1 mL of 25% TCA solution to 1 mL of plasma and kept at 4°C for 1 hour. After centrifugation at 10000g, 30 μL of the supernatant was mixed with 60 μL loading sample buffer (Biochrom 20 reagent kit), and 20 μL was run directly and analyzed by Biochrom 20 plus (Amersham Pharmacia) using a specific physiological amino acid column. Free amino acids standards were run first, followed by the samples. Amino acid quantification was performed using Biochrom 20 Control Software Version 3.05 (Year 2000 Compliant Windows 95/Windows NT) and the results were expressed as % of total area (according the manufacturer's instructions).

Pancreatic islet isolation and static insulin secretion

Wistar rats were decapitated and the islets were isolated by hand-picking under a stereomicroscope after collagenase digestion of the pancreas, following a technique previously described (20). Groups of 5 islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer of the following composition (in mmol/L): NaCl, 115; KCl, 5; $CaCl_2$, 2.56;

$MgCl_2$, 1; $NaHCO_3$,24 and glucose, 5.6, supplemented with 3 g/L of bovine serum albumin and equilibrated with a mixture of 95% O_2 / 5% CO_2, pH 7.4. This medium was then replaced with fresh buffer and the islets incubated for 1h with 5.6; 8.3 or 16.7 mmol/L glucose. The insulin in the medium at the end of the incubation period was measure by RIA (21).

Macroarray analysis

RNA was isolated from islets using the TRIzol reagent-phenol-chloroform procedure (Invitrogen), followed by digestion of genomic DNA by RNase-free DNase. Quality and purity were analyzed by electrophoresis in denaturing gel and PCR. Radiolabeled cDNA was prepared using 5 μg total RNA added in a 10 μL volume containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.5 mmol/L dNTP mixture without dATP, 5 mmol/L dithiothreitol, gene-specific CDS primer mix (mix of primers specific for each different type of atlas array) (Clontech Labs, Palo Alto, CA), and Maloney murine leukemia virus reverse transcriptase (Clontech Labs) in the presence of 35 μCi [α-^{33}P] dATP (3000 Ci/mmol; Amersham, Piscataway, NJ). After incubation at 50°C for 25 min, the reaction was stopped by addition of 0.01 mol/L EDTA (pH 8.0). cDNA generated was purified by column chromatography (Chroma Spin-200 DEPC-H_2O columns - Clontech Labs).

The arrays were performed, in parallel, under identical conditions according to the manufacturer's instructions (Clontech Labs). Atlas rat 1.2 arrays were composed of 1,176 genes spotted on positively charged nylon membranes. Plasmid and bacteriophage DNAs were included as negative controls to confirm hybridization specificity and housekeeping cDNAs were used as positive controls for normalization of mRNA abundance. All the cDNAs and controls immobilized on the membrane were grouped in several clusters according with their functions. The complete list of arrayed genes is available on line (http://www.clontech.com/atlas/genelist/index.shtml).

The membranes were pre-hybridized in ExpressHyb™ buffer containing 0.5 mg heat denatured sheared salmon DNA at 68°C for 30 min. Labeled cDNA probe was added to the pre-hybridization buffer (2-10 X 10^6 cpm/membrane) and hybridization continued overnight at 68°C. The membranes were washed and exposed directly to a storage phosphor screen (Molecular Dynamics). The screens were scanned using Storm 840. Signal intensities attained by Array Vision Evaluation 7.0 software, from each spot were normalized using the intensities of the

housekeeping genes (polyubiquitin, phospholipase A2 group IB and ribosomal protein S29) provided in the array to compare, quantitatively, the signal intensity of each gene on different membranes (LP and NP). Differentially expressed genes were identified using Microsoft ™ Excel XP. The result for each gene was expressed as fold change for LP rats compared with NP controls with were taken as 1. We considered that low protein diet induced differences in gene expression when software showed that the fold change was ≥ 2.0 (up-regulated) or ≤ 0.5 (down-regulated). The experiment was performed twice using cDNA obtained from two different sets of 5 rats and new membranes each time. Genes were classified on different functional clusters based on the putative biological function of the encoded protein, as determined by database searches on Pubmed.

RNA isolation and RT-PCR

Semi-quantitative RT-PCR was performed to validate the findings of gene transcript measurements by the macroarray analysis. Briefly, total RNA was extracted from 300 islets obtained from LP and NP rats using the TRIzol reagent-phenol-chloroform procedure (Invitrogen). Before the reverse transcription, electrophoresis in denaturing gel, DNase treatment and PCR were performed to analyze the quality, purity and genomic DNA contamination, respectly. cDNA was prepared using 2 μg of total RNA and 150 ng of random hexamers in a 20 μL solution containing 50 mM Tris-HCl (pH 8.4), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.125 mM of each dNTP, and 200 units of SuperScript™ II RNase H^ reverse (Invitrogen). After incubation for 1 h at 42°C, the reaction mixture was heated to 70°C for 15 min to inactivate the RT and the cDNA was stored at -20°C.

PCRs were then carried out by mixing 2 μl of reverse transcriptase reaction mixture in a final volume of 50 μL reaction containing 0.2 mM dNTPs, 10 pmol of a pair of oligonucleotide primers (Table 2), 50 mM KCL, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4 and 2 units of *Taq* DNA polymerase (Invitrogen). The number of cycles was selected to allow linear amplification of the cDNA, primer sequences and their respective PCR fragments lengths are shown in Table 1.

We used RNA of a modified Potato Virus X (PVX - Gene Bank D00344) as an external control instead of β-actin, since β-actin was altered under our condition (LP islets). PVX RNA was obtained by in vitro transcription with "RiboMAX™ Large Scale RNA Production System-T7" (Promega), following the manufacturer's instruction. PVX sequence has no homology to any

rat sequence as confirmed by BLAST search and RT-PCR (data not shown). An aliquot of the external control was then thawed on ice and 0.06 μg was mixed with fresh islets (300) before RNA extraction (22).

The amplified products were analyzed by eletrophoresis on 1.8% agarose gels in Tris-borate-EDTA buffer 1X (TBE 1X) and stained with ethidium bromide. All reactions included a negative control. Subsequent digitalization and relative band intensities were performed employing an Eagle Eye II documentation system (Stratagene. La Jolla. Ca). The results were expressed as a ratio of target to standard signals.

Statistical Analysis

Results are expressed as means ± SEM. Student's unpaired t test was generally used to compare normal (NP) and LP groups. Insulin secretion data were log-transformed to correct for heterogeneity in variance and then analyzed by two-way ANOVA. followed by the Tukey-Kramer test to determine significant differences between groups and among glucose and secretagogue concentrations. and to assess the interactions between these factors. Data were analyzed using the Statistica software package (Statsoft. Tulsa. OK) and the level of significance was set at $P<0.05$.

RESULTS

Features of the rats - As observed in multiple studies, protein deprivation induced many functional and morphological alterations (23-27). In our studies, rats submitted to an isocaloric diet containing low protein (6%), for 8 weeks, demonstrated reduced body weigh, higher free fatty acid levels, normoglycemia, decreased total serum protein and albumin, increased liver glycogen and fat contents, and lower plasma insulin (fed) levels when compared to NP ($P<0.05$) (Table 3).

Amino acids profile - Serum analysis of the LP group showed a modified amino acid profile either during fed or fasting periods, as a consequence of deficient protein intake (Table 4). The fasting state was characterized by decreased levels in several amino acids, such as taurine, hydroxyproline, threonine, asparagine, valine, methionine, isoleucine, leucine, phenylalanine and 3-methyl-histidine, whilst increased levels of asparagine, serine, and hydroxylysine were observed. In contrast, the amino acid profile during the fed state demonstrated decreased levels of taurine, threonine, valine, leucine, phenylalanine, hydroxylysine, ornithine and arginine, whereas the levels of serine, glutamine, alanine and beta-alanine were increased.

Insulin secretion - A dose-response curve of insulin secretion was observed when NP islets were exposed to increasing concentrations (5.6, 8.3 and 16.7 mM) of glucose. At the same concentrations of glucose, insulin secretion in LP rats was significantly reduced compared with NP islets (Figure 1).

Gene expression - Of the 1176 genes included in the Atlas rat 1.2 array, 32 genes in islets from LP rats displayed significant changes in expression levels compared with NP islets, in two different experiments, after normalization. Of the 32 genes, 17 displayed a greater than two-fold increase, whilst 15 genes demonstrated decreased expression levels of greater than two-fold (Tables 5 and 6). Alterations in genes related to metabolism, hormones, trafficking and targeting proteins and extracellular kinase network members, amongst others, were observed in rats submitted to the low protein diet.

RT-PCR - Semi-quantitative RT-PCR was performed to validate the findings of gene transcript measurements obtained from atlas cDNA array. For this, we selected the following genes: eukaryotic translation initiation factor 2 alpha subunit (eIF2-alpha), secretogranin precursor, glucose transporter-2 (GLUT2), clusterin and phospholipase A2 group IB (PLA2G1B). All the genes analyzed by RT-PCR confirmed the results obtained in the array (Figure 2). The eIF2-alpha and PLA2G1B mRNA concentrations did not differ in islets from NP and LP groups (Fig 2A, F), however decreases in secretogranin (32%), GLUT-2 (53%) and clusterin (31%) mRNA concentrations were observed ($P<0.05$) (Fig. 2B, C, D). The expression of PVX, which was mixed with fresh islets before RNA extraction, did not differ in LP and NP rats when analyzed by RT-PCR (Fig.2E). This result validates the use of this external standard/control for the normalization of gene expression in RT-PCR.

DISCUSSION

Gene expression was analyzed, herein, in young rats maintained on a low protein diet (6%) for 8 weeks, immediately after weaning. Recent studies have reported that nutritionally adverse conditions during early and postnatal periods of life are linked to alterations in pancreatic endocrine functions (25-28). Nutritional perturbations provoked by low protein diets during pregnancy induce changes in amino acid profiles. Thus, multicellular organisms have to adjust several of their physiological functions involved in adaptation to amino acid limitation by regulating the expression of numerous genes (14).

As expected, LP rats showed typical features of malnutrition, including low body weight, low levels of plasma albumin and insulin, higher liver glycogen and fat contents. Despite a significant reduction in insulinemia, glycemia was unaltered in LP compared to NP rats. These findings may be related to a marked increased in insulin sensitivity, explained by an increase in the phosphorylation of the insulin receptor and insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase (19, 29,30).

Changes in amino acid profile, characterized by decreased levels of valine, isoleucine, methionine, phenylalanine and threonine were observed. These amino acids are classified as essential and alterations in their levels and deficiency in any one of them could lead to a negative nitrogen balance and clinical consequences (13). In addition, the amino acid taurine is reduced either during fasting or the fed state in LP rats. Taurine influences glucose metabolism and, as a consequence, augments insulin secretion. Deficiency in taurine slows down growth, decreases insulin secretion and induces cardiac dysfunction and immunological insufficiency (31-33).

Leucine, a stimulator of insulin secretion is also diminished in LP rats. This amino acid stimulates insulin release by elevation of ATP/ADP ratio produced by its direct metabolization in Krebs' cycle or mitochondrial oxidation in β-cells. (34, 35). Previous results obtained by our group suggest that leucine-induced insulin secretion is affected by a low protein diet (36).

To determine possible global transcriptional modifications produced by low protein diet, the cDNA array technique was selected to monitor and compare differential gene expression. The Atlas cDNA array 1.2, containing 1176 genes, including housekeeping genes and negative controls, was used in this study. Among these, 32 genes in islets from LP rats showed alterations

in expression compared with NP islets. As shown in table 5 and 6, metabolic and structural adaptations occur in pancreatic islets of rats submitted to low protein diet.

The expressions of GLUT-2 gene and its respective protein (measured by Western blot, not shown) were reduced in LP islets. Thorens and collaborators (37), using knockout mice for GLUT-2, showed a total loss of first phase-glucose stimulated insulin secretion and a reduced second phase, during perfusion experiments. In addition, a similar pattern of insulin secretion profile was observed in islets of pups from mothers maintained on low protein diets during pregnancy and lactation (36). These results are consistent with GLUT-2 acting as a glucose sensor and regulator of glucose metabolism.

LP islets present increased expression of muscle phosphofructokinase. PFK is tetramer composed of three subunit isoforms, designated M (muscle), P (platelet) (or C), and L (liver), involved in the glycolytic pathway (38, 39). All three isoforms were detected in pancreatic islets and clonal pancreatic beta-cells (INS-1) (39, 40). The autocatalytic activation of phosphofructokinase by its product fructose 1,6-bisphosphate generates glycolytic oscillations. The overexpression of L-phosphofructokinase in transgenic mice showed defective glucose metabolism, followed by impaired glucose-induced insulin response (41). Thus, it is possible to suggest that alterations in the enzyme subunit composition could leads changes in the enzymatic properties and activity, which may result in decreased glucose-induced insulin secretion.

The voltage-gated ion channel plays an important role in the insulin secretion stimulated by glucose or others insulinotropic agents. Increased levels of the ATP/ADP ratio, due to the metabolism of glucose, closes the K_{ATP} channels, depolarizing the β-cell, leading to the opening of the voltage-dependent calcium channel, allowing calcium flux and triggering insulin granule exocytosis (36, 42, 43). In LP islets, previous data have shown decreased movement of intracellular calcium and reduced expression of PKCα (27, 36, 42). In addition, we observed, herein, diminishing gene expression of voltage-gated K^+ channel proteins, which could explain, at least, in part the poor secretory response, to glucose or other secretagogues in LP rats.

Alterations in mRNA expression of G-protein Rab 26 were observed. Although functions of this protein are not completely understood, some ras-related proteins are implicated in intracellular vesicle traffic along the biosynthetic and endocytic pathways, establishing functional links with SNARE complexes (44-46). Sequence comparison between Rab 26 and other Ras proteins described in the literature revealed closest homology to Rab 8, Rab 3A, Rab 15 and

SEC4 (47). To our knowledge, no information concerning the effect of RAB 26 is available. However, other members of the RAB family such as Rab 3A, Rab 3B and Rab 3D, were described as negative modulators of Ca^{2-}- triggering exocytosis in the B cell (48, 49). Thus, it is conceivable that the increase in RAB 26 expression observed herein may be related to reduction in insulin secretion in LP islets. Conversely, a decreased level in calcium/calmodulin-dependent protein kinase was observed. This protein kinase is related to Ca^{2-} modulation and induction of insulin secretion and appears to be the major Rab 3A-associating protein in pancreatic β-cells (50, 51).

LP rats also showed a 3.3 fold increase in Carboxypeptidase D, an important enzyme present in the trans-Golgi network, responsible for the production of peptides and proteins that transit the secretory and endocytic pathways and cleaving C-terminal residues from the prohormones (52). Interestingly, reduction in transcriptional expression of secretogranin III was observed. This protein is involved in the production or release of peptide hormones from the storage vesicles of neuroendocrine cells involved in the biogenesis of secretory granules (53, 54).

Reduction in mRNA of insulin-like growth factor-I (IGF-I) and IGF-II was observed in β-cells from neonatal rats of mothers maintained on low protein diets during pregnancy. These growth hormones are regulated by the nutritional supply of dietary energy and protein and are able to increase islet cell DNA synthesis and proliferation, providing an important mechanism for linking nutrition and growth (25). In our studies, we observed an increase in IGF-II mRNA, suggesting an increase in proliferation rates.

Elevated levels of matrix metalloproteinase 14 were also observed in our analysis. The matrix metalloproteinase family (MMP) of extracellular proteinase regulates development and physiological events and is important for cell migration, invasion, proliferation and apoptosis, where their main function is presumed to be remodeling of the extracellular matrix (55). However, other genes related to cell growth, differentiation and cellular responses to infection/injury such as STAT3, were concomitantly diminished. (56).

Alterations in signal transduction pathways produced by decreased gene expression were observed in LP, mainly in genes that belong to the intracellular kinase network, such as mitogen-activated protein kinase 3 (MAP3), insulin-stimulated microtube-associated protein kinase 2 (MAP2 kinase), mitogen-activated protein kinase 2. Previous published studies in INS-1 cells and rat islets indicate that MAPS are not involved in insulin secretion, but may be related to other β-

cell functions, such as metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis and differentiation. (50, 57). In addition, a decrease in the mRNA of extracellular signal-regulated kinase 1 and 2 (ERK1, ERK2), whose functions have been related to regulation of meiosis and mitosis in differentiated cells, were identified in our experimental model (58).

Although it should be kept in mind that results obtained by the macroarray technique do not provide information concerning protein levels or enzyme activity, our results suggest that low protein nutritional stimuli, after weaning, associated with changes in amino acid profile, leads to metabolic, structural and physiological adaptations in pancreatic islets. In addition, modifications in expression of several genes from the endocrine pancreas of LP rats could be partly responsible for the decreased insulin secretion observed during islet stimulation by different insulinotropic agents.

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Table 1 - Composition of the normal and low protein diets (g/Kg).

Ingredient	**Normal protein (17% protein)**	**Low protein (6% protein)**
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinized cornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93G-MX)*	35.0	35.0
Vitamin mix (AIN-93-VX)*	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorydrate	2.5	2.5

* For detailed composition see Reeves *et al.*. 1993 (14) - vide anexo

Table 2 - Sequences of PCR primers and PCR conditions for the analysis of specific mRNAs

mRNA	GenBank accession no	Primer sequences (5'-3')	PCR reaction conditions				Size of PCR Product (bp)
			Desn	Ann	Ext	Cycs	
PLA2G1B	D00036	CTGCTGGCTGCTTTGCTCAC ACGGCATAGACAGGAAGTGGG	94	58	72	25	458
eIF2-alpha	J02646	TCTTGCCCATGTTGCTGAGG TAACCACTTTGGGCTCCATCTG	94	57	72	32	503
SGII	M93669	TGCTGAGGCTTCCTTATGGTCC CCCAGATGCTCCTTGATGGC	94	57	72	28	268
Clusterin	M64723	AAACGACTCGCTCCAGGTGG ATCGCAAGGTGGCTTTATTGG	94	57	72	27	506
GLUT-2	J03145	CATTGCTGGAAGAAGCGTATCAG GAGACCTTCTGCTCAGTCGACG	94	55	72	26	408
PVX	D00344	CGATCTCAAGCCACTCTCCG GTAGTTGAGGTAGTTGACCC	94	57	72	23	106

Den, denaturation; Ann, annealing; Ext, extension; Cyc, cycles; PLA2G1B, phospholipase A2 group IB; eIF2-alpha, eukaryotic translation initiation factor 2 alpha subunit; SGII, secretogranin II; GLUT-2, glucose transporter 2; PVX, potato virus X.

Table 3 – Body weight, serum protein, albumin, glucose, insulin, free fatty acid levels and liver glycogen and fatty acid of NP and LP rats after 8-10 weeks of dietary treatment.

Parameters	**NP**	**LP**
Body weight	299.68 ± 7.9	257.58 ± 6.4*
Protein (g/dL)	5.7 ± 0.4	4.9 ± 0.4*
Albumin (g/dL)	3.8 ± 0.2	3.4 ± 0.1*
Glucose (mg/dL)	128.5 ± 6.4	131.2 ± 12.6
FFA (mmol/L)	0.5 ± 0.02	0.7 ± 0.07*
Insulin (ng/mL)	0.92 ± 0.15	0.73 ± 0.22
Insulin (fed) (ng/mL)	1.97 ± 0.8	0.7 ± 0.3*
Liver glycogen (mg glucose/100mg tissue)	0.8 ± 0.2	1.23 ± 0.2*
Liver fat (g FA/100g tissue)	6.5 ± 0.6	13.2 ± 0.8*

* Indicates a significant difference compared with NP rats ($P<0.05$).

Values are mean ± SE, n = 10-15.

Table 4 – Amino acid content (%) present in plasma of male Wistar rats fed low (6%) and normal (17%) protein diets in fed and fasting state.

	Normal Protein (17%)		Low protein (6%)	
	Fed	**Starvation**	**Fed**	**Starvation**
Taurine	4.93 ± 0.15	3.32 ± 0.21	2.87 ± .017[a]	1.52 ± 0.092[b]
Aspartic	0.51 ± 0.086	0.62 ± 0.042	0.63 ± 0.09	0.47 ± 0.07[b]
Threonine	2.21 ± 0.076	1.71 ± 0.42	1.47 ± 0.20[a]	1.52 ± 0.082
Serine	1.99 ± 0.086	4.59 ± 0.17	6.17 ± 0.10[a]	5.15 ± 0.09[b]
Asparagine	4.35 ± 0.23	0.15 ± 0.012	0.53 ± 0.055[a]	0.35 ± 0.018[b]
Glutamic	10.11 ± 0.12	4.47 ± 0.37	2.59 ± 0.12[a]	1.90 ± 0.06[b]
Glutamine	4.35 ± 0.21	16.67 ± 0.72	7.27 ± 0.54[a]	5.23 ± 0.20[b]
Sarcosine	1.12 ± 0.072	0.47 ± 0.019	1.72 ± 0.04[a]	2.07 ± 0.058[b]
Proline	0.92 ± 0.015	1.18 ± 0.01	0.62 ± 0.15[a]	1.03 ± 0.06[b]
Glycine	3.96 ± 0.15	3.66 ± 0.22	5.9 ± 0.40[a]	5.09 ± 0.66[b]
Alanine	3.95 ± 0.95	2.65 ± 0.36	8.83 ± 0.48[a]	5.08 ± 0.66[b]
Citrulline	1.36 ± 0.052	0.87 ± 0.44	1.38 ± 0.086	1.34 ± 0.08
Valine	2.85 ± 0.12	2.25 ± 0.20	2.23 ± 0.12[a]	1.91 ± 0.22
Cysteine	0.05 ± 0.0045	0.08 ± 0.0032	0.076 ± 0.023	0.21 ± 0.092
Methionine	0.93 ± 0.023	0.91 ± 0.057	0.54 ± 0.02[a]	0.53 ± 0.063[b]
Isoleucine	1.36 ± 0.068	0.92 ± 0.032	1.03 ± 0.077[a]	0.88 ± 0.036
Leucine	2.63 ± 0.16	1.59 ± 0.075	2.08 ± 0.12[a]	1.98 ± 0.04[b]
Tyrosine	1.72 ± 0.19	1.49 ± 0.29	1.76 ± 0.015	1.97 ± 0.03[b]
beta-Alanine	0.10 ± 0.023	0.38 ± 0.015	0.18 ± 0.059[a]	0.08 ± 0.012[b]
Aaiba	1.23 ± 0.032	1.38 ± 0.018	1.18 ± 0.032	0.032 ± 0.015
Phenylalanine	1.37 ± 0.047	0.87 ± 0.056	0.87 ± 0.072[a]	0.12 ± 0.032[b]
Homocysteine	0.042 ± 0.012	0.68 ± 0.053	0.032 ± 0.038	0.15 ± 0.05[b]
Ethanolamine	1.13 ± 0.075	0.84 ± 0.065	1.01 ± 0.054	0.53 ± 0.19
Amonium	1.85 ± 0.10	1.12 ± 0.041	2.95 ± 0.18[a]	2.16 ± 0.26[b]
Hydroxylysine	0.35 ± 0.021	0.46 ± 0.006	0.035 ± 0.019[a]	0.68 ± 0.26
Ornithine	1.23 ± 0.085	2.14 ± 0.005	1.16 ± 0.14	0.93 ± 0.019[b]
Lysine	4.18 ± 0.035	3.84 ± 0.046	5.52 ± 0.32[a]	4.5 ± 0.047[b]
Histidine	0.87 ± 0.023	0.99 ± 0.020	0.88 ± 0.042	0.79 ± 0.014[b]
Arginine	1.78 ± 0.045	1.23 ± 0.07	1.35 ± 0.075[a]	1.58 ± 0.032[b]

Values are means ± SEM. n= 4

Different letters indicate a significant difference compared with the respective control. ($p < 0.05$).

Table 5 - Identification of up-regulated genes in rat islets induced by protein restriction diet

Protein gene	Acess no.	Fold Induction
Membrane channels & transporters		
Multidrug resistance protein	X96394	7.5
Simple carbohydrate metabolism		
Cytosolic hydroxymethylglutaryl-CoA synthase	X52625	4.0
Energy metabolism / Other intracellular transducers, effectors & modulators		
Mitochondrial H+ transporting ATP synthase F1 complex alpha subunit isoform 1	X56133	2.7
Muscle Phosphofructokinase	U25651	3.2
Metalloproteinases		
Matrix metalloproteinase 14; membrane-type matrix metalloproteinase 1	X83537	2.0
Oncogenes & tumor suppressors		
Wilms' tumor protein homolog 1 (WT1)	X69716	21.1
Nucleotide metabolism		
cAMP-dependent protein kinase type-I alpha regulatory subunit	M17086	2.5
Growth factors, cytokines & chemokines		
Insulin-like growth factor II (IGF2)	M13969	3.6
Hormone receptors / G protein-coupled receptors		
Thyrotropin-releasing hormone	D17469	15.0
Estrogen receptor beta; ESR2 ; nuclear receptor subfamily 3 group A member 2	U92289	7.3
Calcium-binding proteins		
NVP-3; neural visinin-like Ca^{2+} binding protein	D13126	2.0
Neurotransmitter receptors / Ligand-gated ion channels		
Gamma-aminobutyric acid receptor alpha 2 subunit	L08491	4.3
Gastric inhibitory polypeptide receptor; glucose-dependent insulinotropic polypeptide receptor	L19660	3.1
Amino- & carboxypeptidases / Other trafficking & targeting proteins		
Carboxypeptidase D	D49846	3.3
G proteins / Other trafficking & targeting proteins		
Ras-related protein RAB26	U18771	4.0

Table 6 - Identification of down-regulated genes in rat islets induced by protein restriction diet.

Protein / gene	Acess no.	Fold Induction
Other trafficking & targeting proteins		
Sterol carrier protein 2: SCPX: nonspecific lipid transfer protein	M34728	0.28
Simple carbohydrate metabolism		
Fructose-biphosphate aldolase A	M12919	0.23
Symporters & antiporters		
Solute carrier family 13 member 1	L19102	0.17
Intracellular transducers, effectors & modulator		
Transcription activators & repressors		
Signal transducer & activator of transcription 3 (STAT3)	X91810	0.23
Voltage-gated ion channel		
Voltage-gated K^+ channel protein	M59980	0.24
Intracellular kinase network members		
Mitogen-activated protein kinase 3: extracellular signal-regulated kinase 1 (ERK1): insulin-stimulated microtube-associated protein kinase 2 (MAP2 kinase)	M61177	0.46
Mitogen-activated protein kinase 2: extracellular signal-regulated kinase 2 (ERK2)	M64300	0.22
Calcium/calmodulin-dependent protein kinase type II beta subunit (CAM kinase II beta)	M16112	0.11
Protein phosphatase receptors		
Pheochromocytoma-derivated protein tyrosine phosphatase-like protein	D38222	0.20
Phospholipases & phosphoinositol kinases / Kinase activators & inhibitors		
Amino acid metabolism		
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta/delta: protein kinase C inhibitor protein 1: mitochondrial import stimulation factor S1 subunit	D17615	0.16
Metalloproteinases		
Carboxypeptidase E	M31602	0.27
Cysteine Proteases		
Cathepsin L	Y00697	0.25
Other extracellular communication proteins /Trafficking & targeting proteins		
Secretogranin III	U02983	0.25
Facilitated diffusion proteins		
Glucose transporter	J03145	0.18
Amino & carboxypeptidases		
Metabolism of cofactors, vitamins & related substances		
Dipeptidase (DPEP1)	M94056	0.26
Stress response proteins		
Clusterin	M64723	0.24
Adenylate/guanylate cyclase & diesterases		
Adenylyl cyclase type VI: ATP pyrophosphate-lyase: Ca(2+)-inhibitable adenylyl cyclase	L01115	0.27

FIGURE LEGENDS

Figure 1 - Insulin secretion by islets from NP and LP rats at different concentrations of glucose. Five islets were preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing 5.6 mM glucose for 45 min. Islets insulin-secretory responsiveness was determined after incubation in medium containing 5.6, 8.3 and 16.7 mM glucose. Values are mean +/- SE, n = 10. Different letters indicate a significant difference compared with the respective control ($p < 0.05$).

Figura 2 – Relative expression of mRNA levels of the (A) eukaryotic translation initiation factor 2 alpha subunit; (B) secretogranin; (C) glucose transporter 2; (D) clusterin; (E) potato virus X and (F) phospholipase A2 precursor genes in islets from LP and NP rats. mRNA levels were semiquantified by RT-PCR. Top: representative ethidium bromide-stained agarose gel(s) separation for specific mRNA levels from LP and NP islets. In each agarose gel(s) separation, the upper band corresponds to the cDNA for the specific mRNA indicated, and the lower band corresponds to the external control (PVX). The expression value for the mRNA of each gene from NP and LP was expressed relative to PVX. Values are means ± SE, n = 6-8 (*$P<0.05$).

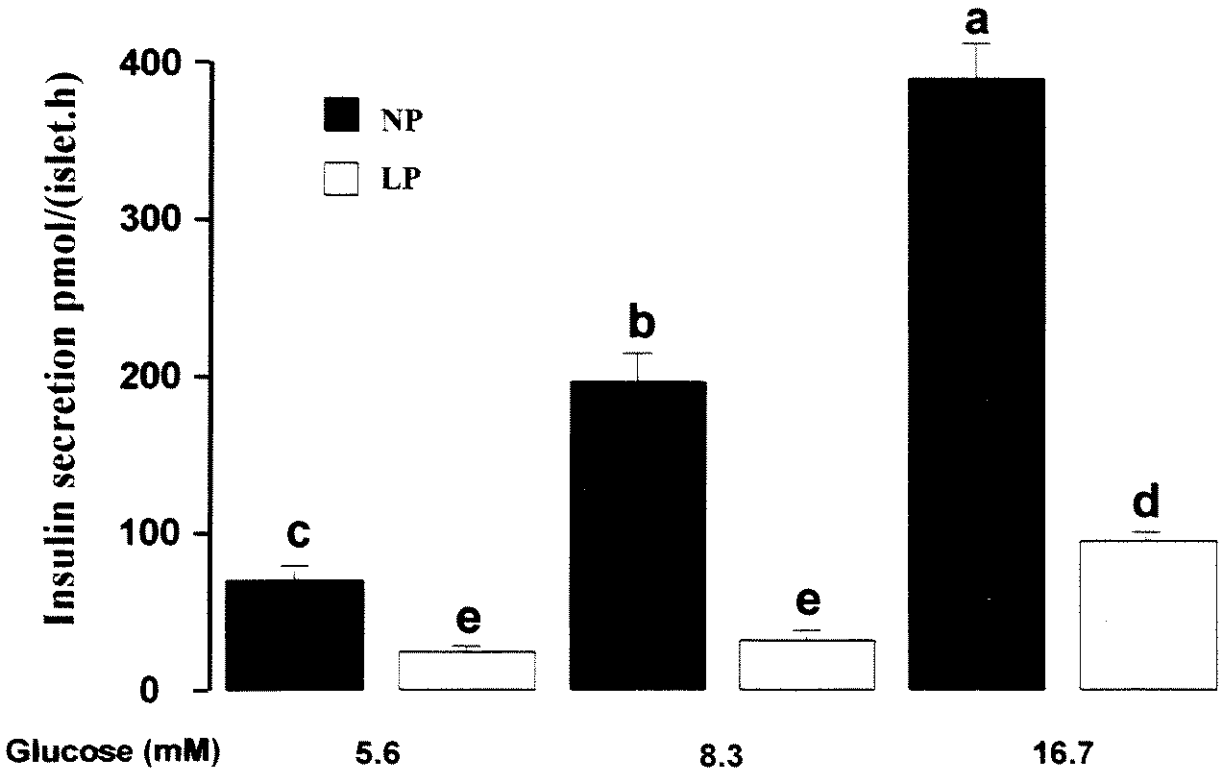

Figure 1

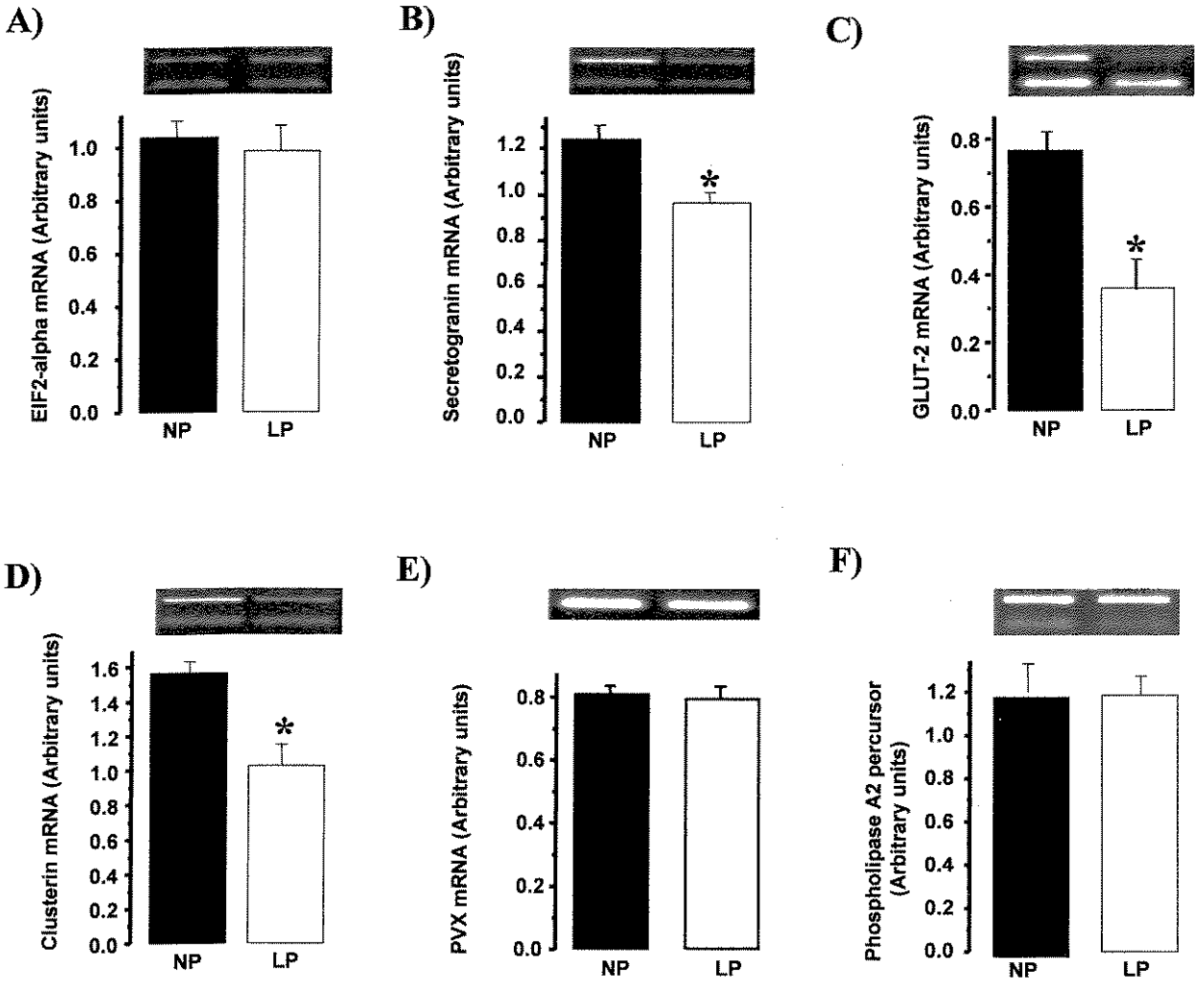
A)
EIF2-alpha mRNA (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
NP
LP
B)
Secretogranin mRNA (Arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NP
LP
C)
GLUT-2 mRNA (Arbitrary units)
0.8
0.6
0.4
0.2
0.0
*
NP
LP
D)
Clusterin mRNA (Arbitrary units)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NP
LP
E)
PVX mRNA (Arbitrary units)
0.8
0.6
0.4
0.2
0.0
NP
LP
F)
Phospholipase A2 percursor (Arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NP
LP

Figure 2

REFERENCES

1- Fowden, A. L. & Hill, D. J. (2001) Intra-uterine programming of the endocrine pancreas. Br Med Bull 60: 123-142.

2- Vaulont, S. & Kahn, A. (1994) Transcriptional control of metabolic regulation genes by carbohydrates. Faseb J 8: 28-35.

3- Towle, H. C. (1995) Metabolic regulation of gene transcription in mammals. J Biol Chem 270: 23235-23238.

4- Foufelle, F., Girard, J. & Ferre, P. (1998) Glucose regulation of gene expression. Curr Opin Clin Nutr Metab Care 1: 323-328.

5- Pegorier, J. P. (1998) Regulation of gene expression by fatty acids. Curr Opin Clin Nutr Metab Care 1: 329-334.

6- Srinivasan, M., Song, F., Aalinkeel, R. & Patel, M. L. (2001) Molecular adaptations in islets from neonatal rats reared artificially on a high carbohydrate milk formula. J Nutr Biochem

7- Harper, A. E. & Rogers, Q. R. (1965) Amino acid imbalance. Proc Nutr Soc 24:173-190.

8- Richardson, L. R., Hale, F. & Ritchey, S. J. (1965) Effect of fasting and level of dietary protein on free amino acids in pig plasma. J Anim Sci 24:368-379.

9- Peng, Y., Meliza, L. L., Vavich, M. G. & Kemmerer, A. R. (1975) Effects of amino acid imbalance and protein content of diets on food intake and preference of young, adult, and diabetic rats. J Nutr 105:1395-1404.

10- Reusens, B., Dahri, S., Snoeck, A., Bennis-Taleb, N., Remacle, C. & Hoet, J. J. (1995) Long term consequences of diabetes and its complications may have a fetal origin: experimental and epidemiological evidence. In: Diabetes (Cowett, R. M., ed.), vol. 35, pp. 187-198. Nestlé Nutrition Workshop Series, Raven Press, New York, NY.

11- Cetin, I. (2001) Amino acid interconversions in the fetal-placental unit: the animal model and human studies in vivo. Pediatr Res 49:148-154.

12- Kilberg, M. S., Hutson, R. G. & Laine, R. O. (1994) Amino acid-regulated gene expression in eukaryotic cells. Faseb J 8: 13-19.

13- Jousse, C., Bruhat, A. & Fafournoux, P. (1999) Amino acid regulation of gene expression. Curr Opin Clin Nutr Metab Care 2: 297-301.

14- Fafournoux, P., Bruhat, A. & Jousse, C. (2000) Amino acid regulation of gene expression. Biochem J 351: 1-12.

15- Snoeck, A., Remacle, C., Reusens, B. & Hoet, J. J. (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate 57: 107-118.

16- Dahri, S., Snoeck, A., Reusens-Billen, B., Remacle, C. & Hoet, J. J. (1991) Islet function in offspring of mothers on low-protein diet during gestation. Diabetes 40 Suppl 2: 115-120.

17- Hoet, J. J., Dahri, S., Snoeck, A., Reusens-Billen, B. & Remacle, C. (1992) Importance of diets and their effect on fetal development: function and structure of the endocrine pancreas following protein deficiency during intrauterine life. Bull Men Acad R Med Belg, 147: 174-181.

18- Latorraca, M. Q., Carneiro, E. M., Mello, M. A. & Boschero, A. C. (1999) Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake. J Nutr Biochem 10:37-43.

19- Reis, M. A., Carneiro, E. M., Mello, M. A., Boschero, A. C., Saad, M. J. & Velloso, L. A. (1997) Glucose-induced insulin secretion is impaired and insulin-induced phosphorylation of the insulin receptor and insulin receptor substrate- 1 are increased in protein-deficient rats. J Nutr 127: 403-410.

20- Bordin, S., Boschero, A. C., Carneiro, E. M. & Atwater, I. (1995) Ionic mechanisms involved in the regulation of insulin secretion by muscarinic agonists. J Membr Biol 148: 177-184.

21- Scott, A. M., Atwater, I. & Rojas, E. (1981) A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia 21: 470-475.

22- Eickhoff, B., Korn, B., Schick, M., Poustka, A. & van der Bosch, J. (1999) Normalization of array hybridization experiments in differential gene expression analysis. Nucleic Acids Res 27: e33.

23- Hales, C. N., Desai, M., Ozanne, S. E. & Crowther, N. J. (1996) Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. Biochem Soc Trans 24:341-350.

24- Berney, D. M., Desai, M., Palmer, D. J., Greenwald, S., Brown, A., Hales, C. N. & Berry, C. L. (1997) The effects of maternal protein deprivation on the fetal rat pancreas: major structural changes and their recuperation. J Pathol. 183: 109-115.

25- Petrik, J., Reusens, B., Arany, E., Remacle, C., Coelho, C., Hoet, J. J. & Hill, D. J. (1999) A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. Endocrinology 140: 4861-4873.

26- Cherif, H., Reusens, B., Dahri, S. & Remacle, C. (2001) A protein-restricted diet during pregnancy alters in vitro insulin secretion from islets of fetal Wistar rats. J Nutr 131: 1555-1559.

27- Ferreira, F., Filiputti, E., Arantes, V. C., Stoppiglia, L. F., Araujo, E. P., Delghingaro-Augusto, V., Latorraca, M. Q., Toyama, M. H., Boschero, A. C. & Carneiro, E. M. (2003) Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase calpha expression. J Nutr 133: 695-699.

28- Arantes, V. C., Teixeira, V. P., Reis, M. A., Latorraca, M. Q., Leite, A. R., Carneiro, E. M., Yamada, A. T. & Boschero, A. C. (2002) Expression of PDX-1 is reduced in pancreatic islets from pups of rat dams fed a low protein diet during gestation and lactation. J Nutr 132: 2030-3035.

29- Latorraca, M. Q., Carneiro, E. M., Boschero, A. C. & Mello, M. A. (1998) Protein deficiency during pregnancy and lactation impairs glucose- induced insulin secretion but increases the sensitivity to insulin in weaned rats. Br J Nutr 80: 291-297.

30- Rao, R. H. (1988) Diabetes in the undernourished: coincidence or consequence? Endocr Rev 9: 67-87.

31- Lake, N., Wright, E. D. & Lapp, W. S. (1992) Effects of taurine deficiency on immune function in mice. Adv Exp Med Biol 315: 241-243.

32- Eley, D. W., Lake, N. & ter Keurs, H. E. (1994) Taurine depletion and excitation-contraction coupling in rat myocardium. Circ Res 74: 1210-1219.

33- Cherif, H., Reusens, B., Ahn, M. T., Hoet, J. J. & Remacle, C. (1998) Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. J Endocrinol 159: 341-348.

34- Gao, Z., Young, R. A., Li, G., Najafi, H., Buettger, C., Sukumvanich, S. S., Wong, R. K., Wolf, B. A. & Matschinsky, F. M. (2003) Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. Endocrinology 144: 1949-1957.

35- Li, C., Najafi, H., Daikhin, Y., Nissim, I. B., Collins, H. W., Yudkoff, M., Matschinsky, F. M. & Stanley, C. A. (2003) Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. J Biol Chem 278: 2853-2858.

36- Latorraca, M. Q., Reis, M. A., Carneiro, E. M., Mello, M. A. & Boschero, A. C. (1999) Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake. J Nutr Biochem 10: 37-43.

37- Thorens, B. (2003) A gene knockout approach in mice to identify glucose sensors controlling glucose homeostasis. Pflugers Arch 445: 482-490.

38- Dunaway, G. A. & Kasten, T. P. (1987) Nature of the subunits of the 6-phosphofructo-1-kinase isoenzymes from rat tissues. Biochem J 242: 667-671.

39- Yaney, G. C., Schultz, V., Cunningham, B. A., Dunaway, G. A., Corkey, B. E. & Tornheim, K. (1995) Phosphofructokinase isozymes in pancreatic islets and clonal beta-cells (INS-1). Diabetes 44: 1285-1289.

40- Eto, K., Sakura, H., Yasuda, K., Hayakawa, T., Kawasaki, E., Moriuchi, R., Nagataki, S., Yazaki, Y. & Kadowaki, T. (1994) Cloning of a complete protein-coding sequence of human platelet-type phosphofructokinase isozyme from pancreatic islet. Biochem Biophys Res Commun 198: 990-998.

41- Knobler, H., Weiss, Y., Peled, M. & Groner, Y. (1997) Impaired glucose-induced insulin response in transgenic mice overexpressing the L-phosphofructokinase gene. Diabetes 46: 1414-1418.

42- Carneiro, E. M., Mello, M. A., Gobatto, C. A. & Boschero, A. C. (1995) Low protein diet impairs glucose-induced insulin secretion from and ^{45}C uptake by pancreatic rat islets. Nutr Biochem 6: 314-318.

43- Miki, T., Nagashima, K. & Seino, S. (1999) The structure and function of the ATP-sensitive K+ channel in insulin- secreting pancreatic beta-cells. J Mol Endocrinol 22: 113-123.

44- Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E. & Sollner, T. (1994) A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell 78: 937-948.

45- Johannes, L., Doussau, F., Clabecq, A., Henry, J. P., Darchen, F. & Poulain, B. (1996) Evidence for a functional link between Rab3 and the SNARE complex. J Cell Sci 109: 2875-2884.

46- Lupashin, V. V. & Waters, M. G. (1997) t-SNARE activation through transient interaction with a rab-like guanosine triphosphatase. Science 276: 1255-1258.

47- Wagner, A. C., Strowski, M. Z., Goke, B. & Williams, J. A. (1995) Molecular cloning of a new member of the Rab protein family, Rab 26, from rat pancreas. Biochem Biophys Res Commun 207: 950-956.

48- Easom, R. A. (2000) Beta-granule transport and exocytosis. Semin Cell Dev Biol 11: 253-266.

49- Kajio, H., Olszewski, S., Rosner, P. J., Donelan, M. J., Geoghegan, K. F. & Rhodes, C. J. (2001) A low-affinity Ca^{2+}-dependent association of calmodulin with the Rab3A effector domain inversely correlates with insulin exocytosis. Diabetes 50: 2029-2039.

50- Jones, P. M. & Persaud, S. J. (1998) Ca(2+)-induced loss of Ca2+/calmodulin-dependent protein kinase II activity in pancreatic beta-cells. Am J Physiol 274: E708-715.

51- Coppola, T., Perret-Menoud, V., Luthi, S., Farnsworth, C. C., Glomset, J. A. & Regazzi, R. (1999) Disruption of Rab3-calmodulin interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis. Embo J 18: 5885-5891.

52- Sidyelyeva, G. & Fricker, L. D. (2002) Characterization of Drosophila carboxypeptidase D. J Biol Chem 277: 49613-49620.

53- Rong, Y. P., Liu, F., Zeng, L. C., Ma, W. J., Wei, D. Z. & Han, Z. G. (2002) Cloning and characterization of a novel human secretory protein: secretogranin III. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) 34: 411-417.

54- Taupenot, L., Harper, K. L. & O'Connor, D. T. (2003) Mechanisms of disease: the chromagranin family. N Engl J Med. 348: 1134-1149.

55- Vu, T. H. & Werb, Z. (2000) Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev 14: 2123-2133.

56- Bromberg, J. & Darnell, J. E., Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19: 2468-2473.

57- Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. (2002) The protein kinase complement of the human genome. Science 298: 1912-1934.

58- Johnson, G. L. & Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298: 1911-1912.

ARTIGO 2

Modulation of gene and protein expression involved in the mechanism of insulin secretion in pancreatic islets from rats submitted to protein restriction

Viviane Delghingaro-Augusto, Fabiano Ferreira, Maria Esméria Corezola do Amaral, Eliana Pereira Araújo, Antonio Carlos Boschero, Everardo Magalhães Carneiro

Departamento de Fisiologia e Biofisica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), SP, Brazil.

Correspondence to: Dr. A Carlos Boschero, Departamento de Fisiologia e Biofisica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brazil. Phone: 55 19 3788 6202; FAX: 55 19 3289 3124
Email: boschero@unicamp.br

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Running title: Protein restriction gene expression and insulin secretion

Key words: insulin secretion, gene and protein expression, pancreatic islets, protein restriction

Abbreviations: cDNA, complementary deoxynucleic acid; DAG, diacylglycerol; EDTA, ethylenediamine tetraacetic acid; FFA, free fatty acid; IP_3, 1, 4, 3 inositol triphosphate; IR, insulin receptor; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; LP, low protein; mRNA, messenger ribonucleic acid; NP, normal protein; PI3-K, phosphatidylinositol 3-kinase; PKAα, protein kinase cAMP dependent catalytic subunit alpha; PKB, protein kinase B; PKC, protein kinase c; PCR, polymerase chain reaction; PVX, potato virus x; RT-PCR, reverse transcriptase-polymerase chain reaction, K^+, potassium.

ABSTRACT

The maintenance of rodents in a protein deficient diet leads to changes in the development of endocrine pancreas producing beta cell dysfunction. As a consequence, insulin secretion in response to different secretagogues is reduced as well as the number of beta cells and size and vascularization of the islets. In the present study, we analyzed the expression of some genes and proteins related to glucose metabolism and insulin secretion in islets from rats maintained on a low (6%, LP) or control (17%, NP) protein diet. The islets from LP rats showed a 57 % reduction in the total islet and pancreas insulin contents associated with a 30% reduction of insulin mRNA. Lower D-[U^{14}C]-glucose oxidation to $^{14}CO_2$ and a marked reduction in PKAα and PKCα expression were also observed in LP compared to NP islets. These alterations were accompanied by a significant reduction in insulin secretion in response to glucose, leucine, arginine and high concentrations of K^+. In addition, several components involved in insulin signaling systems such as IR and IRS-1 were also altered in LP compared to NP islets. Taken together, these results indicate that the final reduction in insulin synthesis and secretion in islets from rats maintained on a low protein diet is a consequence of alterations in several proteins belonging to different signal transduction pathways in pancreatic islet cells.

INTRODUCTION

Stimulus applied at a critical or sensitive period of development can produce changes in the structure and/or function of several organs predisposing the development of degenerative diseases, such as diabetes, hypertension, obesity and cardiac dysfunctions in adulthood (1-2).

Nutritional alterations during fetal and early postnatal periods, induced by either low protein or caloric restriction diets, interfere with beta cell development leading to alterations in cell number, size, pancreas vascularization and disturbed insulin secretion stimulated by glucose or other secretagogues (3-6). Since altered concentrations of specific amino acids in the plasma have been observed in malnourished rats (7) some studies have associated those alterations in endocrine pancreas to amino acid limitation. Thus, deficiency of any one of the essential amino acids can lead to a negative nitrogen balance and clinical symptoms (8).

Several adaptative responses at biochemical, cellular and molecular levels were reported in the pancreas and other tissues of rats maintained on nutritional interventions (9-16).

The biosynthesis and insulin secretion by pancreatic B-cells are stimulated by glucose in a dose-dependent manner. Other substances such as amino acids, fatty acids, peptide hormones and cyclic nucleotides are also able to potentiate glucose-induced insulin secretion (17).

In rodents, glucose, the major physiological regulator of insulin secretion, transported into the B-cells through GLUT-2, is phosphorylated by glucokinase undergoing to glycolysis and oxidation. This results in augmentation in the ATP/ADP ratio that, in turn, inhibits K^{-}_{ATP} channels, inducing depolarization of the plasma membrane and the consequent opening of voltage-dependent Ca^{2-} channels, culminating with Ca^{2-} influx (18-22). Elevation of $[Ca^{2-}]_i$ triggers exocytosis by both the fusion of a readily-releasable pool of plasma membrane-bound insulin granules (primed granules) and trafficking/mobilization of intracellular stored insulin granules to the cell surface (23, 24).

Insulin secretion can also be activated by augmentation in the phosphoinositide turnover, which increases DAG and IP_3 production, leading to activation of PKC isoforms, resulting in mobilization of stored Ca^{2-} and increased $[Ca^{2-}]_i$, which may directly trigger fusion of the secretory granules to the plasma membrane. Glucose metabolism coupled to increased activity of adenylyl cyclase-cAMP activates PKA, which enhances insulin secretion at a distal site through

the elevation of $[Ca^{2+}]_i$ from endoplasmic reticulum, sensitizing the exocytotic machinery to Ca^{2+} (25).

Furthermore, recent reports have indicated the presence of components involved in the insulin signaling pathways in B-cell, such as the IR (26-28), IRS-1 and IRS-2 (29-31), PI3-K (32, 33) and PKB (34) proteins. Several studies using knockout animals for any one of these proteins have shown the importance of this pathway for initiating insulin synthesis at both transcriptional and translational levels, increasing insulin cellular content and secretion (31-33). Thus, defects in any of these components could be also involved in impaired insulin secretion and resistance, as seen in type 2 diabetes (36, 38, 39).

Based on our previous results, obtained by cDNA array analysis, which indicated significant changes at the transcription level of several genes in pancreatic islets from rats submitted to protein restriction, we decided to investigate gene and protein expression of some key molecules involved in the mechanism of insulin secretion in islets from LP and NP rats.

MATERIALS AND METHODS

Animals and diet

The Institutional (UNICAMP) Committee for Ethics in Animal Experimentation (São Paulo-Brazil) approved all the experiments described herein. Male Wistar rats (28 days old) from the breeding colony at UNICAMP were maintained at 24°C with a 12 h light dark cycle and had free access to food and water. The rats were distributed randomly and were fed with 17% (normal protein diet, NP) or 6% (low protein diet, LP), during eight weeks, as described elsewhere (40, 41). The calorie difference between the two diets was balanced with additional carbohydrate instead of protein in the low protein diet. At the end of the experimental period, animals' nutrition status was evaluated. The rats were anesthetized by intraperitoneal injection of sodium-amo-barbital (15 mg/kg body weight) and, following the loss of corneal and pedal reflexes, the animals were killed by decapitation. Blood samples were colleted and the sera were stored at -20°C for the subsequent measurement of total serum protein (Bio-Rad Laboratories GmbH, Munchen, Germany), albumin (42), glucose (DiaSys Diagnostic Systems, Holzheim, Germany), free fatty acids (FFA) levels (Nonsterified Fatty Acid C kit, Wako Chemicals, Neuss, Germany). Part of the serum was stored at -20°C for the subsequent measurement of insulin by RIA (43).

Pancreas insulin content

Following decapitation, the pancreas of four rats were removed after 10 mL of acid ethanol mixture injection (75% ethanol: 1.5% HCl) and incubated overnight at 4°C. After homogenization using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA), the tissue extracts were centrifuged at 664 X g at 4°C for 20 min, and the supernatant used for determination of insulin content by RIA (43).

Pancreatic islet isolation and measurement of insulin secretion

Islets were collected under a stereomicroscope after collagenase digestion of the pancreas, following a technique previously described (44). Groups of 5 islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer of the following composition (in mmol/L): NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, and glucose 5.6, supplemented with 3 g/L of bovine serum albumin and equilibrated with a mixture of 95% O_2 / 5% CO_2, pH 7.4. This medium was

then replaced with fresh buffer and the islets incubated for 1 h with 2.8; 8.3; 16.7 or 27 mmol glucose/L. The insulin in the medium at the end of the incubation period was measured by RIA (43). A second series of insulin secretions experiments were performed using medium containing 2.8 mM glucose plus the following secretagogues (in mmol/L): K^+, 5, 20, 80; leucine, 20; arginine, 20. Total islet insulin content was accessed by addition of 1 mL of acid ethanol mixture (75% ethanol: 1.5% HCl).

Glucose Metabolism – Glucose oxidation in pancreatic islets was measured as conversion of [U-^{14}C]-labeled glucose into $^{14}CO_2$ (45). Briefly, groups of 15 islets were placed in wells containing Krebs-bicarbonate buffered media (50 µL) supplemented with trace amounts of D-[U-^{14}C] glucose (10 µCi/mL) plus non-radioactive glucose to a final concentration of 2.8 or 16.7 mM. The wells were suspended in 20 mL scintillation vials, which were gassed with 5% O_2 and 95% CO_2 and capped airtight with rubber membranes. The vials were shaken continuously for 2 h at 37°C in a water bath. After incubation, 0.1 mL HCl (0.2N) and 0.2 mL hyamine hydroxide were injected through the rubber cap into the glass cup containing the incubation media and into the counting vial, respectively. After 1 h at room temperature, 6 mL of scintillation fluid was added to the hyamine and the radioactivity was counted. The rate of glucose oxidation was expressed as pmol/islet.2h.

RNA isolation and RT-PCR

Total RNA was extracted from 500 islets obtained from the respective pool of 3 rats (LP and NP) using TRIzol reagent (Invitrogen). Reverse transcription was performed on 2 µg of total RNA using Moloney murine leukemia virus-reverse transcriptase (SuperSript ™ II) and random hexamers, according to manufacturer's instructions (Invitrogen). PCRs were then carried out using *Taq* DNA polymerase, recombinant (Invitrogen) containing 10 pmol of each primer in a master mix of 50 µL. The number of cycles was selected to allow linear amplification of the cDNA. Primer sequences and their respective PCR fragment lengths are shown in Table 1.

To determinate the expression level of the selected genes, the relative amounts of respective products obtained by RT-PCR were normalized by an external control [RNA of a modified Potato Virus X (PVX - Gene Bank D00344)], as described (41). PCR samples were analyzed by electrophoresis on 1.8% agarose gels and visualized by ethidium bromide staining.

Subsequent digitalization and relative band intensities were performed using Scion Image Analysis Software. The results were expressed as a ratio of target to standard signals.

Immunoblotting and immunoprecipitation - After isolation, islets were homogenized in 300 μL of solubilization buffer (10% Triton-X 100, 100 mmol Tris/L (pH 7.4), 10 mmol sodium pyrophosphate/L, 100 mmol sodium fluoride/l, 10 mmol EDTA/l, 10 mmol sodium vanadate/L, 2 mmol PSMF/L, and 0.1 mg/ml aprotinin) for 30 s using a Polytron PT 1200 C homogenizer (Brinkman Instruments, NY, USA). Insoluble material was removed by centrifugation during 20 min at 15000 X g and the supernatant was used for protein determination (Bradford method) and for immunoprecipitation. Samples containing 70 μg total protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies for PKAα, PKCα and GLUT-2. For immunoprecipitation and following immunoblotting of IR and IRS-1, NP and LP groups of islets were exposed to experimental conditions and technical procedures according to previously described procedures (46). Visualization of specific protein bands was detected by incubating membranes with ^{125}I-protein A followed by exposure to autoradiography. Band intensities were quantified by optical densitometry (Scion Image Analysis Software).

Immunohistochemistry - Pancreata from six rats (NP and LP) were examined to determine the expression and tissue distribution of insulin. Tissues were dissected and fixed by immersion in 4% buffered formaldehyde. After embedding in paraffin, consecutive sections, 5 μm thick, were cut and stained by the avidine-peroxidase method. These were incubated for 30 min with 2% of fetal calf serum at room temperature, and then exposed for 12 h in a moister chamber at 4°C with the primary antibody against insulin (produced in guinea-pig) (1/50). For the avidine-peroxidase method, biotinylated secondary antibody was used in incubations for two hours at room temperature, followed by incubation for 1 h with ready-to-use avidine-coupled peroxidase from Vector. The resulting immunocomplexes were detected with 50 mg/100 ml diaminobenzidine - 4 M HCl/0.01 ml/100 ml H_2O_2 dissolved in 5 mM Tris, pH 7.6. Analysis and photo-documentation were performed using an Olympus BX60 Microscope.

Statistical Analysis

The results are expressed as means ± SEM Student's unpaired t test was generally used to compare NP and LP groups. Insulin secretion data were log-transformed to correct for heterogeneity in variance and then analyzed by two-way ANOVA, followed by the Tukey-Kramer test to determine significant difference between groups and among glucose and secretagogue concentrations, and to assess the interactions between these factors. The data were analyzed using the Statistica software package (Statsoft, Tulsa, OK). The level of significance was set at $P<0.05$.

RESULTS

Rat characteristics – All features related to undernutrition, such as low body weight, high free fatty acid levels, reduced total serum protein and albumin, were demonstrated in rats LP ($P<0.05$). In addition, normal glucose levels and decreased levels of plasma insulin, during fed state, were observed in LP rats when compared to NP. A significant reduction in total pancreas and islet insulin contents were also observed in LP rats versus NP ($P<0.05$) (Table 2).

Insulin secretion – Insulin secretion by islets from the LP and NP groups were accessed in response to several stimuli. The insulin secretion stimulated by different concentrations of glucose showed that islets from LP rats had a dose-response curve shifted to the right compared to NP islets. Insulin secretion from LP islets was lower than islets from NP islets ($P < 0.05$). Maximal release obtained at 16.7 mM glucose was higher than basal secretion in both types of islets; however, the rise in insulin secretion was greater in NP than LP islets (Fig. 1A). In addition, the stimulation of insulin secretion by the association of glucose (2.8 mM) and different concentrations of K^+ (5, 20 and 80 mM) was lower in LP compared to NP islets (Fig 1B). As shown in Figure 1C, the insulin secretion in response to glucose (2.8 mM) and leucine (20 mM) was significantly lower in LP, compared to NP islets. Similar results were obtained when insulin secretion was obtained with 2.8 mM glucose plus arginine (20 mM) (Fig 1D).

Glucose Metabolism – In the presence of 16.7 mM glucose, the D-[U-^{14}C]-glucose oxidation to $^{14}CO_2$ was significantly lower in LP compared to NP islets (75.7 ± 15.6 and 149.7 ± 10.3 pmol/islet.2h, respectively; $P < 0.05$). No differences between LP and NP were observed in the presence of 2.8 mM glucose (22.1 ± 0.4 and 21.1 ± 0.6, respectively).

Immunohistochemistry – The presence of insulin in pancreatic islets from LP and NP rats was demonstrated using specific polyclonal antibody against insulin. The central region of the islets showed a higher labeling for the hormone than other areas, according to the pattern of staining obtained in three independent experiments, and based on the evaluation of at least 10 islets per section. In addition, the area staining for insulin was smaller in LP compared to NP islets (Fig. 2).

Gene and protein expression – Based on the reduction of insulin content (islets and pancreas), decreased insulin secretion, in response to different secretagogues and the reduction in glucose metabolism, we investigated the expression of proteins involved in insulin secretion, such as insulin, GLUT-2, PKAα, PKCα, IR and IRS-1. The RT-PCR analysis showed a significant decreased of insulin (30%), GLUT-2 (53%), PKAα (33%), PKCα (54%) and IRS-1 (42%) mRNA (Fig. 3A-E). There was no alteration in PVX gene expression in the LP and NP groups, validating the use of this gene as an external control (Fig. 3F). Densitometry of the bands, obtained from total protein extract and immunoprecipitation analysis for GLUT-2, PKAα, PKCα, and IRS-1, also showed reduced expression of these proteins in LP compared to NP islets ($P<0.05$) (Fig. 4), except for IR, which showed increased levels of expression.

DISCUSSION

Protein restriction in rats reduces body weight, decreases total serum protein, diminishes insulin (fasting) and serum albumin levels and leads to long-lasting functional deficiencies in the endocrine pancreas. In this study, LP rats showed decreased levels of total insulin content (pancreas and islets), which may reflect alterations in insulin biosynthesis at transcriptional and/or translational levels. However, during the fed state, serum insulin and glucose levels were similar in both groups, probably as a consequence of an increased sensitivity to insulin of peripheral tissues in the LP rats (40, 47).

Insulin secretion is stimulated by glucose and other substances including amino acids, fatty acids and peptide hormones, among others. Glucose and leucine metabolism stimulate insulin secretion by increasing the ATP:ADP ratio, which induces depolarization of the beta cell membrane through the closure of K^+-ATP channels and an increase in $[Ca^{2+}]_i$ by opening the Ca^{2+} voltage sensitive channels. The decreased rate of glucose oxidation, under stimulatory conditions, in LP islets with the consequent decrease in ATP generation, may explain, partially, the reduced insulin secretion in response to these secretagogues.

B cell membrane depolarization can also be elicited by crescent concentrations of K^+ and arginine, ultimately leading to insulin secretion. Amino acids can also be metabolized, generating polyamines, which can be used as substrates for transglutaminases or for activation of protein kinases, culminating in insulin secretion (48). Thus, the defective insulin secretion observed in LP islets in response to these secretagogues could be the sum of reduced glucose and leucine metabolism associated with defective ion fluxes and generation of polyamines. We have also observed a reduction in forskolin-induced insulin secretion in LP islets (not shown). Thus, the altered pattern of secretion observed in LP islets could also be due to a defect in the cAMP/PKA pathway.

Although the amount of insulin is reduced in LP compared to NP islets, as judged by immunohistochemistry and RT-PCR analysis, we have to consider that the insulin released during a short period of stimulation represents only a small fraction of the total insulin content of these islets. Thus, other components of the stimulus-secretion coupling mechanism may be altered in the LP islet. In this sense, we observed a reduction of GLUT-2 mRNA and protein expression in LP rats, which, probably, contributed to the decreased glucose-induced insulin

secretion. LP islets also demonstrated a decreased gene and protein expression of PKCα and PKAα. PKCα phosphorylates some cytoskeletal elements, inducing their translocation towards the cytoplasmic membrane, whereas PKA increases insulin granule trafficking, favouring insulin secretion (49).

Mice deficient for IRS-1 present growth retardation and mild insulin resistance, islet hyperplasia, defects in insulin secretion and reduced islet insulin content (37). The analysis of gene and protein expression of components involved in insulin signaling systems showed that pancreatic islets from LP have increased protein levels of IR and decreased gene and protein expression of IRS-1. Thus, alterations in insulin signaling pathway may also contribute to the reduced insulin secretion in LP islets.

In conclusion, these results indicate that the final reduction in insulin synthesis and secretion in islets from rats maintained on a low protein diet is a consequence of alterations in several proteins belonging to different signal transduction pathways in pancreatic islet cells.

ACKNOWLEDGMENTS

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Table 1 - Composition of the normal and low protein diets (g/Kg).

Ingredient	**Normal protein (17% protein)**	**Low protein (6% protein)**
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinized cornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93G-MX)*	35.0	35.0
Vitamin mix (AIN-93-VX)*	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorydrate	2.5	2.5

* For detailed composition see Reeves *et al.*, 1993 (50).

Table 2 - Sequences of PCR primers and PCR conditions for the analysis of specific mRNAs

Mrna	GenBank accession n°	Primer sequences (5'-3')	PCR reaction conditions				Size of PCR product (bp)
			Desn	Ann	Ext	Cycles	
GLUT-2	J03145	CATTGCTGGAAGAAGCGTATCAG GAGACCTTCTGCTCAGTCGACG	94	55	72	26	408
Insulin	J00747	ATTGTTCCAACATGGCCCTGT TTGCAGTAGTTCTCCAGTT	94	57	72	23	340
PKAα	X53261	CCAAGAGAGTCAAGGGCAGGAC CAACCTTTCTCGGTAAATCGC	94	55	72	32	369
PKCα	X07286	CCTGCTCTACGGACTTATC TGTAGTATTCACCCTCCTC	94	57	72	33	501
IRS-1	X58375	TATGCCTTCTTCACGATGC GGCATCATCTCTGTATATTC	94		72		539
PVX	D00344	CGATCTCAAGCCACTCTCCG GTAGTTGAGGTAGTTGACCC	94	57	72	23	100

Den, denaturation; Ann, annealing; Ext, extension; Cyc, cycles.

Table 3 – Serum protein, albumin, glucose, insulin and free fatty acid levels of NP and LP rats after 8-10 weeks of dietary treatment.

Parameters	**NP**	**LP**
Body weight (g)	268 ± 0.6	184 ± 0.3*
Protein (g/dL)	5.0 ± 0.3	4.2 ± 0.1*
Albumin (g/dL)	3.6 ± 0.2	3.1 ± 0.1*
Glucose (mg/dL)	127.5 ± 5.9	130.1 ± 7.6
FFA (mmol/L)	0.49 ± 0.02	0.67 ± 0.07*
Insulin (pmol/L)	140 ± 16	109 ± 21
Insulin (fed) (pmol/L)	272 ± 84	94 ± 28*
Total pancreas insulin (μg/mL)	75.2 ± 11.7	32.5 ± 4.6*
Total islet insulin content (ng/mL)	235.7 ± 15.2	99 ± 13.4*

* Indicates a significant difference compared with NP rats ($P<0.05$).

Values are mean ± SE, n = 10-15

FIGURE LEGENDS

Figure 1 – Insulin secretion in response to glucose and non-fuel stimuli in isolated islets from rats fed normal (NP) or low (LP) protein diets. Isolated rat islets were incubated for 45 min in Krebs solution containing 5.6 mM glucose. After this period islets were washed and then incubated for 1 hour in Krebs solution 2.8 mM glucose without or with the non-fuel stimuli in different concentrations leucine (20 mM); arginine (20 mM), potassium (5, 20 and 80 mM) or at 8.3, 16.7 and 27 mM glucose The columns represent the cumulative 1 hour insulin secretions and are means ± SD, n = 8 independent experiments. $P < 0.05$.

Figure 2 - Immunolocalization of insulin hormone present in islets from rats fed NP (left) and LP (right) protein diets for 8 weeks. Magnification 200X.

Figure 3 – Relative expression of mRNA levels of the (A) insulin; (B) protein kinase A alpha subunit; (C) protein kinase C alpha subunit; (D) glucose transporter 2; (E) IRS-1; (F) potato virus X in islets from LP and NP rats. mRNA levels were semi-quantified by RT-PCR. Top: representative ethidium bromide-stained agarose gel(s) separation for specific mRNA levels from LP and NP islets. In each agarose gel(s) separation, the upper band corresponds to the cDNA for the specific mRNA indicated, and the lower band corresponds to the external control (PVX). The expression value for the mRNA of each gene from NP and LP was expressed relative to PVX. Values are means ± SE, n = 6-8 (*$P<0.05$).

Figure 4 – GLUT-2 (A), PKAα (B), PKCα (C), IR (D) and IRS-1 (E) protein content in islets from rats fed normal (NP) and low (LP) protein diets for 8 weeks. * Values are means ± SE, n = 3 (*$P<0.05$).

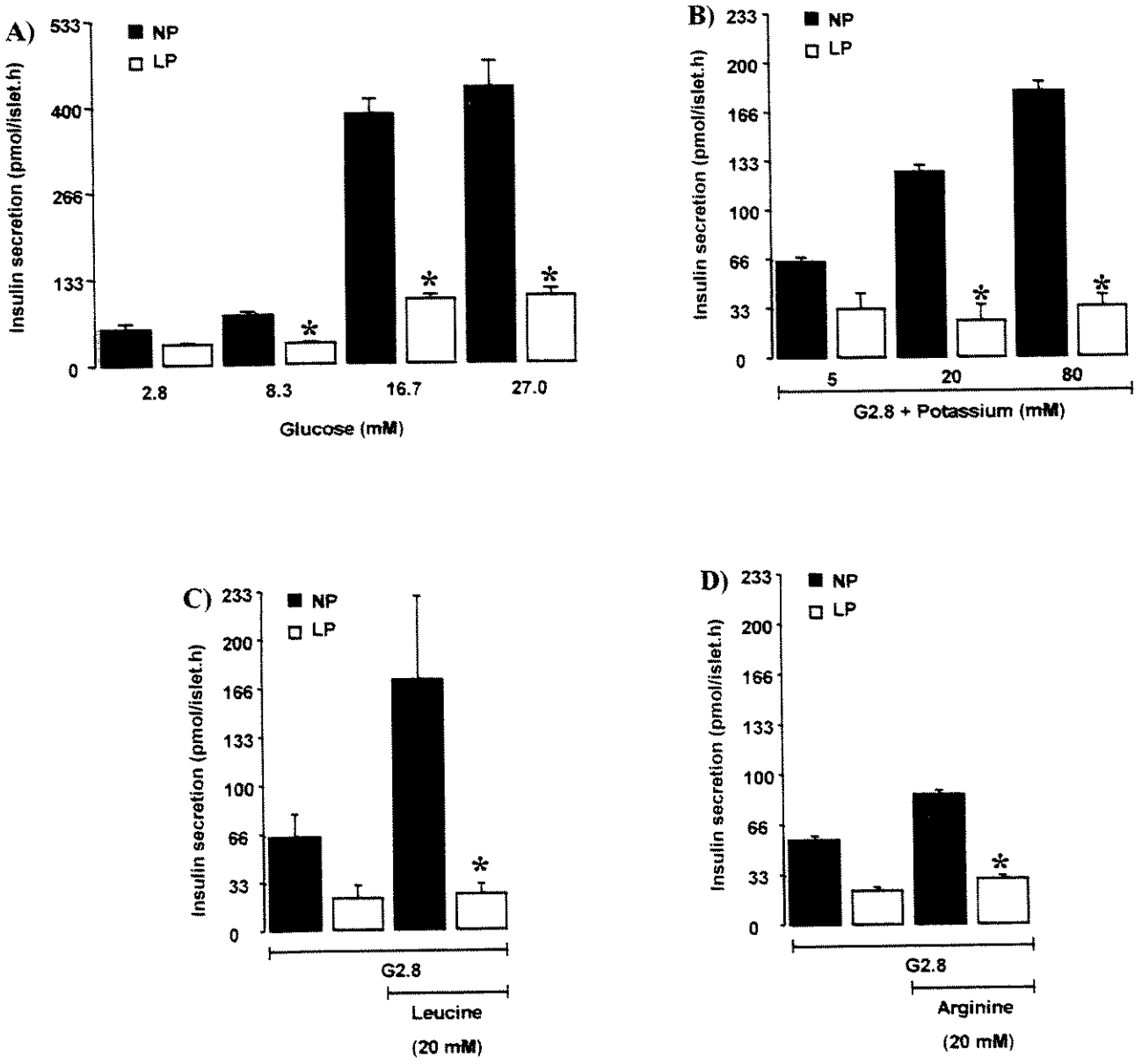
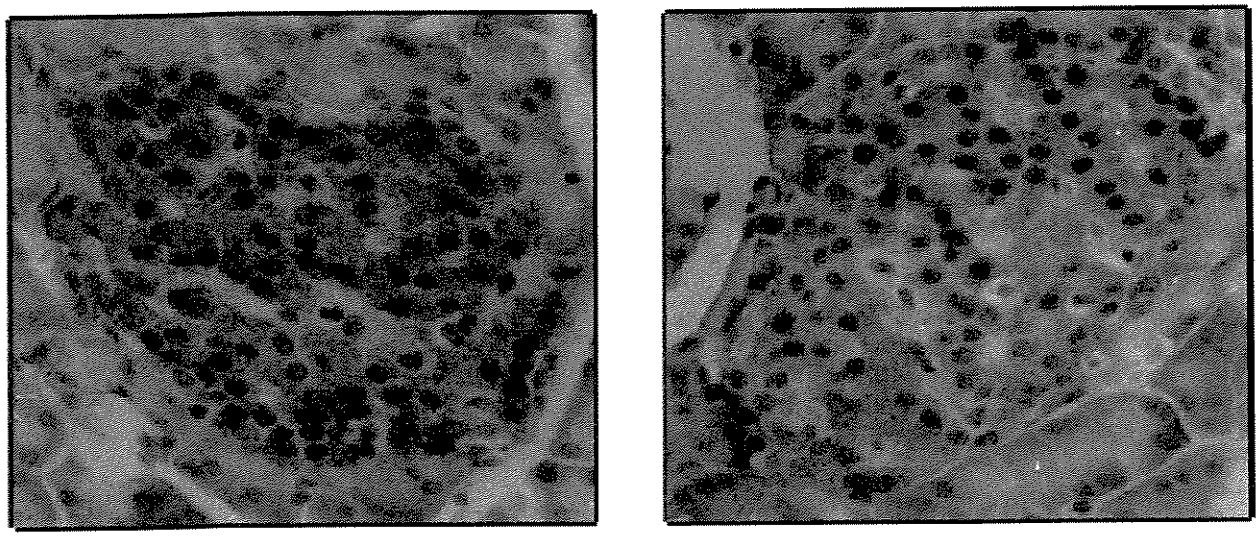

A)
NP
LP
Insulin secretion (pmol/islet.h)
533
400
266
133
0
2.8
8.3
16.7
27.0
Glucose (mM)
B)
233
200
166
133
100
66
33
0
5
20
80
G2.8 + Potassium (mM)
C)
G2.8
Leucine
(20 mM)
D)
G2.8
Arginine
(20 mM)

Figure 1



NP LP

Figure 2

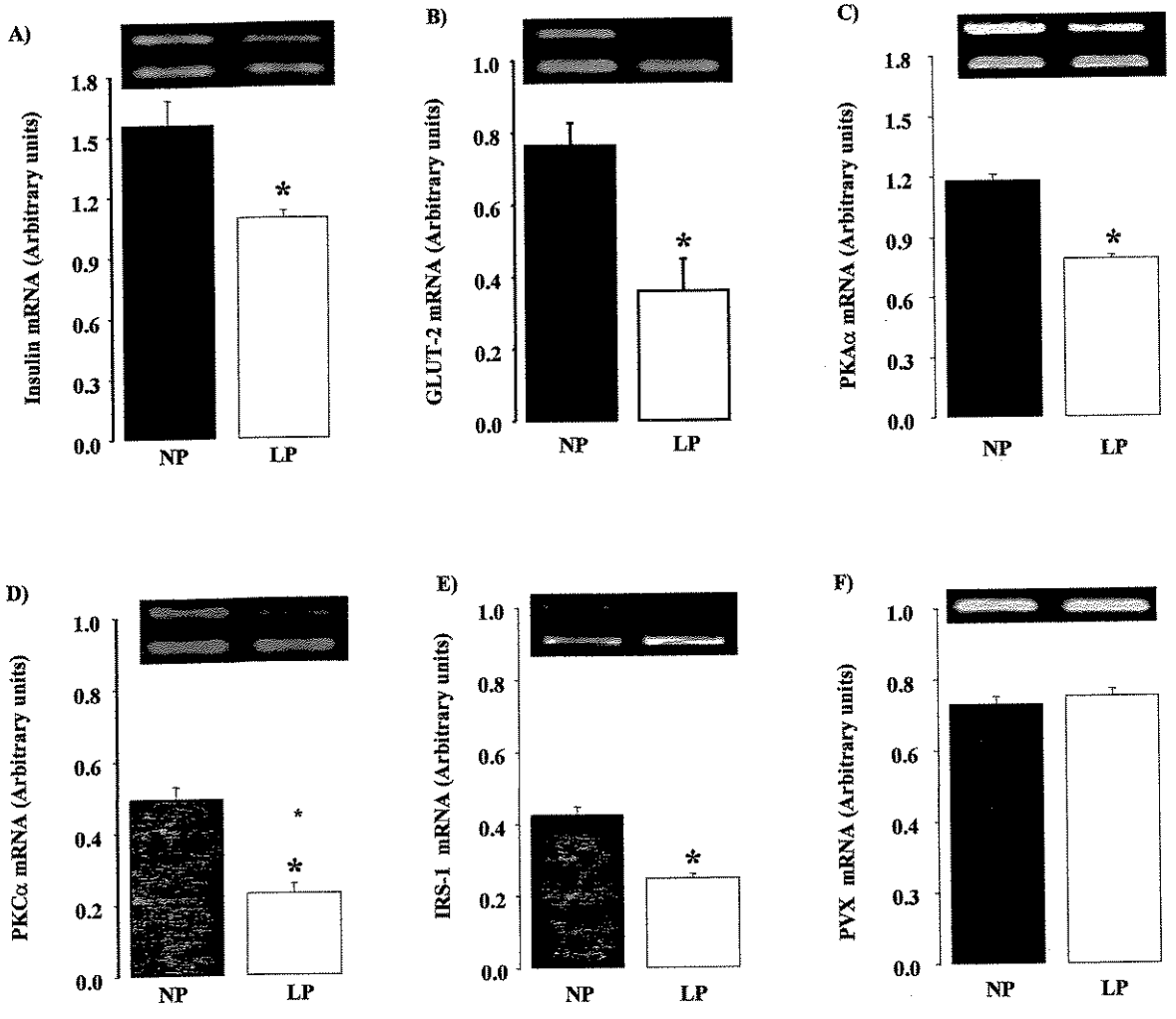

A)
Insulin mRNA (Arbitrary units)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
NP
LP
B)
GLUT-2 mRNA (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
NP
LP
C)
PKAα mRNA (Arbitrary units)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
NP
LP
D)
PKCα mRNA (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
NP
LP
E)
IRS-1 mRNA (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
NP
LP
F)
PVX mRNA (Arbitrary units)
1.0
0.8
0.6
0.4
0.3
0.0
NP
LP

Figure 3

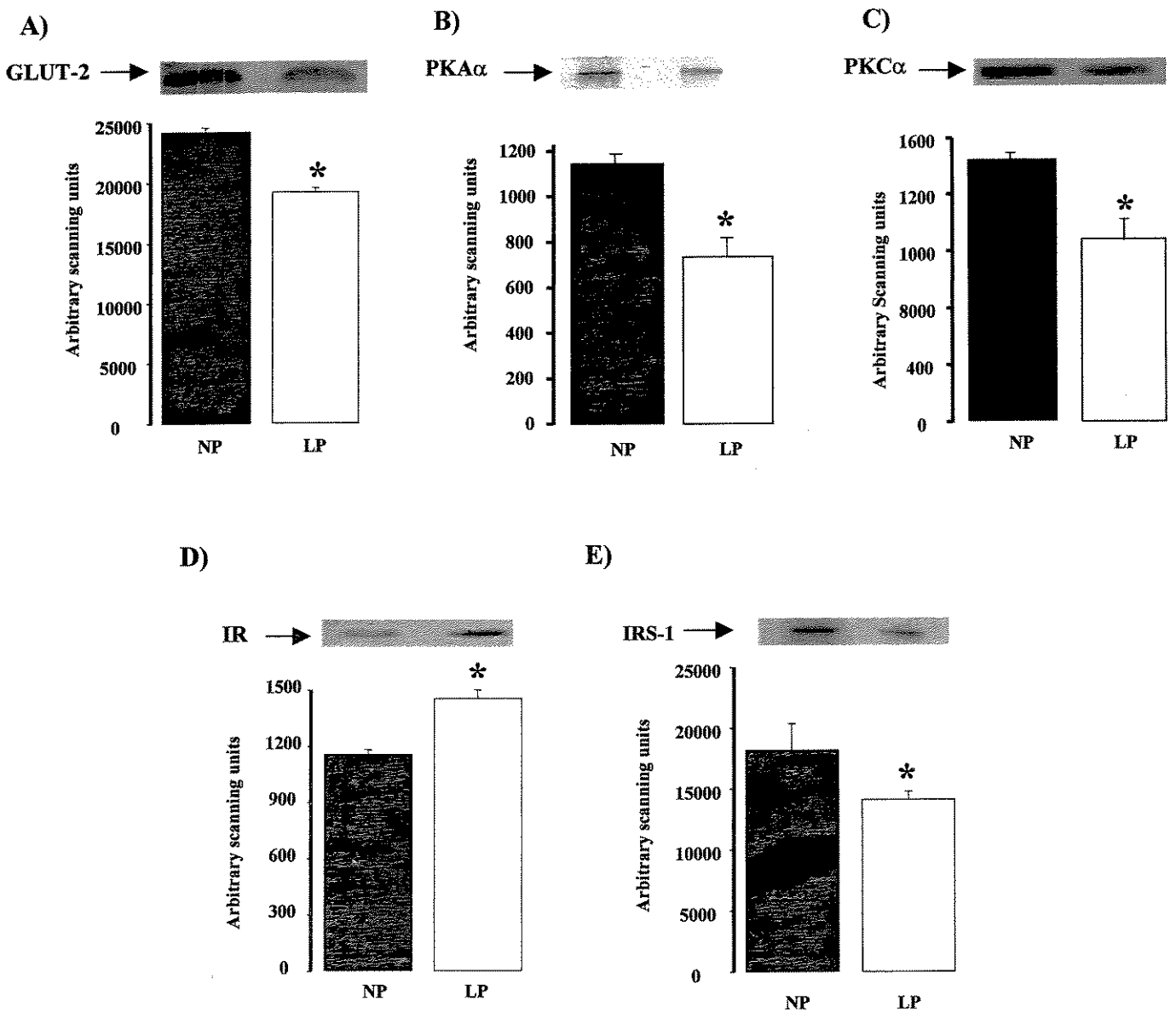

A)
GLUT-2
Arbitrary scanning units
25000
20000
15000
10000
5000
0
NP
LP
B)
PKAα
Arbitrary scanning units
1200
1000
800
600
400
200
0
NP
LP
C)
PKCα
Arbitrary Scanning units
1600
1200
1000
800
400
0
NP
LP
D)
IR
Arbitrary scanning units
1500
1200
900
600
300
0
NP
LP
E)
IRS-1
Arbitrary scanning units
25000
20000
15000
10000
5000
0
NP
LP

Figure 4

REFERENCES

1- Barker, D. J. P., Hales, C. N., Fall, C. H. D., Osmond, C., Phillips K., & Clark, P. M. S. (1993) Type II (non-insulin dependent) diabetes mellitus, hypertension and hyperlipidemia (syndrome X) relation to reduced fetal growth. Diabetologia 36:65-67.

2- Barker, D. J. P. (1995) Fetal origins of coronary heart disease. B M J 311:171-174.

3- Snoeck, A., Remacle, C., Reusens, B., & Hoet, J. J. (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate 57:107-118.

4- Dahri, S., Snoeck, A., Reusens-Billen, B., Remacle, C., & Hoet, J. J. (1991) Islet function in offspring of mothers on low-protein diet during gestation. Diabetes 40 Suppl 2:115-120.

5- Berney, D. M., Desai, M., Palmer, D. J. (1997) The effects of maternal protein deprivation on the fetal rat pancreas: major structural changes and their recuperation. J Pathol 183:109-115.

6- Latorraca, M. Q., Reis, M. A., Carneiro, E. M., Mello, M. A. & Boschero, A. C. (1999) Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake. J Nutr Biochem 10:37-43.

7- Dahri, S., Reusens-Billen, B., Remacle, C., & Hoet, J. J. (1995) Nutritional influences on pancreatic development and potential links with non-insulin-dependent diabetes. Proc Nutr Soc 54:345-356.

8- Fafournoux, P., Bruhat, A., & Jousse, C. (2000) Amino acid regulation of gene expression. Biochem J 351:1-12.

9- Song, F., Srinivasan, M., Aalinkeel, R., & Patel, M. S. (2001) Use of a cDNA array for the identification of genes induced in islets of suckling rats by a high-carbohydrate nutritional intervention. Diabetes 50(9):2053-60.

10- Lee, C-K.; Klopp R. G.; Weindruch, R., & Prolla, T. A. (1999) Gene expression profile of aging and its retardation by caloric restriction. Science 285:1390-1393.

11- Girard, J., Perdereau, D., Foufelle, F., Prip-Buus, C., & Ferré, P (1994) Regulation of lipogenic enzyme gene expression by nutrients and hormones. FASEB J 8:36-42.

12- Gurney, A. L., Pak, E. A. Liu, M., Giralt, M. M., McGrane, Y. M., Patel, D. R., Crawford, S. E., Nizielski, S., Savon, S., & Hanson, R. W. (1994) Metabolic regulation of gene transcriptions. J. Nutri 124:1533S-1539S.

13- Towle, H. C. (1995) Metabolic regulation of gene transcription in mammals. J Biol Chem 270:23235-23238.

14- Vaulont, S., & Kahn, A. (1994) Transcriptional control of metabolic regulation genes by carbohydrates. FASEB J 8:28-35.

15- Foufelle, F., Girard, J., & Ferre, P. (1998) Glucose regulation of gene expression. Curr Opin Clin Nutr Metab Care 1:323-328.

16- Latorraca, M. Q., Reis, M. A., Carneiro, E. M., Mello, M. A., Velloso, L. A., Saad, M. J., & Boschero, A. C. (1998) Protein deficiency and nutritional recovery modulate insulin secretion and the early steps of insulin action in rats. J Nutr 128:1643-1649.

17- Vuguin, P., Ma, X., Yang, X., Surana, M., Liu, B., & Barzilai, N. (2001) Food deprivation limits insulin secretory capacity in postpubertal rats. Pediatric Research 49(4):468-473.

18- Ashcroft, F. M., Harrison, D. E., & Ashcroft, S. J. (1984) Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. Nature 312:446-448.

19- Cook, D. L., & Hales, C. N. (1984) Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. Nature 311:271-273.

20- Satin, L. S., & Cook, D. L. (1985) Voltage-gated Ca^{2-} current in pancreatic B-cells. Pflugers Arch 404:385-387.

21- Meglasson, M. D., & Matschinsky, F. M. (1986) Pancreatic islet glucose metabolism and regulation of insulin secretion. Diabetes Metab Rev 2:163-214.

22- Prentki, M., & Matschinsky, F. M. (1987) Ca^{2-}, cAMP, and phospholipid-derived mensagers in coupling mechanisms of insulin secretion. Physiol Rev 67:1185-1248.

23- Henquim, J. C. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes 49:1751-1760.

24- Rorsman, P. (1997) The pancreatic β-cell as a fuel sensor: an electrophysiologist's viewpoint. Diabetologia 40:487-495.

25- Yajima, H., Komatsu, M., Schermerhorn, T., Aizawa, T, Kaneko, T., Nagai, M., Sharp, G. W. G., & Hashizume, K. (1999) cAMP enhances insulin secretion by an action on the ATP-sensitive K^{-} channel-independent pathway of glucose signaling in rat pancreatic islets. Diabetes 48:1006-1012.

26- Verspohl, E. J., & Ammon, H. P. T. (1980) Evidence for the presence of insulin receptors in rat islets of Langerhans J Clin Invest 65:1230-1237.

27- Gazzano, H., Halban, P. Prentki, M. Ballotti, R., Brandenburg, D., Fehlmann, M., & Van Obberghen, E. (1985) Identification of insulin receptors on membranes from an insulin-producing cell line RINm5F. Biochem J 226:867-872.

28- Rothenberg, P. L., Willison, L. D., Simon, J., & Wolf, B. A. (1995) Glucose-induced insulin receptor tyrosine phosphorylation in insulin-secreting β cells. Diabetes 44:802-809.

29- Velloso, L. A., Carneiro, E. M., Crepaldi, S. C., Boschero, A. C., & Saad, M. J. A (1995) Glucose- and insulin -induced phosphorylation of the insulin receptor and its primary substrates IRS-1 and IRS-2 in rat pancreatic islets. FEBS Lett 377:353-357.

30- Harbeck, M. C., Louie, D. C., Howland, J., Wolf, B. A., & Rothenberg, P. L. (1996) Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet β-cells. Diabetes 45:711-717.

31- Sun, X. J., Pons, S., Wang, L. M., Zhang, Y. T., Yenush, L., Burks, D., Myers, M. G., Glasheen, E., Copeland, N. G., Jerkins, N. A., Pierce, J. H., & White, M. F. (1997) The IRS-2 gene on murine chromosome 8 encodes a unique signaling adapter for insulin and cytokine action. Mol Endocrinol 11:251-262.

32- Alter, C. A., & Wolf, B. A. (1995) Identification of phosphatidylinositol 3, 4, 5-triphosphate in pancreatic islets and insulin-secreted β-cells. Biochem Biophys Res Commun 208:190-197.

33- Gao, Z. -Y., Konrad, R. J., Collins, H., Matschinsky, F. M., Rothenberg, P. L., & Wolf, B. A. (1996) Wortmannin inhibits insulin secretion in pancreatic islets and β-TC3 cell independent of its inhibition of phosphatidylinositol 3-kinase. Diabetes 45:854-862.

34- Holst, L. S., Mulder, H., Manginiello, V., Sundler, F., Ahren, B., Holm, C., & Degerman, E. (1998) Protein kinase B is expressed in pancreatic beta cells and activated upon stimulation with insulin-like growth factor I. Biochem Biophys Res Commun 250:181-186.

35- Xu, G., Marshall, C. A., Lin, T. A., Kwon, G., Munivenkatappa, R. B., Hill, J. R., Lawrence, J. C., & McDaniel, M. L. (1998) Insulin mediates glucose-stimulated phosphorylation of PHAS-I by pancreatic beta cells – an insulin-receptor mechanism for autoregulation of protein synthesis by translation. J Biol Chem 273:4485-4491.

36- Kulkarni, R. N., Brüning, J. C., Winnay, J. N., Postic, C., Magnuson, M. A., & Kahn, R. (1999) Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329-339.

37- Kulkarni, R. N., Winnay, J. N., Daniels, M., Brüning, J. C., Flier, S. N., Hanahan, D., & Kahn, R. (1999) Altered function of insulin receptor substrate-1-deficint mouse islets and cultured β-cell lines. J Clin Invest 104:R69-R75.

38- Leibiger, I. B., Leibiger, B., Moede, T., & Berggren, P. O. (1998) Exocytosis of insulin promotes insulin gene transcription via the insulin receptor PI-3 kinase p70 s6 kinase and CaM kinase pathways. Mol Cell 1:933-938.

39- Aspinwall, C. A., Lakey, J. R., & Kennedy, R. T. (1999) Insulin-stimulated insulin secretion in single pancreatic beta cells. J Biol Chem 274:6360-6365.

40- Reis, M. A., Carneiro, E. M., Mello, M. A., Boschero, A. C., Saad, M. J., & Velloso, L. A. (1997) Glucose-induced insulin secretion is impaired and insulin-induced phosphorylation of the insulin receptor and insulin receptor substrate- 1 are increased in protein-deficient rats. J Nutr 127:403-410.

41- Ferreira, F., Filiputti, E., Arantes, V. C., Stoppiglia, L. F., Araujo, E. P., Delghingaro-Augusto, V., Latorraca, M. Q., Toyama, M. H., Boschero, A. C., & Carneiro, E. M. (2003) Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase c alpha expression. J Nutr 133:695-699.

42- Doumus, B. T., Watson, W. A., & Biggs, H. G. (1971) Albumin standards and measurements of serum albumin with bromocresol green. Clin Chim Acta 31:87-96.

43- Scott, A. M., Atwater, I., & Rojas, E. (1981) A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia 21:470-475.

44- Bordin, S., Boschero, A. C., Carneiro, E. M., & Atwater, I. (1995) Ionic mechanisms involved in the regulation of insulin secretion by muscarinic agonists. J Membr Biol 148:177-184.

45- Malaisse, W. J., Sener, A., & Mahy, M. (1974) The stimulus-secretion coupling of glucose-induced insulin release. Sorbitol metabolism in isolated islets. Eur J Biochem 47:365-370.

46- Velloso, L. A., Folli, F., Sun, X. J., White, M. F., Saad, M. J. A., & Kahn, C. R. (1996) Cross-talk between the insulin and angiotensin signaling systems. Proc Natl Acad Sci 93:12490–12495.

47- Latorraca, M. Q., Carneiro, E. M., Boschero, A. C., & Mello, M. A. (1998) Protein deficiency during pregnancy and lactation impairs glucose- induced insulin secretion but increases the sensitivity to insulin in weaned rats. Br J Nutr 80:291-297.

48- Lindsay, M. A., Bungay, P. J., & Griffin, M. (1990) Transglutaminase involvement in the secretion of insulin from electropermeabilised rat islets of Langerhans. Biosci Rep 10:557-561.

49- Wei, Y., Tae, N., Tatsuya, F., Hiroyoshi, H., Takao, S., Yasuharu, S., & Ighiro, N. (2000) Synergism of protein kinase A, protein kinase C, and myosin light-chain kinase in the secretory cascade of the pancreatic B-cell. Diabetes 49:945-952.

50- Reeves, P. G., Nielsen, F. H., & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123: 1939-1951.

CONCLUSÕES

CONCLUSÕES

❖ Ilhotas de ratos submetidos à restrição protéica, pós-desmame, apresentam modificações na expressão gênica de diversas proteínas, sendo a maior parte relacionada aos processos de biossíntese/secreção de insulina e remodelamento celular.

❖ Dieta hipoprotéica causa reduções na expressão de RNAm e conteúdo total de insulina (pâncreas e ilhota).

❖ Restrição protéica induz redução na expressão gênica de proteínas relacionadas ao processo de secreção de insulina.

❖ Modificações na expressão de vários genes do pâncreas endócrino, podem ser parcialmente responsáveis pela redução da secreção de insulina, estimulada por diferentes agentes insulinotrópicos, observada neste modelo animal.

REFERÊNCIAS BIBLIOGRÁFICAS

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ALTER, C.A.; WOLF, B.A. Identification of phosphatidylinositol 3, 4, 5-triphosphate in pancreatic islets and insulin-secreted β-cells. **Biochem Biophys Res Commun**, v.208, p.190-197, mar. 1995.

ARAUJO, E.P.; et al. Blockade of IRS1 in isolated rat pancreatic islets improves glucose-induced insulin secretion. **FEBS Lett**, v.531, n.3, p.437-442, nov. 2002.

ASHCROFT, F.M.; HARRISON, D.E.; ASHCROFT, S.J. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. **Nature**, v.312, n.5993, p.446-448, nov-dec. 1984.

ASPINWALL, C.A.; LAKEY, J.R.; KENNEDY, R.T. Insulin-stimulated insulin secretion in single pancreatic beta cells. **J Biol Chem**, v.274, n.10, p.6360-6365, mar. 1999.

BARKER, D.J.; et al. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. **Diabetologia**, v.36, n.1, p.62-67, 1993.

BECKER, D.J.; et al. Insulin secretion in protein-calorie malnutrition. 1. Quantitative abnormalities and response to treatment. **Diabetes**, v.20, n.8, p.542-551, aug. 1971.

BERNEY, D.M.; et al. The effects of maternal protein deprivation on the fetal rat pancreas: major structural changes and their recuperation. **J Pathol**, v.183, n.1, p.109-115, sept. 1997.

BRATANOVA-TOCHKOVA, T.K.; et al. Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion. **Diabetes**, v.51, Supp.1, p.S83-S90, feb. 2002.

CARDOZO, A.K.; et al. Identification of novel cytokine-induced genes in pancreatic b-cells by high-density oligonucleotide arrays. **Diabetes**, v.50, n.5, p.909-920, may 2001.

CARNEIRO, E.M.; et al. Low protein diet impairs glucose-induced insulin secretion from and ^{45}Ca uptake by pancreatic rat islets. **J Nutr Biochem**, v.6, p.314-318, 1995.

CHERIF, H.; et al.. A protein-restricted diet during pregnancy alters in vitro insulin secretion from islets of fetal Wistar rats. **J Nutr**, v.131, n.5, p.1555-1559, may 2001.

COOK, C.G. Glucose tolerance after kwashiorkor. **Nature**, 215:1295-1296, 1967.

COOK, D.L.; HALES, C.N. Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. **Nature**, v.311, n.5983, p.271-273, sept., 1984.

DAHRI, S.; et al. Nutritional influences on pancreatic development and potential links with non-insulin-dependent diabetes. **Proc Nutr Soc**, v.54, n.2, p.345-356, jul. 1995.

DAHRI, S.; et al. Islet function in offspring of mothers on low protein diet during late gestation. **Diabetes**, v.40, Suppl 2, p.115-120, dec. 1991.

EXPERT COMMITTEE ON THE DIAGNOSIS AND CLASSIFICATION OF DIABETES MELLITUS: Report of the expert committee on the diagnosis and classification of diabetes mellitus. **Diabetes Care**, v.26, Suppl.1, S5-S20, jan. 2003.

FAFOURNOUX, P.; BRUHAT, A; JOUSSE, C. Amino acid regulation of gene expression. **Biochem. J**, v.351, Pt1, p.1-12, oct. 2000.

FOUFELLE, F.; GIRARD, J.; FERRE, P. Glucose regulation of gene expression. **Curr Opin Clin Nutr Metab Care**, v.1, n.4, p.323-328, jul. 1998.

FOWDEN, A.L. Endocrine regulation of fetal growth. **Reprod Fertil Dev**, v.7, n.3, p.351-363, 1995

GAO, Z. -Y.; et al. Wortmannin inhibits insulin secretion in pancreatic islets and β-TC3 cell independent of its inhibition of phosphatidylinositol 3-kinase. **Diabetes**, v.7, n.3, p.854-862, 1996.

GAZZANO, H.; et al. Identification of insulin receptors on membranes from an insulin-producing cell line RINm5F. **Biochem J**, v.226, n.3, p.867-872, mar. 1985.

GEPTS, W.; LECOMPT, P.M. The pancreatic islets in diabetes. **Am J Med**, v.70, n.1, p.105-109, 1981.

GIRARD, J.; et al. Regulation of lipogenic enzyme gene expression by nutrients and hormones. **FASEB J**, v.8, n.1, p.36-42, jan. 1994.

GRODSKY, G.M. Kinetics of insulin secretion: current implications. In: LE ROITH, D., TAYLOR, S. I., & OLEFSKY, J.M. **Diabetes Mellitus.** Philadelphia: Lippincott-Raven, 1996. p.12-20.

GURNEY, A.L.; et al. Metabolic regulation of gene transcriptions. **J. Nutr**, v.124, Supp8, p.1533S-1539S, aug. 1994.

HALES, C.N.; BARKER, D.J.P. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. **Diabetologia**, v.35, n.7, p.595-601, jul. 1992.

HARBECK, M.C.; et al. Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet β-cells. **Diabetes**, v.45, n.6, p.711-717, jun. 1996.

HEARD, C.R.; TURNER, M.R. Glucose tolerance and related factors in dogs fed diets of suboptimal protein value. **Diabetes**, v.16, n.2, p.96-107, feb. 1967.

HOET, J.J.; et al. Importance of diets and their effect on fetal development: function and structure of the endocrine pancreas following protein deficiency during intrauterine life. **Bull Mem Acad R Med Belg**, v.147, n.3-5, p.174-181, 1992.

HOLST, L.S.; et al. Protein kinase B is expressed in pancreatic beta cells and activated upon stimulation with insulin-like growth factor I. **Biochem Biophys Res Commun**, v.250, n.1, p.181-186, sept. 1998.

KULKARNI, R.N.; et al. Altered function of insulin receptor substrate-1-deficient mouse islets and cultured β-cell lines. **J Clin Invest**, v.104, n.12, p.R69-R75, 1999.

LATORRACA, M.Q.; et al. Protein deficiency during pregnancy and lactation impairs glucose-induced insulin secretion but increases the sensitivity to insulin in weaned rats. **Br J Nutr**, v.80, n.3, p.291-297, sept. 1998.

LATORRACA, M.Q.; et al. Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake. **J Nutr Biochem**, v.10, p.37-43, sept. 1999.

LEAHY, J.L. Detrimental effects of chronic hyperglycemia on the pancreatic β-cell. In: LE ROITH, D., TAYLOR, S. I., & OLEFSKY, J.M. **Diabetes Mellitus.** Philadelphia: Lippincott-Raven, 1996. p.12-20.

LEE, C-K.; et al. Gene expression profile of aging and its retardation by caloric restriction. **Science**, v.285, n.5432, p.1390-1393, aug. 1999.

LEIBIGER, B.; et al. Glucose-stimulated insulin biosynthesis depends on insulin-stimulated insulin gene transcription. **J Biol Chem**, v.275, n.39, p.30153-30156, sept. 2000.

MEGLASSON, M.D.; MATSCHINSKY, F.M Pancreatic islet glucose metabolism and regulation of insulin secretion. **Diabetes Metab Rev**, v.2, n.3-4, p.163-214, 1986.

PETRIK, J.; et al. A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. **Endocrinology**, v.140, n.10, p.4861-4873, oct. 1999.

PRENTKI, M.; Matschinsky, F.M. Ca^{2+}, cAMP, and phospholipid-derived mensagers in coupling mechanisms of insulin secretion. **Physiol Rev**, v.67, n.4, p.1185-1248, oct. 1987.

RAO, R.H. Diabetes in the undernourished: coincidence or consequence? **Endocrine Rev**, v.9, n.1, p.67-87, feb. 1988.

REAVEN, G.M. Role of insulin resistance in human disease. **Diabetes**, v.37, n.12, p.1595-1607, 1988.

REEVES, P.G.; NIELSEN, F.H.; FAHEY JR, G.C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **J Nutr**, v.123, p.1939-1951, 1993.

RORSMAN, P.; et al. The cell physiology of biphasic insulin secretion. **News Physiol Sci**, v.15, p.72-77, apr. 2000.

ROTHENBERG, P.L.; et al. Glucose-induced insulin receptor tyrosine phosphorylation in insulin-secreting β cells. **Diabetes**, v.44, n.7, p.802-809, jul. 1995.

SATIN, L.S.; COOK, D.L. Voltage-gated Ca^{2+} current in pancreatic B-cells. **Pflugers Arch**, v.404, n.4, p.385-387, aug. 1985.

SMITH, S.R.; et al. Insulin secretion and glucose tolerance in adults with protein-calorie malnutrition. **Metabolism**, v.24, n.9, p.1073-1084, sept. 1975.

SNOECK, A.; et al. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. **Biol Neonate**, v.57, n.2, p.107-118, 1990.

SONG, F.; et al. Use of a cDNA array for the identification of genes induced in islets of suckling rats by a high-carbohydrate nutritional intervention. **Diabetes**, v.50, n.9, p.2053-60, 2001.

SUN, X.J.; et al. The IRS-2 gene on murine chromosome 8 encodes a unique signaling adapter for insulin and cytokine action. **Mol Endocrinol**, v.11, n.2, p.251-262, feb. 1997.

SWENNE, I; CRACE, C.J.; MILNER, R.D. Persistent impairment of insulin secretory response to glucose in adult rats after limited period of protein-calorie malnutrition early in life. **Diabetes**, v.36, n.4, p.454-458, apr. 1987.

TOWLE, H.C. Metabolic regulation of gene transcription in mammals. **J Biol Chem**, v.270, n.42, p.23235-23238, oct. 1995.

VAN ASSCHE, F.A.; AERTS, L. The fetal endocrine pancreas. **Contrib Gynecol Obstet**, v.5, p.44-57, 1979.

VAULONT, S.; KAHN, A. Transcriptional control of metabolic regulation genes by carbohydrates. **FASEB J**, v.8, n.1, p.28-35, jan. 1994.

VELLOSO, L.A.; et al. Glucose- and insulin -induced phosphorylation of the insulin receptor and its primary substrates IRS-1 and IRS-2 in rat pancreatic islets. **FEBS Lett**, v.377, n.3, p.353-357, dec. 1995.

VERSPOHL, E.J.; AMMON, H.P.T. Evidence for the presence of insulin receptors in rat islets of Langerhans. **J Clin Invest**, v.65, n.5, p.1230-1237, 1980.

VUGUIN, P.; et al. Food deprivation limits insulin secretory capacity in postpubertal rats. **Pediatric Research**, v.49, n.4, p.468-473, apr. 2001.

WEBB, G.C.; et al. Expression profiling of pancreatic beta cells: glucose regulation of secretory and metabolic pathway genes. **PNAS**, v.97, n.11, p.5773-5788, may 2000.

WEI, Y.; et al. Synergism of protein kinase A, protein kinase C, and myosin light-chain kinase in the secretory cascade of the pancreatic B-cell. **Diabetes**, v.49, n.6, p.945-952, jun. 2000.

WILSON, M.R.; HUGHES, S.J. The effect of maternal protein deficiency during pregnancy and lactation on glucose tolerance and pancreatic islet function in adult rat offspring. **J Endocrinol**, v.154, n.1, p.177-185, jul. 1997.

WITHERS, D.J.; et al. Disruption of IRS-2 causes type 2 diabetes in mice. **Nature**, v.391, n.6670, p.900-904, feb. 1998.

XU, G.G.; ROTHENBERG, P.L. Insulin receptor signaling in the beta-cell influences insulin gene expression and insulin content: evidence for autocrine beta-cell regulation. **Diabetes**, v.47, n.8, p.1243-1252, aug. 1998.

YAJIMA, H.; et al. cAMP enhances insulin secretion by an action on the ATP-sensitive K^+ channel-independent pathway of glucose signaling in rat pancreatic islets. **Diabetes**, v.48, n.5, p.1006-1012, may 1999.

ANEXO

Quadro 1 - Composição das dietas (g/Kg).

Ingredientes	**Dieta NP (17% proteína)**	**Dieta LP (6% proteína)**
Caseína (84% proteína)*	202.0	71.5
Amido	397.0	480.0
Dextrina	130.5	159.0
Sucrose	100.0	121.0
Óleo de soja	70.0	70.0
Fibra (microcelulose)	50.0	50.0
Mistura de minerais (AIN-93G-MX)	35.0	35.0
Mistura de vitaminas (AIN-93-VX)	10.0	10.0
L-Cistina	3.0	1.0
Cloreto de colina	2.5	2.5

* Valores corrigidos em função do conteúdo de proteína na caseína.

Quadro 2 - Mistura de vitaminas (AIN-93G-VX)

	VITAMINAS	g/Kg
1	Ácido nicotínico	3.0
2	Pantotenato de cálcio	1.6
3	Piridoxina-HCl	0.7
4	Tiamina-HCl	0.6
5	Riboflavina	0.6
6	Ácido fólico	0.2
7	d-biotina	0.02
8	Vitamina B_{12} (cianocobalamina) 0.1% em manitol	2.5
9	Vitamina E (acetato de α-tocoferol) (500 UI/g)	15.0
10	Vitamina A (retinil palmitato) (500.000 UI/g)	0.8
11	Vitamina D3 (colecalciferol) (400.000 UI/g)	0.25
12	Vitamina K (filoquinose)	0.075
13	Sacarose	974.65

Quadro 3 - Mistura de minerais (AIN-93G-MX)

	Sais	g/Kg
1	Carbonato de cálcio anidro	357.0
2	Fosfato de potássio monobásico	196.0
3	Citrato de potássio tribásico monohidratado	70.78
4	Cloreto de sódio	74.0
5	Sulfato de potássio	46.6
6	Óxido de magnésio	24.0
7	Citrato férrico	6.06
8	Carbonato de zinco	1.65
9	Carbonato manganoso	0.63
10	Carbonato cúprico	0.30
11	Iodato de potássio	0.01
12	Selenato de sódio anidro	0.01025
13	Paramobilidato de amônio.$4H_2O$	0.00795
14	Meta-silicato de sódio $12H_2O$	1.45
15	Sulfato de crômio e potássio $12H_2O$	0.275
16	Cloreto de lítio	0.0174
17	Ácido bórico	0.0815
18	Fluoreto de sódio	0.0635
19	Carbonato de níquel	0.0318
20	Vanato de amônio	0.0066
21	Sacarose	221.026