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VANESSA CRISTINA ARANTES

# EXPRESSÃO DO FATOR DE TRANSCRIÇÃO PDX-1 EM ILHOTAS DE RATOS SUBMETIDOS À RESTRIÇÃO PROTÉICA DURANTE A FASE FETAL E LACTAÇÃO



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**Orientador: Prof. Dr. Antonio Carlos Boschero**

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**Banca Examinadora**

Prof. Dr. Antonio Carlos Boschero _____

Prof. Dr. Everardo Magalhães Carneiro _____

Profª. Drª. Silvana Auxiliadora Bordin da Silva _____

Profª. Drª. Márcia Queiroz Latorraca _____

Aos meus pais, irmãos e sobrinhas

Ao Luciano

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## ABREVIATURAS E SÍMBOLOS

| | |
|---|---|
| °C | degrees centigrades (graus centígrados) |
| ANOVA | analysis of variance (análise de variância) |
| C group | control group (grupo controle) |
| cDNA | complementary deoxyribonucleic acid ( ácido deoxiribonucléico complementar) |
| dl | deciliter (decilitro) |
| DTT | dithiothreitol (ditiotreitol) |
| EDTA | ethylenediaminetetraacetic acid ( ácido etilendiaminotetracético) |
| EGTA | ethylene glycol-bis (β-aminoethyl ether) (etileno-glicol-bis(β-aminoetil éter) |
| FFA | free fatty acids (ácidos graxos llivres) |
| GLUT-2 | glucose transporter 2 (transportador de glicose facilitador isoforma 2) |
| IGF-II | insulin-like growth factor-i (fator de crescimento semelhante à insulina-i) |
| IR | insulin receptor (receptor de insulina) |
| IRS-1 | insulin receptor substrate-1 (substrato 1 do receptor de insulina) |
| kDa | kilodaltons (quilodatons) |
| L ou l | liter (litro) |
| LP group | low protein group (grupo hipoprotéico) |
| μg | microgram (micrograma) |
| $\mu m^2$ | micrometers (micrômetros quadrados) |
| mg | milligram (miligrama) |

| | |
|---|---|
| min | minute (minuto) |
| mL | milliliter (mililitro) |
| mmol | millimolar (milimolar) |
| MODY4 | maturity onset diabetes of the young ( diabetes tipo 1 com início na idade adulta) |
| mRNA | messenger ribonucleic acid ( ácido ribonucléico) |
| p38/SAPK2 | stress activated protein kinase 2 (proteína quinase ativada por stress) |
| PBS | phosphate buffered saline ( tampão fosfato salina) |
| PCR | polymerize chain reaction |
| PDX-1 | pancreatic-duodenal homeobox (pancreato-duodenal homeobox 1) |
| PHAS-1 | phosphorylated heat- and acid-stable protein regulated by insulin (proteína termo-ácido estável fosforilada regulada pela insulina) |
| PI3-kinase | phosphatidylinositol 3-kinase (fosfatidilinositol 3-quinase) |
| PMSF | Phenylmethylsulfonylfluoride (fenilmetilsulfonilfluoreto) |
| PPI | preproinsulin (pré-pró-insulina) |
| R group | recovered group (grupo recuperado) |
| RNA | ribonucleic acid (ácido ribonicléico) |
| RT | reverse transcription (transcrição reversa) |
| SDS | sodium dodecyl sulphate ( sulfato dodecil de sódio) |
| SEM | standard error of the mean (erro padrão da media) |
| TBE | tris borate EDTA (tris borato EDTA) |
| TTBS | tris tween 20 buffered saline (tampão salina tris-tween 20) |
| v | volum (volume) |

## RESUMO

Em animais, a desnutrição intra-uterina exerce efeitos marcantes sobre o desenvolvimento fetal e pós-natal. Além disso, baixo peso ao nascer está associado à secreção alterada de insulina, resistência à insulina e área diminuída da ilhota pancreática. Neste trabalho, verificamos a expressão do fator de transcrição PDX-1, a área da ilhota e a secreção de insulina em ilhotas de ratos neonatos e recém-desmamados (28 dias de vida), mantidos durante o período fetal e da lactação com uma dieta normoprotéica (17% de proteína) ou hipoprotéica (6% de proteína). Esses parâmetros também foram avaliados em ratos com 28 dias, recuperados da desnutrição intra-uterina. Após o desmame, a secreção de insulina em ilhotas isoladas em resposta 2,8 e 16,7 mmol/L de glicose foi reduzida em ratos desnutridos. Ao nascer e após 28 dias, a área da ilhota e a expressão protéica do PDX-1 também foram diminuídas, enquanto os níveis de mRNA em ilhotas de ratos desnutridos recém desmamados foram semelhantes aos níveis encontrados em ratos controles. A expressão protéica do PDX-1 e a área da ilhota, bem como a secreção de insulina, foram restauradas em ratos recuperados, enquanto o mRNA do PDX-1 foi maior do que em ratos controles. Esses resultados sugerem associação entre expressão protéica do PDX-1 diminuída, redução no tamanho da ilhota e secreção diminuída de insulina em ratos desnutridos. A reintrodução de dieta normal, logo após o nascimento, restaurou todos os parâmetros avaliados.

## ABSTRACT

Intra-uterine and early postnatal malnutrition has profound consequences on fetal and postnatal development, both in humans and animals. In addition, low birth weight has been reported to be associated with impaired insulin secretion, insulin resistance and diminished area of pancreatic islets. To assess the expression of the transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) in pancreatic B-cells, and to examine the islet area and glucose-induced insulin secretion by islets from rats maintained on a low protein diet. PDX-1 expression and islet area were assessed in rats maintained on a low (6%) or normal (17%) protein diet during fetal life. PDX-1 protein and mRNA levels, as well as insulin secretion and islet area were also measured after 28 days of life in normal, low protein, and recovered rats which received a normal protein diet after birth. Insulin secretion by isolated islets in response to 2.8 and 16.7 mmol glucose/L was reduced in 28-day-old low protein rats ($P < 0.0001$). At birth and after 28 days of life, the islet area and PDX-1 protein expression were also reduced ($P < 0.01$). In contrast, PDX-1 mRNA levels in islets from 28-day-old low protein rats was similar to normal rats. PDX-1 protein expression in B-cells, the area of islets and insulin secretion were restored in recovered rats, whereas PDX-1 mRNA was higher than normal rats ($P < 0.05$). These results suggest a link between diminished PDX-1 protein expression, a reduction in islet area and impaired insulin secretion in low protein rats. The reintroduction of a normal diet early in life restored islet area and cell physiology.

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# INTRODUÇÃO

O número de indivíduos portadores de diabetes mellitus no mundo tem aumentado em ritmo alarmante. Esse número, estimado em 124 milhões em 1997, deverá atingir 221 milhões de pessoas em 2010 (Amos e cols, 1997).

O diabetes mellitus do tipo 2 é uma síndrome caracterizada por resistência à insulina, deficiência relativa de insulina, aumento da produção de glicose pelo fígado e redução da utilização de glicose pelo tecidos periféricos. A forma mais comum de diabetes tipo 2 está freqüentemente combinada com obesidade, hipertensão arterial, dislipoproteinemia e doença isquêmica do coração. Essa associação é chamada de "síndrome de resistência à insulina" ou "síndrome X" (Reaven, 1988).

Em 1992, Hales & Barker formularam a hipótese do "thrifty phenotype" para explicar a gênese do diabetes tipo 2 e da "síndrome X". De acordo com essa hipótese, enfermidades que se manifestam tardiamente como o diabetes mellitus, a hipertensão arterial e a doença isquêmica do coração são determinadas por fatores ambientais e possivelmente originadas *in utero* ou durante a infância. Admite-se que a desnutrição intra-uterina e no primeiro ano de vida produz mudanças morfológicas e funcionais em vários tecidos e órgãos. O baixo peso ao nascer parece estar associado à diminuída secreção de insulina e resistência à insulina. Essas anormalidades metabólicas e funcionais, observadas na vida adulta, dependem da fase da vida em que a desnutrição ocorreu, do tipo e intensidade da carência nutricional. Durante o desenvolvimento existem períodos críticos que, freqüentemente coincidem com fases de rápida divisão celular quando os tecidos e órgãos estão se diferenciando. O déficit de crescimento, provocado nesses períodos por desnutrição materna, pode resultar em perda do número

de células (Holness e cols, 2000). Na Inglaterra, foi observada estreita relação entre indivíduos intolerantes à glicose ou mesmo portadores de diabetes mellitus tipo 2 com baixo peso ao nascer (Hales e cols, 1991), ou com déficit de crescimento durante a vida fetal (Phillips e cols, 1994).

Embora muitos fatores podem influenciar o crescimento e desenvolvimento do feto (Purdy e Metzger, 1996), o estado nutricional e hormonal materno exerce efeito importante. Em humanos, a ingestão de uma dieta rica em carboidratos no início da gestação e hipoprotéica no final, está associada à menor placenta e baixo peso ao nascer (Godfrey e cols, 1996). Os aminoácidos são determinantes no desenvolvimento fetal, pois a concentração de alguns deles encontra-se diminuída no plasma de fetos humanos considerados pequenos para a idade gestacional (Kalkhoff, 1991). Taurina, um β aminoácido, derivado do metabolismo da metionina e da cisteína é o aminoácido livre mais abundante nas células de mamíferos (Jacobsen & Smith 1968), sendo encontrado em altas concentrações no pâncreas endócrino (Bustamante e cols, 1998). Interessante notar que os níveis de taurina estão marcadamente reduzidos no plasma de fetos de mães que foram submetidas à restrição protéica durante a gestação (Reusens e cols, 1995). A suplementação de taurina na dieta dessas mães, normalizou a secreção de insulina (Cherif e cols, 1998), bem como a capacidade de proliferação das células beta desses animais (Boujendar e cols, 2000). Em ratos, o peso corporal e os tecidos muscular, adiposo, hepático e pancreático, por exemplo, são significativamente alterados pela restrição protéica durante os períodos críticos de crescimento fetal e pós-natal. No entanto, ainda não se sabe se a associação entre desnutrição durante o crescimento e

subseqüente diabetes mellitus é mediada por alterações na ação da insulina, defeitos na secreção de insulina, alterações genéticas ou uma combinação desses fatores.

A desnutrição, tanto em humanos quanto em animais experimentais, está associada à secreção reduzida de insulina e à homeostasia da glicose alterada (James & Coore 1970; Okitolonda e cols, 1987). Em neonatos submetidos à dieta hipoprotéica, durante a fase fetal, constatou-se diminuição no tamanho da ilhota pancreática, assim como alterações na capacidade de proliferação das células (Snoeck e cols, 1990). Entretanto, em ratos recém-desmamados, submetidos à restrição protéica durante a fase intra-uterina e lactação, observou-se que a capacidade proliferativa das células beta permanece normal, embora a massa de célula beta estivesse diminuída. Essas observações suportam a idéia que os mecanismos pelos quais a desnutrição afeta o desenvolvimento da célula beta podem variar com o período em que ela foi imposta (Garofano e cols, 1997; Holnnes e cols,2000).

Além da redução da massa de células beta, deve-se considerar que o padrão de anormalidades secretórias observado na desnutrição indica alterações em mecanismos celulares envolvidos na secreção de insulina. Nesse sentido, ratos submetidos à restrição protéica logo após o desmame apresentaram secreção reduzida de insulina em resposta à glicose (Carneiro e cols, 1995). Resultados semelhantes foram encontrados em ratos submetidos à restrição protéica durante a gestação e lactação (Latorraca e cols, 1998). Além disso, uma sensibilidade aumentada à insulina , representada por maior fosforilação do receptor de insulina (IR), substrato-1 do receptor de insulina (IRS-1) e a associação do IRS-1 com a fosfatidilinositol 3-kinase (PI3-Kinase) tem sido observada nesse mesmo modelo experimental de desnutrição (Reis e cols, 1997).

As bases genéticas da relação entre desnutrição e desenvolvimento do diabetes na

vida adulta têm sido estudadas e, recentemente, alguns resultados quanto à identificação de genes de susceptibilidade ao diabetes tipo 2 têm sido relatados (Elbein, 1997; Neel, 1999).

Como já descrito anteriormente, a hipótese do "thrifty phenotype" considera o ambiente intrauterino como fator preponderante no desenvolvimento de doenças (ex: diabetes) na vida adulta. No entanto, muitos geneticistas assumem que a susceptibilidade para desenvolver o diabetes tipo 2 é, em grande parte, geneticamente determinada. A formulação inicial da hipótese do "thrifty genotype" propõe que a explosão da prevalência de diabetes nas sociedades ocidentais deve-se não somente a fatores ambientais, como também à seleção de genes candidatos ao desenvolvimento do diabetes tipo 2 (Neel, 1962). Esses genes proporcionariam uma vantagem seletiva em um ambiente de escassez de alimento, mas tornar-se-iam prejudiciais quando o suprimento alimentar passasse a ser adequado ou abundante. Nos tempos atuais, quando indivíduos geneticamente susceptíveis são expostos a fatores de risco como consumo elevado de alimentos calóricos e sedentarismo, desenvolvem obesidade, hiperinsulinemia, resistência à insulina com conseqüente descompensação das células beta pancreáticas e expressão do diabetes (Zimmet e cols, 1990). Os mesmos fatores genéticos que provocam redução na secreção e resistência aumentada à insulina, podem alterar o crescimento intrauterino e a tolerância à glicose na vida adulta, possibilitando uma ligação entre eles. Para investigar se o peso ao nascer poderia ser modificado por alteração no gene da glicoquinase e provocar secreção diminuída e resistência à insulina, Terauchi e cols (2000) avaliaram camundongos com mutação no gene da glicoquinase e observaram baixo peso ao nascer e susceptibilidade ao diabetes tipo 2. Corroborando essas observações, camundongos com mutação no gene do GLUT-2 (transportador de

glicose isoforma 2) perderam a primeira fase de secreção de insulina (Guillam e cols, 1997).

As células beta do pâncreas respondem ao aumento dos níveis circulantes de glicose, secretando mais insulina e promovendo o controle glicêmico através do aumento da captação de glicose pelos tecidos periféricos (principalmente pelos tecidos muscular e adiposo). Entretanto, a insulina deve ser reposta por ressíntese. À curto prazo, isso é possível através da tradução de moléculas de mRNA da insulina pré-existentes, mas a longo prazo esse processo é possível somente através da estimulação da transcrição do gene da insulina (Macfarlane e cols, 1997).

O fator de transcrição PDX-1 é responsável pela diferenciação das células da ilhota pancreática e transcrição do gene da insulina. Sua expressão é limitada às células beta e algumas células que produzem somatostatina nas ilhotas de Langherans, entretanto, existem períodos do desenvolvimento em que o PDX-1 é expresso nas células do ducto pancreático (McKinnon e cols, 2001). O PDX-1 também é conhecido como IPF-1, IDX-1, STF-1, IUF-1, GSF e se liga a 4 sítios (A1, A2, A3, A4) com a seqüência consenso C (C/T) TAATG no promotor da insulina humana (Clark e cols, 1993). Em pâncreas de camundongos adultos, o PDX-1 é seletivamente expresso na célula β pancreática; liga-se e transativa o promotor da insulina, promovendo a transcrição do gene da insulina (Ohlsson e cols, 1993). Jonsson e cols (1994), mostraram que camundongo homozigoto para uma mutação seletiva no gene PDX-1 apresenta agenesia pancreática. Esses neonatos mutantes sobrevivem ao desenvolvimento fetal, mas morrem poucos dias após o nascimento. Muitos estudos indicam que o PDX-1 regula genes envolvidos na manutenção da função e identidade da

célula beta. Esses genes são a insulina, GLUT-2, a glicoquinase e polipeptídeo amilóide da ilhota (Gremlich e cols, 1997). PDX-1 está envolvido no mecanismo pelo qual a glicose estimula a transcrição do gene da insulina. Elevadas concentrações de glicose em ilhotas maduras, proporcionam a fosforilação do PDX-1 e subseqüente translocação para o núcleo, onde se liga ao promotor do gene da insulina e promove a transcrição do mesmo. Essa ativação, dependente de glicose, ocorre através de um mecanismo que envolve a PI3-Kinase (Fosfatidilinositol 3 kinase) e deve envolver a p38/SAPK2 (Macfarlane e cols, 1999). Outros nutrientes metabolizáveis que induzem a secreção de insulina (incluindo frutose, piruvato e xilitol) e até mesmo a própria insulina ativam o PDX-1 (Wu e cols, 1999). Rafiq e cols (2000), demonstraram que as células β de ilhotas pancreáticas expostas à glicose, responderam ao estímulo com a transcrição do gene da insulina, através da translocação do PDX-1 da periferia do núcleo para o nucleoplasma. Também concluíram que a ativação da PI3-kinase é importante para a regulação do PDX-1, uma vez que, utilizando inibidores da PI3-kinase não há ativação do PDX-1.

A função do PDX-1 no diabetes mellitus tem sido documentado na última década. Mutação negativa no PDX-1 mostrou causar diabetes tipo 1, porém com aparecimento na idade adulta, conhecido como MODY4. Resultados semelhantes foram encontrados em uma população da Inglaterra (Macfarlane e cols, 1999) e mesmo em camundongos knockout para o PDX-1 nas células beta (Ahlgren e cols, 1998).

Assim como o PDX-1 é ativado pela presença de glicose no meio, é inibido pela presença de ácidos graxos livres (AGL). Altos níveis de AGL induzem um estado de resistência à insulina em músculo cardíaco e esquelético, através de uma diminuição no metabolismo da glicose via ciclo inibitório glicose-ácidos graxos (Randle e cols, 1988).

Coincidentemente, ilhotas pancreáticas de ratos submetidos a altas concentrações de ácidos graxos, apresentaram redução de 70% no mRNA e na proteína PDX-1 (Gremlich e cols, 1997).

As evidências encontradas na literatura mostram as influências do estado nutricional nas fases fetal e neonatal sobre os eventos que controlam a homeostasia glicêmica. Portanto, propomos estudar, em ratos, os efeitos da restrição protéica durante a vida intra-uterina e lactação sobre a morfometria da ilhota, secreção de insulina e expressão do PDX-1.

# OBJETIVOS

## 1-Objetivo geral

- Verificar a expressão do PDX-1 em ilhotas de ratos submetidos à restrição protéica durante a fase fetal e lactação e recuperados da desnutrição intra-uterina durante a lactação

## 2- Objetivos específicos

- Verificar a secreção de insulina por ilhotas de ratos recém-desmamados submetidos à desnutrição intra-uterina e durante a lactação.
- Verificar a secreção de insulina por ilhotas de ratos recém-desmamados submetidos à desnutrição intra-uterina e recuperados durante a lactação.
- Avaliar a morfometria da ilhota de ratos neonatos e recém-desmamados submetidos à desnutrição intra-uterina e durante a lactação.
- Avaliar a expressão do fator de transcrição do gene da insulina, PDX-1, em ilhotas de ratos neonatos e recém-desmamados, submetidos à desnutrição intra-uterina e durante a lactação.
- Avaliar a expressão do PDX-1 em ilhotas de ratos submetidos à desnutrição intra-uterina e recuperados durante a lactação.

# Reduced expression of PDX-1 in pancreatic B-cells from rats maintained on a low protein diet during gestation and suckling

Vanessa C Arantes[1], Vicente PA Teixeira[3], Márcia Q Latorraca[4], Everardo M Carneiro[1], Áureo T Yamada[2], and Antonio C Boschero[1]*

[1]Departamento de Fisiologia e Biofísica e [2]Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brasil. [3]Departamento de Patologia Geral, Faculdade de Medicina do Triângulo Mineiro, Uberaba, MG, Brasil. [4]Departamento de Nutrição e Dietética, Faculdade de Enfermagem e Nutrição, Universidade Federal de Mato Grosso, Cuiabá, MT, Brasil.

*To whom correspondence should be addressed: Departamento de Fisiologia e Biofisica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), 13083-970, Campinas, SP, Brasil.

Phone: 55 (19) 3788 6198

FAX: 55 (19) 3289 3124

E-mail: boschero@unicamp.br

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## ABSTRACT

**Background:** Intra-uterine and early postnatal malnutrition has profound consequences on fetal and postnatal development, both in humans and animals. In addition, low birth weight has been reported to be associated with impaired insulin secretion, insulin resistance and diminished area of pancreatic islets.

**Objective:** To assess the expression of the transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) in pancreatic B-cells, and to examine the islet area and glucose-induced insulin secretion by islets from rats maintained on a low protein diet.

**Design**: PDX-1 expression and islet area were assessed in rats maintained on a low (6%) or normal (17%) protein diet during fetal life. PDX-1 protein and mRNA levels, as well as insulin secretion and islet area were also measured after 28 days of life in normal, low protein, and recovered rats which received a normal protein diet after birth.

**Results:** Insulin secretion by isolated islets in response to 2.8 and 16.7 mmol glucose/L was reduced in 28-day-old low protein rats ($P < 0.0001$). At birth and after 28 days of life, the islet area and PDX-1 protein expression were also reduced ($P < 0.01$). In contrast, PDX-1 mRNA levels in islets from 28-day-old low protein rats was similar to normal rats. PDX-1 protein expression in B-cells, the area of islets and insulin secretion were restored in recovered rats, whereas PDX-1 mRNA was higher than normal rats ($P < 0.05$).

**Conclusion:** These results suggest a link between diminished PDX-1 protein expression, a reduction in islet area and impaired insulin secretion in low protein rats. The reintroduction of a normal diet early in life restored islet area and cell physiology.

## INTRODUCTION

The appropriate maternal metabolic environment is essential for development of the fetus, including its endocrine pancreas (1). In rapidly growing organisms, malnutrition in early life is a serious challenge to which the system tries to adjust. The quantity and/or quality of nutrition in this critical period has permanent consequences for later life. One of the mechanisms of adaptation to an inadequate supply of nutrients is to slow down the rate of cell division in tissues and organs, which may lead to altered "programming" of the structure and function of the system (2-4).

Based on epidemiological evidence, malnutrition has been envisaged as an etiological and/or a precipitating factor for diabetes in malnourished individuals in developing countries (5, 6). The prevalence of type 2 diabetes is much higher in adults with a low weight at birth (7, 8) and malnutrition is associated with impaired insulin secretion (8). Alteration in the maternal metabolic milieu during pregnancy influences the development and functional maturation of B-cells (9). Fetal malnutrition in rat pups results in a reduced number of B-cells, a diminution in the proliferation of islet cells, reduced islet size and a marked decrease in islet vascularization (10).

Glucose homeostasis in mammals requires the proper regulation of insulin secretion from pancreatic B-cells. The primary signal for secretion is an elevation in blood glucose concentrations that induces the release of stored insulin. Glucose also regulates the transcription (11-14) and translation (15-18) of preproinsulin (PPI) and stabilizes PPI mRNA (19). However, the mechanisms by which glucose influences the expression of the preproinsulin gene are less well understood than those that control insulin secretion acutely.

The 5' flanking region of the PPI gene has been extensively studied and a number of important regulatory elements and *trans*-acting factors have been identified (20). One of these factors is the homeodomain transcription factor PDX-1 (pancreatic duodenal homeobox-1), which plays an important role in lineage determination in the developing endocrine pancreas (20, 21). In addition, PDX-1 transactivates insulin, GLUT-2, glucokinase, and somatostatin genes (22). There is strong evidence supporting a role for PDX-1 in the mechanism by which glucose stimulates insulin gene transcription (23-25). Glucose stimulates the conversion of PDX-1 from a 31 kDa unphosphorylated to a 46 kDa phosphorylated form in human isolated islets. In B-cells, this event appears to be dependent on insulin signaling via the PI 3-kinase pathway (26).

In this study, we evaluated the insulin secretion, islet size and PDX-1 expression in islets from rats maintained on a protein deficient diet during gestation and lactation, and examined the influence of nutritional recovery on these parameters. PDX-1 mRNA levels were similar whereas PDX-1 protein levels were reduced in islets from rats on a low protein diet, indicating possible alterations in the translational process which may have been associated with a reduced islet area and insulin secretion. The reposition of dietary protein early in life increased PDX-1 mRNA levels and restored PDX-1 protein expression, as well as the islet area and insulin secretion in undernourished rats.

## MATERIALS AND METHODS

### Animals

The animal experiments were approved by the institutional (UNICAMP) Committee for Ethics in Animal Experimentation. Virgin female Wistar rats (80-90 days old) were obtained from the breeding colony at UNICAMP. Mating was done by housing females with males overnight, and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained from the first day of pregnancy until the end of lactation on an isoenergetic diet containing 6% protein (low protein diet, LP) or 17 % protein (control diet) as described previously (27). Some of the control and low protein diet rats were sacrificed at birth for immunohistochemistry. The remaining rats were distributed in three groups: *1)* a control group (C) consisting of rats born to and suckled by mothers fed a control diet during pregnancy and lactation, 2*)* an LP group consisting of the offspring of mothers fed an LP diet during both pregnancy and lactation and 3*)* a recovered group (R) consisting of the offspring of mothers fed an LP diet during pregnancy but fed a control diet during lactation. During the experimental period, the rats were fed their respective diets *ad libitum* and had free access to water. The rats were housed under standard lighting (12 h light-dark cycle) at a temperature of 24°C. At the end of the experimental period (28 days of life), the rats were killed by decapitation. Serum glucose and free fatty acids were measured immediately. Part of the serum was stored at –20°C for the subsequent measurement of insulin by radioimmunoassay (28).

**Insulin secretion**

Islets were isolated by collagenase digestion. Briefly, the pancreas was inflated with Hanks solution containing 0.7-0.9 mg collagenase/mL, excised and then maintained at 37°C for 20 min. The digested tissue was harvested and the islets were collected by handpicking. Group of five islets were first incubated for 45 min at 37°C in 0.5 ml of Krebs-bicarbonate buffer of the following composition (in mmol/L): NaCl 115, KC 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24, and glucose 5.6, supplemented with 3 mg bovine serum albumin/mL 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 90 min in the presence of 2.8 or 16.7 mmol glucose/L. The insulin content of the medium at the end of the incubation period was measured by radioimmunoassay (28).

**Morphometry and immunohistochemistry**

The pancreata were excised and dissected free of surrounding tissues, weighed and fixed by immersion in 10% formaldehyde-PBS solution. The fixed tissue was embedded in paraffin using a standardized protocol, then cut in 6 μm thick sections and mounted on glass slides. The sections were counterstained with hematoxylin-eosin for morphometric analysis. The islet area was measured by planimetry using a MOP-Videoplan image analysis software (Kontron Eletronik, Germany). The sectional area of islets was measured at a magnification of 40X.

Every tenth section from each pancreas was deparaffinized, rehydrated, and heated in 10 mmol citrate buffer/L (pH 6.0) at 92°C for 10 min in a microwave oven for antigen retrieval. After cooling at room temperature, the sections were washed three

times with phosphate–buffered saline, and endogenous peroxidase was blocked by a 30 min incubation with 3% $H_2O_2$ at room temperature. For PDX-1 immunohistochemistry, sections were incubated overnight at 4°C with polyclonal rabbit anti-PDX-1 antibody diluted 1:1000. Detection was with a streptavidin-biotin-peroxidase complex developed with aminoethylcarbazol (Zymed, San Francisco, CA).

**Western blot**

A pool of at least 1000 islets from each experimental group was homogenized by sonication (15 sec) in an anti-protease cocktail (10 mmol imidazole/L, pH 8.0, 4 mmol EDTA/L, 1 mmol EGTA/L, 0.5 μg pepstatin A/mL, 200 KIU aprotinin/mL, 2.5 μg leupeptin/mL, 30 μg trypsin inhibitor/mL, 200 μmol DL-Dithiothreitol (DTT)/L, 200 μmol PMSF/L). After sonication, an aliquot of extract was collected and the total protein content was determined by the dye-binding protein assay kit (Bio-Rad Laboratories). Samples of crude membrane preparations from each experimental group containing 70 μg of protein were incubated for 5 min at 80°C with 5x concentrated Laemmli sample buffer (1mmol sodium phosphate/L, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% mercaptoethanol) (4:1, v/v). The samples were then run on 10% polyacrylamide gels. Electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was done for 60 min at 120 V (constant) in buffer containing methanol and SDS. After checking the efficiency of transfer by Ponceau S staining, the membranes were blocked with 5% dry skimmed milk in TTBS (10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) overnight at 4°C. PDX-1 was detected in the membrane after a 2h incubation at room temperature with a rabbit polyclonal antibody against PDX-1 (diluted 1:2500 in

TTBS plus 3% dry skimmed milk). The membrane was then incubated with a rabbit anti-mouse IgG (diluted 1:1500 in TTBS plus 3% dry skimmed milk) followed by a further 2 h incubation at room temperature with $I^{125}$-labeled protein A (diluted 1:1000 in TTBS plus 1% dry skimmed milk). Radiolabeled protein bound to the antibody was detected by autoradiography. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

**mRNA expression**

Total RNA from 400 islets was extracted using TRIzol reagent (Life Technologies, UK). For polymerase chain reaction (PCR) analysis, the equivalent to 2 µg of RNA was reverse-transcribed using oligo(dT). The resulting cDNA were amplified by PCR using oligonucleotides complementary to sequences in the PDX-1 gene (5'- CCGAATGGAACCGAGACTGG -3' and 5'- AGGTGGTGGCTTTGGCAATG -3') ( 429 bp) and Phospholipase A2 gene (5'- CTGCTGGCTGCTTTGCTCAC -3'and 5'- ACGGCATAGACAGGAAGTGGG –3') (458 bp), the latter used as internal control. The PCR was done in a 25 µl reaction volume containing 1 µl cDNA equivalent to 2 µg of RNA, 10 mmol cold dNTPs/L (dATP, dCTP, dGTP, dTTP), 50 mmol $MgCl_2$ /L, 10 x PCR buffer, 10 µmol of appropriate oligonucleotides primers/L, and 2 units of Taq polymerase (Life Technologies, UK). The PCR amplification conditions for PDX-1 and Phospholipase A2 were: 2 min at 94°C followed by 29 cycles (30 s each) at 94°C, 59°C and 72°C, and 2 min at 94°C followed by 25 cycles (30 s each) at 94°C, 58°C and 72°C, respectively. The PCR products were separated on a 1.5% agarose gel in Tris borate EDTA buffer 1x

(TBE 1x) and stained with ethidium bromide. All PCRs included a negative control. The absence of genome contamination in the RNA samples was confirmed by the RT-negative RNA samples. The relative band intensities were determined by densitometry and the ratio of PDX-1 to Phospholipase A2 was calculated for each sample.

### Statistical analysis

The results are expressed as the mean ± SEM, as appropriate. When comparing the normal (control) and low protein groups, Student's two tailed unpaired *t* test was used. When comparing the changes in insulin secretion, Levene's test for homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric ANOVA. The data were analyzed using the software Statistic for Windows (version 4.3).

## RESULTS

### Characteristics of the rats

At birth, pups from mothers fed an LP diet during pregnancy, showed low body and pancreas weights. The serum glucose, but not insulin, levels were reduced, whereas the serum levels of FFA were high when compared to pups from mothers fed a normal diet during pregnancy. After 28 days, LP rats had low body and pancreas weights, reduced plasma glucose and insulin levels, and increased plasma FFA levels compared to C and R rats ($P < 0.001$ between LP and C and R rats for all parameters). Except for body and pancreas weights, which were higher in R than in C rats ($P < 0.001$), in the R group all parameters were normalized (Table 1).

**Insulin secretion**

Insulin secretion (Fig. 1) in the presence of a subthreshold glucose concentration (2.8 mmol/L) was 0.54 ± 0.15, 0.26 ± 0.05 and 0.42 ± 0.05 ng/islet per 90 min for the C, LP and R groups, respectively. At 16.7 mmol glucose/L, insulin secretion was 17.32 ± 1.8, 5.4 ± 1.1 and 14.0 ± 1.1 ng/islet per 90 min for the C, LP and R groups, respectively ($P < 0.0001$ LP vs C and R).

**Islet area**

The area of islets from C and LP rats at birth were 5430 ± 192 $\mu m^2$ and 3627 ± 213 $\mu m^2$, respectively (Fig 2A; $P<0.0001$) and 6192 ± 321$\mu m^2$, 4578 ± 323 $\mu m^2$ and 6873 ± 452 $\mu m^2$ for C, LP and R islets, respectively, at 28 days (Fig. 2B; $P < 0.001$ LP vs C and R).

**PDX-1 mRNA and protein expression**

PDX-1 expression in islets from 28-day-old C, LP and R rats was analyzed by immunoblotting using a polyclonal anti-PDX-1 antibody (Fig 3). A 46kDa protein representing PDX-1 was detected in all three tissue extracts. Densitometry of the bands showed that the amount of PDX-1 in the extract from LP islets was approximately 50% lower than in extracts from C and R islets ($P < 0.01$). The presence of PDX-1 in B-cells was confirmed by immunocytochemistry. As shown in Figure 4 (upper panels), PDX-1 labeling was located in the nucleus of the islet cells and showed a bright, punctuate pattern within the nucleus. The number of positive cells for PDX-1 was higher in C than in LP islets from neonates (Fig. 4A, B). PDX-1 labeling was also detected in pancreatic

duct cells (Fig 4 A). Figure 4 (C-E) shows that PDX-1 expression in islets from 28-day-old LP rats (D) was lower than in C islets (C) and was normalized in R islets (E). The number of aggregates positive for PDX-1 (Fig. 5 A, B) in LP was approximately 5-fold lower than in C islets at birth and after weaning ($P < 0.05$). The number of positive aggregates in R islets was similar to that in C islets.

In contrast, the PDX-1 mRNA concentrations in LP and C islets from 28-day-old rats were similar but increased in R islets ($P < 0.05$, R vs C and LP) (Fig. 6). These results indicated that the reduced expression of PDX-1 protein in LP rats was due to alterations in the translational process.

**TABLE 1.** Body and pancreas weight, serum glucose, insulin and free fatty acids of control (C), low protein (LP) and recovered rats 1 day and 28 days after birth.

| Group | 1 Day | | 28 Days | | |
|---|---|---|---|---|---|
| | C | LP | C | LP | R |
| Body weight (g) | 7.1 ± 0.08 | 4.9 ± 0.27* | 54.4 ± 1.42 | 26.04 ± 0.97* | 63.55 ± 1.74** |
| Pancreas weight (mg) | 19.8 ± 0.5 | 18.0 ± 0.8* | 278.0 ± 12.5 | 104.0 ± 6.1* | 353.0 ± 15.0** |
| Serum glucose (mg/dL) | 98.2 ± 0.5 | 73.2 ± 0.3* | 144.7 ± 3.3 | 120.0 ± 6.7* | 141.9 ± 3.0 |
| Serum insulin (ng/mL) | 0.15 ± 0.02 | 0.12 ± 0.04 | 0.70 ± 0.07 | 0.29 ± 0.04* | 0.76 ± 0.07 |
| Free fatty acids (mmol/L) | 1.44 ± 0.3 | 2.22 ± 0.2* | 0.39 ± 0.05 | 0.85 ± 0.05* | 0.34 ± 0.07 |

*$P<0.001$ for C vs LP

** $P< 0.001$ for R vs C and LP

The values are the mean ± SEM of 30-40 rats

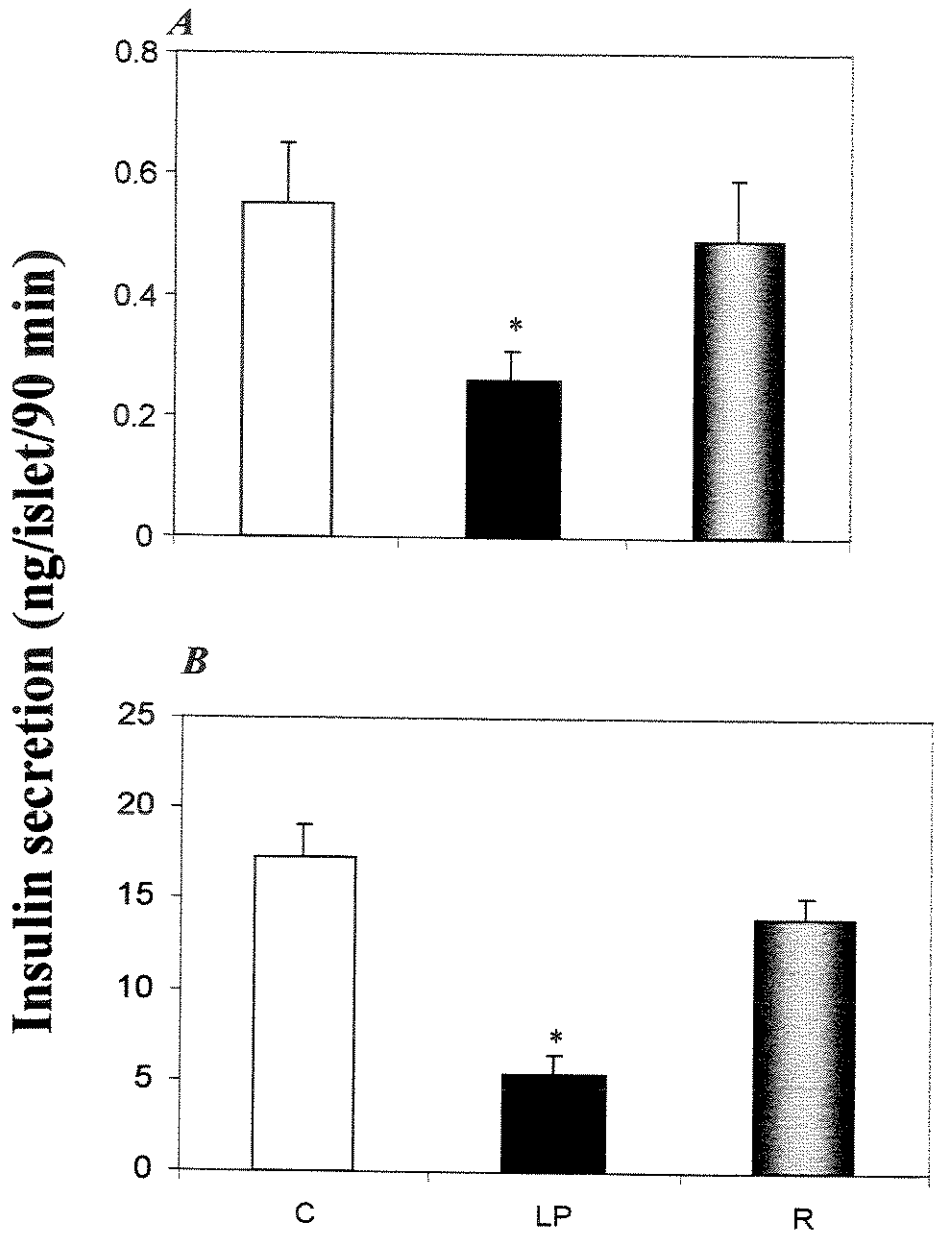


**Figure 1.** Glucose stimulation of insulin secretion in islets from control (C), low protein (LP) and recovered (R) rats. Groups of five islets were incubated for 45 min in Krebs-bicarbonate medium containing 5.6 mmol glucose/L, after which the medium was replaced with Krebs solution containing 2.8 mmol (**A**) or 16.7 mmol glucose/L (**B**). The columns represent the cumulative 90 min insulin secretion and are the means ± SEM of 10-20 groups of islets. $*P < 0.0001$ compared to the respective control.

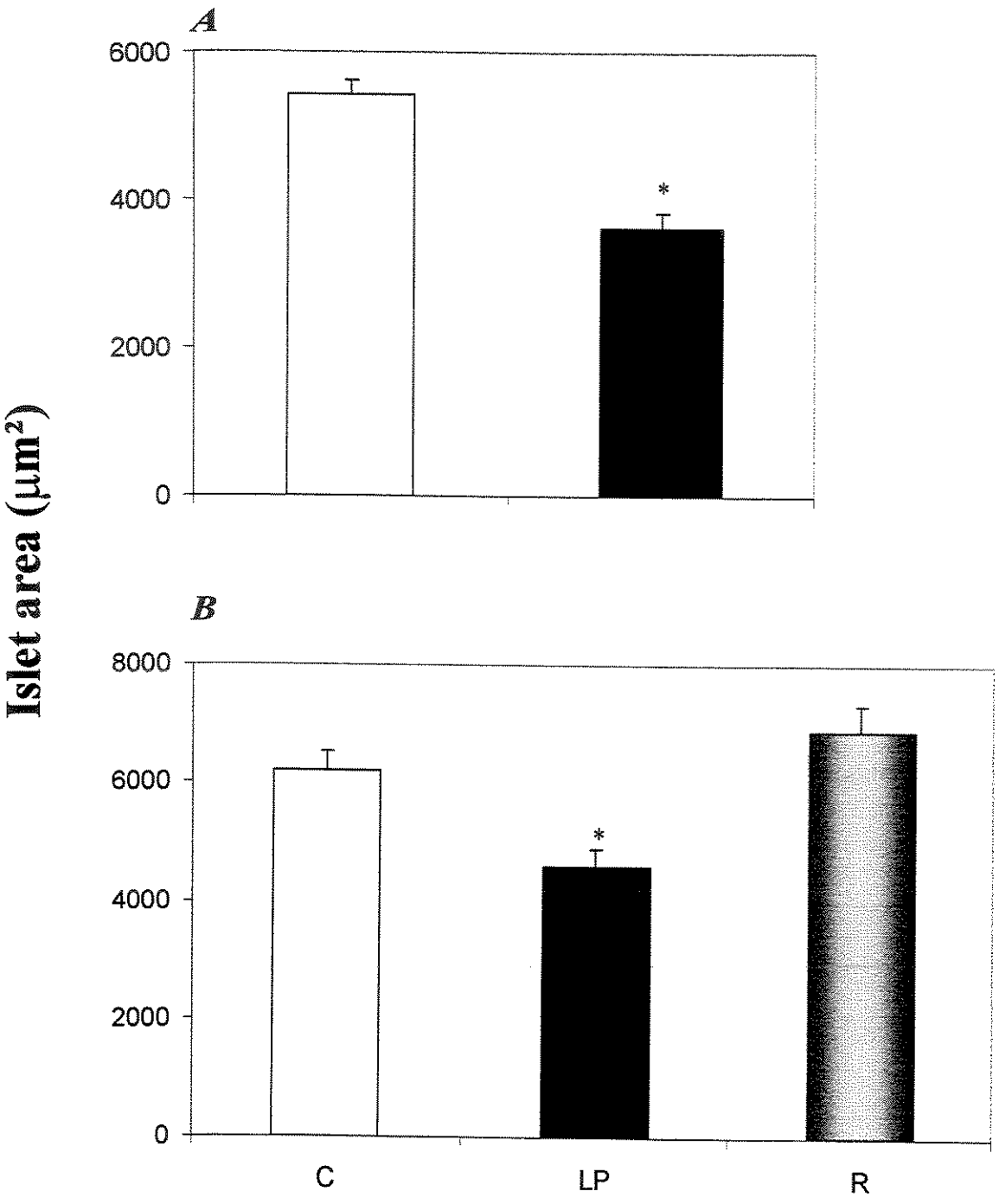


**Figure 2**. Areas of islets from control (C), low protein (LP) and recovered (R) rats. A section of the pancreas was counterstained with hematoxylin-eosin for morphometric analysis. The area of the islets was measured by planimetry as described in Methods. A, area of islets from C and LP neonatal rats. B, area of islets from control, low protein and recovered rats at 28 days of age. Data are the mean ± SEM of 200-400 islet sections. $*P < 0.001$ compared to the respective control group.

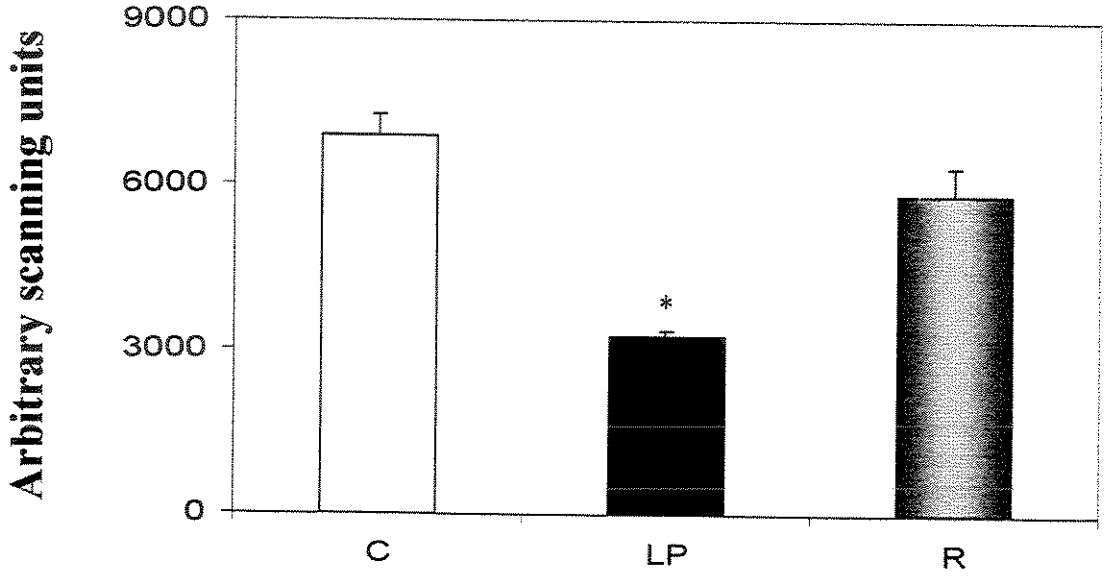


**Figure 3.** PDX-1 protein content in islets from control (C), low protein (LP) and recovered (R) rats. Each lane contained 70 μg of protein extracted from isolated islets from 28-day-old rats. PDX-1 was detected by immunobloting with polyclonal anti-PDX-1 antibody. Note that the C and R groups show a significantly higher expression of this protein than LP islets. The densitometry values are the mean ± SEM of 3 independent experiments. * $P < 0.001$ LP for C vs LP.

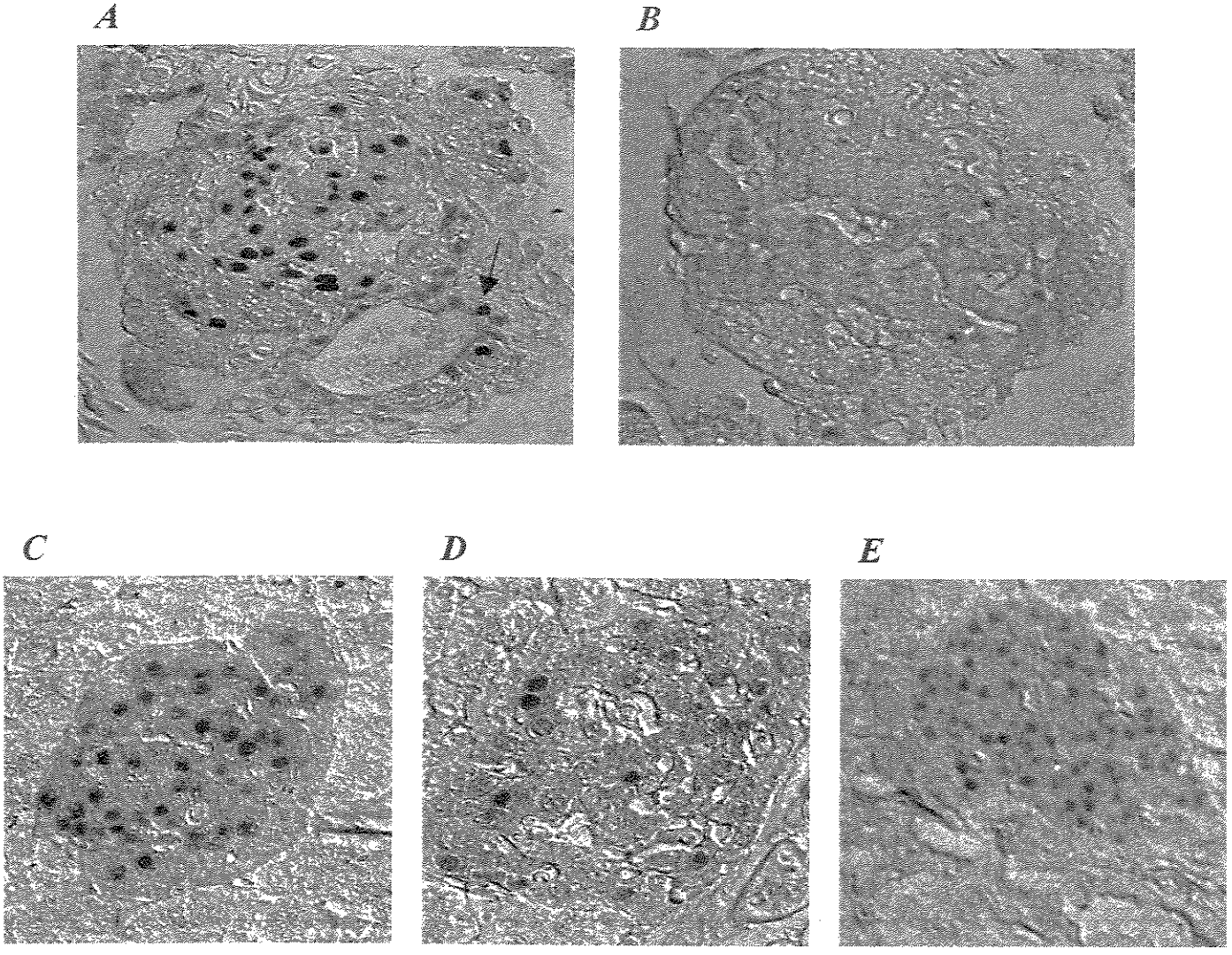


**Figure 4.** Immunolocalization of PDX-1 in pancreatic islet cells. The images show indirect immunofluorescence staining for PDX-1 in C (**A**) and LP islets (**B**) at birth and in control (**C**), low protein (**D**) and recovered (**E**) islets after weaning. Note the higher amount of PDX-1 positive granules in islets from control and recovered rats, and the preferential location of PDX-1 in the nucleus. PDX-1 positive granules were also detected (arrow) in cells from pancreatic ducts (**A**). Magnification, 40X.

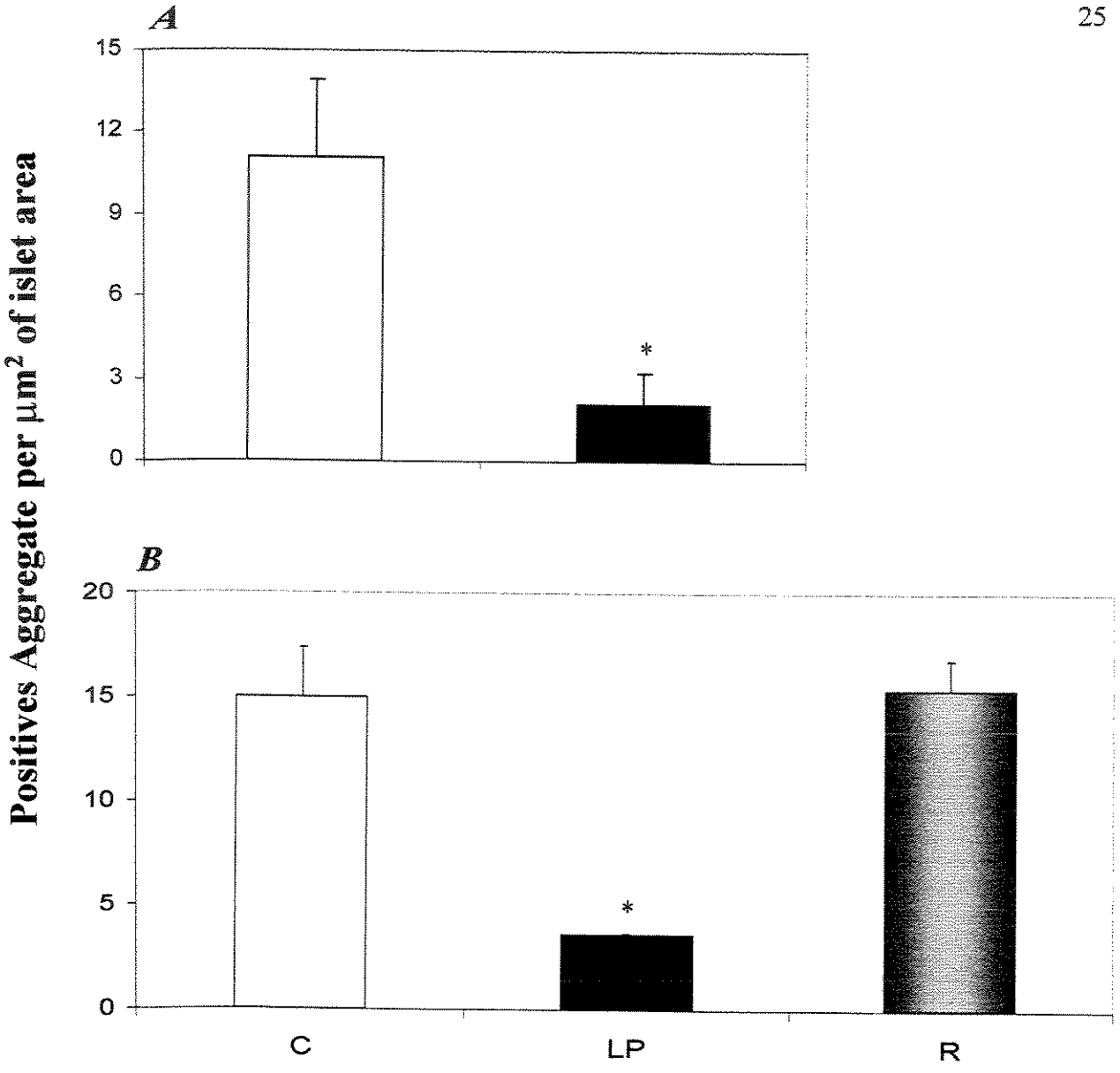


**Figure 5.** PDX-1 positive aggregates in islets from control (C), low protein (LP) and recovered (R) rats. The figure illustrates the number of PDX-1 positive aggregates/μm² in islets from newborn control and low protein rats (**A**) and in islets from 28-day-old control, low protein and recovered rats (**B**). The columns are the mean ± SEM 60-100 islet sections. *$P < 0.001$ compared to the respective control.

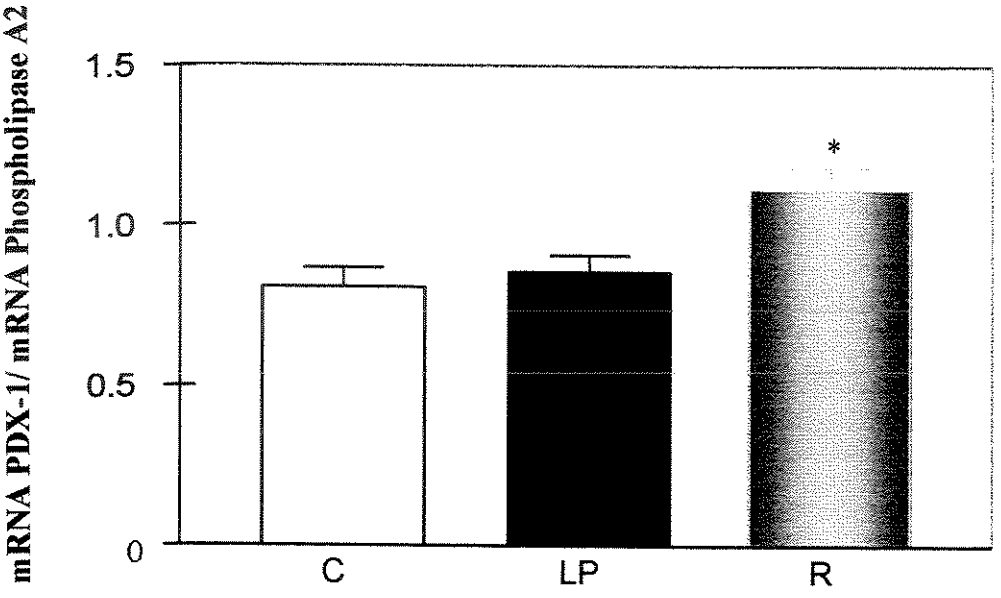


**Figure 6**. PDX-1 mRNA levels in pancreatic islets from 28-day-old rats. Total RNA was extracted from ~ 400 islets for each group and reverse–transcribed. Equal amounts of cDNA were subjected to PCR using primers complementary to the 5' and 3' ends of the PDX-1 and Phospholipase A2 genes. The mRNA concentrations of PDX-1 was expressed relative to Phospholipase A2 mRNA. The columns are the mean ± SEM of 4 independent experiments. * $P < 0.05$ for R compared with C and LP.

## DISCUSSION

In this study we investigated the effect of a low protein diet during fetal life and lactation on insulin secretion, islet area and PDX-1 expression. Newborn and weaned LP rats showed typical features of protein malnutrition, including a low body weight and high FFA. A reduction in body weight was associated with low serum glucose levels in pups from LP dams when compared to those from C dams (8). As previously reported, low maternal glycemia caused by malnutrition accounted for the reduced liver glycogen concentration and consequent low serum glucose levels of the offspring (29). The reduced body weight of LP rats may also have been related to the reduced serum insulin levels. Insulin exerts a profound effect on the growth of organs and on fetal metabolism, as judged by the hyperinsulinemia and macrosomia of neonates from non-compensated diabetic mothers (30). In weaned LP rats, the post prandial serum glucose levels were lower than in C and R rats. These results were consistent with an increased sensitivity to insulin in the target tissues of these animals (31). In R rats, all features were normalized, except for the body and pancreas weights which were still higher than in C rats. These findings confirmed and extended previous observations that no obvious alterations persisted at weaning when the protein deficiency was restricted to fetal life (32-34).

Protein deficiency impairs insulin secretion (8), and this was confirmed by our experiments with isolated islets. LP islets showed reduced glucose-induced insulin secretion in the presence of a high concentration of glucose (16 mmol/L) when compared to the C and R groups. It is conceivable that different steps in the mechanism of insulin secretion may be altered, such as a reduction in $Ca^{2+}$ uptake by the islets (35).

A reduction in islet size, a decrease in B-cell proliferation, and a reduction in the size of the islet vascular bed have been related to protein restriction during pregnancy

(10). At birth, pups from LP mothers had a much lower pancreas weight and islet size compared to the controls. After weaning (28 days old), these alterations were still present in LP rats, but were fully normalized in R rats, showing that the endocrine pancreas has considerable plasticity and is able to adapt to environmental changes during development, but only when the protein deficiency is restricted to fetal life (34). Thus, independent of the increased sensitivity to insulin in target tissues, the reduced insulin secretion in LP islets may result from intrinsic alterations in islets such as a smaller size and/or a reduced volume of B-cells (36).

One explanation for the reduced size of islets in LP rats could be the decrease in serum glucose which could signal to the B-cells that less insulin is required, so that the latter would not need to replicate or increase in size (37). In addition, amino acids, particularly branched-chain amino acids, may promote B-cell proliferation by stimulating the phosphorylation of PHAS-1 (a regulator of translation initiation during mitogenesis) and by facilitating the proliferative effects mediated by growth factors (38). Since branched-chain amino acids are most depleted in the plasma of fetuses from LP mothers (39), this could explain the lower rate of B-cell proliferation in the pancreas of LP pups. A reduced expression of IGF-II may also contribute to the lower B-cell proliferation rate and increased apoptosis observed in the fetus and neonate after feeding an LP diet (40).

Alterations such as a reduced insulin secretion and a smaller size or reduced cell volume for B-cells may also result from a decrease in insulin synthesis (10). PDX-1 is an important B-cell-specific transcription factor (41) that links glucose metabolism to the regulation of insulin gene transcription (42). Glucose actives PDX-1 through an insulin-dependent cell signaling pathway involving PI3-kinase. The stimulation of this

pathway leads to phosphorylation and the activation of a cytoplasmic form of PDX-1 that translocates to the nucleus (26). Thus, lower levels of circulating glucose observed in LP rats ultimately reduce PDX-1 expression.

The exposure of isolated islets to high concentrations of palmitic acid provoked a significant reduction in PDX-1 mRNA and protein expression as well as in PDX-1 binding activity for its *cis* regulatory elements in the insulin and GLUT-2 genes (22). However, in islets from LP and C islets the PDX-1 mRNA levels were similar, indicating that the change in FFA levels in vivo cannot account for the altered PDX-1 mRNA expression. The reduced PDX-1 protein expression in LP islets was probably related to alterations in the translation process, as recently proposed (43).

Taken together, these results indicate that a low protein diet during the fetal and lactation periods leads to alterations in islet size and functionality. These effects are linked to a reduction in PDX-1 protein, but not PDX-1 mRNA expression. The reintroduction of a normal diet immediately after birth normalizes the expression of PDX-1 protein and restores the islets cell mass and B-cell secretory capacity.

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## CONCLUSÕES

- A restrição protéica durante a gravidez e lactação comprometeu o crescimento da prole e produziu alterações típicas da desnutrição, como baixo peso corpóreo, menor peso do pâncreas e aumento da concentração de ácidos graxos livres.
- Ratos desnutridos neonatos e recém-desmamados apresentaram glicemia reduzida, assim como baixas concentrações de insulina sérica.
- Ilhotas isoladas de ratos desnutridos recém desmamados apresentaram secreção de insulina reduzida em resposta à glicose.
- Ilhotas de ratos desnutridos neonatos e recém-desmamados apresentaram menor tamanho.
- Ilhotas de ratos desnutridos recém-desmamados apresentaram diminuída expressão protéica do PDX-1.
- Os níveis de mRNA do PDX-1 apresentaram-se semelhantes em ilhotas de ratos desnutridos e controles.
- Em ilhotas de ratos recuperados os níveis de mRNA do PDX-1 apresentaram-se aumentados.
- Ratos recuperados da desnutrição intra-uterina apresentaram normalização da secreção de insulina e da expressão do PDX-1.

Esses resultados sugerem que a desnutrição durante a vida intra-uterina e lactação alteram a secreção de insulina, a área da ilhota e a expressão protéica do PDX-1. A resposta insulínica alterada frente à estimulação com glicose e o menor tamanho da ilhota podem estar associados à diminuída expressão protéica do PDX-1. Os níveis normais de mRNA do PDX-1 em ilhotas de ratos

desnutridos, sugerem alteração no processo de tradução da proteína. Em ratos recuperados, todos os parâmetros foram normalizados.

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