MÁRCIA QUEIROZ LATORRACA

ALTERAÇÕES DA SECREÇÃO E DA SENSIBILIDADE À INSULINA
ASSOCIADAS À DEFICIÊNCIA PROTÉICA DURANTE A VIDA
INTRA-UTERINA E A LACTAÇÃO

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Ciências Biológicas, área de Fisiologia

ORIENTADOR(A): Profª. Drª. Maria Alice Rostom de Mello
CO-ORIENTADOR: Prof. Dr. Antonio Carlos Boschero

CAMPINAS - 1998
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Aos meus pais, irmãos e sobrinhos

À Carlos Henrique

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ABREVIATURAS E SÍMBOLOS

ANOVA - analysis of variance (análise de variância)
Arg - arginine (arginina)
BSA - bovine serum albumin (albumina bovina sérica)
cAMP - adenosina 3', 5'-cyclic monophosphate (adenosina 3', 5'-monofosfato cíclica)
[Ca^{2+}]_i - cytosolic calcium (cálcio citosólico)
C group - control group (grupo controle)
°C - degrees centigrades (graus centígrados)
d - day (dia)
DAG - diacylglycerol (diacilglicerol)
df - degrees of freedom (graus de liberdade)
dl - deciliter (decilitro)
DNA - deoxyribonucleic acid (ácido desoxirribonucleico)
DTT - dithiothreitol (ditiotreitol)
ΔG - area under the serum glucose concentration curve (área sob a curva de concentração de glicose sérica)
ΔI - area under the serum insulin concentration curve (área sob a curva de concentração de insulina sérica)
EC_{50} - glucose concentration producing half-maximal insulin secretion (concentração de glicose que produz a secreção semimáxima de insulina)
EDTA - ethylenediaminetetraacetic acid (ácido etilendiaminotetracético)
EGTA - ethylene glycol-bis (β-aminoethyl ether) (etileno-glicol-bis(β-aminoetil eter)
F - variance ratio (razão das variâncias)
FFA - free fatty acids (ácidos graxos livres)
g - gram (grama)
GH - growth hormone (hormônio do crescimento)
Gluc - glucose (glicose)
GLUT2 - glucose transporter 2 (transportador de glicose facilitador do tipo 2)
GLUT4 - glucose transporter 4 (transportador de glicose facilitador do tipo 4)
h - hour (hora)
HEPES - N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (ácido hidroxietil piperazina etano sulfônico)
IGF-I - insulin-like growth factor-I (fator de crescimento semelhante à insulina-I)
IP₃ - inositol 1,4,5-triphosphate (inositol 1,4,5-trifosfato)
IR - insulin receptor (receptor de insulina)
IRS-1 - insulin receptor substrate-1 (substrato-1 do receptor de insulina)
ITT - insulin-tolerance test (teste de tolerância à insulina)
K₅ATP - ATP-sensitive K⁺ channel (canal de potássio sensível ao ATP)
kg - kilogram (quilograma)
Kᵣₑₜ - rate constant for serum glucose disappearance during insulin tolerance test (velocidade de desaparecimento da glicose sérica durante o teste de tolerância à insulina)
l ou L - liter (litro)
Leu - leucine (leucina)
LP group - low-protein group (grupo hipoprotéico)
mEq - milliequivalent (miliequivalente)
min - minute (minuto)
mg - milligram (miligrama)
ml - milliliter (mililitro)
mM - millimolar (milimolar)
mmol - millimole (milimol)
MODY - maturity-onset diabetes of the young (diabetes tipo maturidade do jovem)
MU - milliunit (miliumidade)
µCi - microCurie (microCurie)
µl - microliter (microlitro)
n - number of experiments or observations (número de experimentos ou observações)
nmol - nanomole (nanomol)
NEFA - non-esterified fatty acids (ácidos graxos não esterificados)
NP - normal protein group (grupo normoprotéico)
OGTT - oral glucose-tolerance test (teste de tolerância à glicose oral)
P - probability or level of significance (probabilidade ou nível de significância)
PDGF - platelet-derived growth factor (fator de crescimento derivado das plaquetas)
pg - picogram (picograma)
PI 3-kinase - phosphatidylinositol 3-kinase (fosfatidilinositol 3-quinase)
PKC - protein kinase C (proteinoquinase C)
PL - placental lactogen (lactogênio placentário)
pM - picomole (picomol)
PMSF - phenylmethylsulfonylfluoride (fenilmetilsulfonilfluoreto)
RIA - radioimmunoassay (radioimunoensaio)
R group - recovered group (grupo recuperado)
RNA - ribonucleic acid (ácido ribonucleico)
s - second (segundo)
SDS-PAGE - sodium dodecyl-polyacrylamide gel eletrophoresis (sulfato dodecil de sódio- eletroforese em gel de poliacrilamida)
SEM - standard error of the mean (erro padrão da média)
TPA - 5-tetradecanoyl-13-phorbol acetate (tetradecanoil acetato de forbol)
Tris - tris(hydroxymethyl)amino-methane (tris (hidroximetil) aminometano)
v - volum (volume)
w - weight (peso)
RESUMO

Estudos epidemiológicos têm sugerido que a desnutrição intra-uterina e durante a infância é um dos principais determinantes da suscetibilidade a doenças como o diabetes do tipo 2 e a síndrome de resistência à insulina. Presume-se que a desnutrição materna afete o crescimento inicial da prole, altere a secreção de insulina e a sensibilidade a esse hormônio. No presente trabalho estudamos a influência da restrição protéica durante a vida intra-uterina e a lactação sobre a secreção e a ação da insulina em ratos recém-desmamados e em ratos adultos recuperados da desnutrição. A secreção de insulina e a homeostasia da glicose foram examinadas em crias recém-desmamadas (28 dias de vida) de ratas Wistar alimentadas durante a gravidez e a lactação com dieta contendo 17% de proteína (grupo NP) e 6% de proteína (grupo LP). Durante teste de tolerância à glicose oral, ratos do grupo LP apresentaram áreas sob as curvas de concentração de glicose e de insulina séricas menores em relação aos ratos do grupo NP. A velocidade de desaparecimento da glicose durante teste subcutâneo de tolerância à insulina (Km) foi maior no grupo LP do que no grupo NP, indicando aumento da sensibilidade à insulina. Ilhotas pancreáticas isoladas de ratos do grupo LP apresentaram redução da secreção de insulina frente à estimulação com glicose, arginina e leucina. Essas ilhotas apresentaram redução da primeira e segunda fases de secreção de insulina em resposta à glicose. Finalmente, nas ilhotas pancreáticas do grupo LP a incorporação de $^{45}$Ca após 5 ou 90 minutos de incubação em meio contendo glicose (que refletem principalmente a entrada e a retenção de Ca$^{2+}$, respectivamente) foi menor em relação às ilhotas do grupo NP. A secreção de insulina e a sensibilidade ao hormônio foram avaliadas em ratos adultos (90 dias de vida) mantidos com uma dieta contendo 17% de proteína (grupo C) ou 6% de proteína (grupo LP).
durante a vida intra-uterina, a lactação e após o desmame, e em ratos que receberam uma dieta contendo 6% de proteína durante a vida fetal e a lactação e uma dieta contendo 17% de proteína após o desmame (grupo R). Durante o teste de tolerância à glicose oral, as áreas sob as curvas de concentração de glicose sérica foram similares nos três grupos. Ratos dos grupo LP e R apresentaram áreas sob as curvas de concentração de insulina sérica significativamente reduzidas em relação aos ratos do grupo C. A velocidade de desaparecimento da glicose durante o teste intravenoso de tolerância à insulina (Km) foi maior nos ratos do grupo LP em relação aos ratos dos grupos C e R. Em ilhotas isoladas a secreção de insulina em resposta à glicose foi diferente entre os grupos testados, sendo maior no grupo C e menor no grupo LP. Em músculo gastrocnêmio de ratos do grupo LP foi observado aumento dos níveis de receptor de insulina, da fosforilação de substrato-1 do receptor de insulina (IRS-1) e maior associação entre o substrato-1 do receptor de insulina e fosfatidilinositol 3-quinase (PI 3-quinase) em relação aos animais do grupo C. No grupo R as alterações dos passos iniciais da ação da insulina foram parcialmente restauradas. Esses resultados tomados em conjunto sugerem que a desnutrição durante a vida intra-uterina e a lactação altera permanentemente a secreção de insulina. A resposta insulínica deficiente frente à estimulação com glicose e aminoácidos pode estar associada à incapacidade das células beta pancreáticas em “manejar” adequadamente os íons cálcio. Em ratos desnutridos e recuperados, a homeostasia glicêmica é mantida, pelo menos em parte, pelo aumento da sensibilidade à insulina, resultante das alterações nos passos iniciais da via de sinalização do referido hormônio.
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INTRODUÇÃO

O diabetes mellitus não-dependente de insulina ou 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 da glicose pelos tecidos periféricos. Portanto, as células beta pancreáticas, o fígado e os tecidos muscular e adiposo estão envolvidos na sua patogênese (Beck-Nielsen et al. 1995). A forma mais comum de diabetes tipo 2 está frequentemente 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).


O aumento do número de casos de diabetes tipo 2 no mundo ocidental e em certos grupos étnicos tem sido explicado pela hipótese do “thrifty genotype” (Neel, 1962). Essa
hipótese atribui o desenvolvimento do diabetes a um genótipo que conferiu uma vantagem seletiva em um ambiente de escassez de alimento, mas tornou-se prejudicial quando o suprimento alimentar passou a ser adequado ou abundante. De acordo com essa hipótese, entre os caçadores-coletores das culturas primitivas sobreviviam aqueles que conseguiam estocar energia com a máxima eficiência. Esses indivíduos tinham menor capacidade de sintetizar glicogênio na presença de insulina e estocavam energia na forma de gordura, especialmente em depósitos de rápida renovação no abdômen. Nos tempos atuais, quando indivíduos geneticamente suscetíveis são expostos a fatores de risco como consumo elevado de alimentos calóricos e sedentarismo, desenvolvem obesidade, hiperinsulinemia, resistência à insulina com consequente descompensação das células beta pancreáticas e expressão do diabetes (Zimmet et al. 1990).

Os argumentos a favor da determinação genética da doença baseiam-se principalmente na frequência incontestavelmente aumentada do diabetes nas famílias de diabéticos e em estudos que mostram uma maior taxa de concordância para o diabetes tipo 2 em gêmeos monozigotos do que em dizigotos (veja revisão de Guillausseau et al. 1997). Existem, no entanto, críticas a esses estudos, visto que gêmeos também possuem determinantes ambientais idênticos denominados fatores ambientais in utero (Phillips, 1993). Além disso, o modo de herança permanece desconhecido na maioria das famílias, exceto em alguns sub-típos monogênicos que representam de 10 a 15% de todos os casos da doença: MODY2- relacionado à mutação no gene da glucoquinase; MODY1 e MODY3- secundários à mutação de fatores nucleares hepáticos; e diabetes resultante da mutação do DNA mitocondrial (veja revisão de Guillausseau et al. 1997). Alguns genes ligados à ação da insulina, por exemplo o gene da insulina, o gene do receptor de insulina, o gene do transportador de glicose e o gene da
glicogênio sintase, têm sido examinados, mas as alterações encontradas são responsáveis por cerca de 1% de todos os casos (Kahn 1994). A heterogeneidade do fenótipo clínico e biológico sugere que o diabetes é uma desordem “multifatorial”, isto é, resultante da interação de fatores ambientais com o efeito aditivo de múltiplos genes (veja revisão de Guillaumeau et al. 1997).

Recentemente Hales & Barker (1992) 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 tardivamente como o diabetes, a hipertensão arterial e a doença isquêmica do coração são determinadas por fatores ambientais e são originadas in utero ou durante a infância. Admite-se que a desnutrição intra-uterina e no primeiro ano de vida produzem mudanças morfológicas e funcionais em vários tecidos e órgãos. As anormalidades metabólicas e funcionais observadas na vida adulta como intolerância à glicose, hipertensão arterial, dislipidemias, resistência à insulina, dependem da fase da vida em que a desnutrição ocorre, do tipo e da intensidade da carência nutricional. Presume-se que a restrição protéica imposta durante a vida intra-uterina é capaz de produzir danos morfológicos e funcionais irreversíveis nas células beta pancreáticas, nos hepatócitos e nos tecidos muscular e adiposo. Tais alterações causam redução da secreção de insulina e da sensibilidade a esse hormônio. Quando a resistência à insulina é agravada pela obesidade, inatividade física e idade, o pâncreas não consegue suprir a demanda aumentada de insulina e o diabetes se instala (Hales & Barker 1992).

Os estudos epidemiológicos realizados nas duas última décadas têm fornecido provas de que influências ambientais adversas, inclusive nutricionais, que resultam em retardo de crescimento fetal, estão relacionadas à alta incidência de diabetes tipo 2 e da “síndrome X”. As primeiras indicações vieram de um estudo realizado no Reino Unido que mostrou uma maior
incidência de diabetes tipo 2 em grupos populacionais de baixo nível sócio-econômico (Barker et al. 1982). Posteriormente, estudos mais detalhados realizados em Hertfordshire e em Preston mostraram que o baixo peso ao nascer e durante o primeiro ano de vida estavam associados a um maior risco de morte por doença isquêmica do coração (Barker et al. 1989), à hipertensão arterial na vida adulta (Barker et al. 1990), e ao aumento da concentração de fibrinogênio plasmático (Barker et al. 1992). Nessas populações o risco de desenvolver intolerância à glicose ou diabetes tipo 2 foi maior entre os indivíduos com baixo peso ao nascer e no primeiro ano de vida (Hales et al. 1991, Phipps et al. 1993). A relação entre os índices de crescimento fetal e infantil e o aumento de risco de desenvolver o diabetes tipo 2 ou a “síndrome X” na vida adulta tem sido confirmada em tipos étnicos bastante distintos, como os índios Pima do Arizona (McCance et al. 1994), os americanos de origem mexicana (Athens et al. 1993) e suecos (McKeigue et al. 1994). Em estudo recente com gêmeos monozigotos e dizigotos discordantes para o diabetes tipo 2 verificou-se que o portador da enfermidade tinha o peso ao nascer significativamente menor do que os indivíduos que apresentavam tolerância à glicose normal. Esses resultados sugerem que fatores intra-uterinos não genéticos são os principais determinantes do diabetes tipo 2 (Poulsen et al. 1997).

Um corpo crescente de evidências clínicas e experimentais têm reforçado a hipótese do “thrifty phenotype”. Em modelos animais, Widdowson & McCance (1975) demonstraram que durante a vida fetal existem “períodos críticos” de rápida divisão celular. Esses “períodos críticos” diferem entre os vários tecidos. O “período crítico” para o rim e para o pâncreas, por exemplo, é a semana imediatamente anterior ao nascimento. O crescimento depende do suprimento de oxigênio e de nutrientes e o feto adapta-se à sua redução diminuindo a taxa de divisão celular, especialmente naqueles tecidos que se encontram no “período crítico”. A taxa

O peso corporal e os tecidos muscular, adiposo, hepático e pancreático, por exemplo, são significativamente afetados pela restrição protéica durante os períodos críticos de crescimento fetal e pós-natal. O déficit de peso corporal e de órgãos como o figado e o pâncreas é corrigido por uma alimentação normal (Desai et al. 1996a), mas as alterações do tecido muscular (Winick & Noble 1966, Widdowson 1971, Feagle et al. 1975, Desai et al. 1996a) e do tecido adiposo (Shepherd et al. 1997) permanecem após a recuperação nutricional.

O restabelecimento do peso do pâncreas parece não refletir a recuperação da função desse órgão. Pelo menos três possibilidades devem ser consideradas ao avaliarmos a disfunção pancreática causada pela desnutrição: 1) a redução do número das células β pancreáticas; 2) o defeito funcional da massa de células β pancreáticas; ou 3) a combinação de redução da massa de células β com alterações funcionais das células β remanescentes. A última possibilidade parece ser a mais aceitável, a julgar por resultados de estudos que mostram a influência do
ambiente materno sobre o crescimento e a maturação funcional das células β (Hellerström & Swenne 1991).

intra-uterina não são revertidas pelo aumento das células β durante a recuperação nutricional (Garofano et al. 1997).

À parte a redução da massa de células β e da vascularização das ilhotas pancreáticas, o padrão de anormalidades secretórias observadas na desnutrição indica alterações em mecanismos celulares envolvidos na secreção de insulina. Estudos têm mostrado que ilhotas fetais e neonatais de ratos desnutridos apresentam redução significativa da quantidade de insulina liberada em resposta à glicose, aminoácidos (leucina, arginina, taurina, glutamina) e a estímulos não-nutrientes (TPA: ester de forbol, forskolin, teofila, acetilcolina, KCl) (Dahri et al. 1991, 1994b; Cherif et al. 1996, 1997). A normalização do conteúdo de AMP cíclico por teofila e por forskolin não corrige o déficit secretório (Dahri et al. 1994a), mas a resposta insulínica é restaurada quando essas ilhotas são estimuladas com citocinazina-B e com Ba²⁺(Cherif et al. 1997), indicando que o fluxo de cálcio pode estar envolvido na alteração secretória. Ilhotas neonatais de ratos desnutridos apresentam oxidação mitocondrial da glicose normal e taxa de utilização desse substrato reduzida, sinal de um possível defeito na via glicolítica (Wilson & Hughes 1997).

As alterações secretórias não são revertidas por uma nutrição normal, a julgar pelos resultados de três estudos que indicam alterações dos eventos metabólicos e da ativação dos mensageiros intra-celulares (AMP cíclico e proteínoquinase C). O primeiro estudo demonstrou a incapacidade da glicose em estimular o fluxo de fosfato inorgânico em ilhotas isoladas de ratos adultos submetidos à restrição protética durante a lactação (Barbosa et al. 1993). O segundo, realizado por Hales et al. 1996, mostrou que a atividade da glicoxinase pancreática, e possivelmente o fluxo glicolítico na célula beta, encontram-se reduzidos em animais recuperados da desnutrição protética durante a vida intra-uterina e lactação. Finalmente, o
terceiro estudo demonstrou que ilhotas de animais adultos recuperados da desnutrição intra-uterina têm uma resposta reduzida à estimulação com nutrientes (glicose e arginina) e com os ativadores exógenos da proteínaquinase C e do AMP cíclico (teofilina, TPA e forskolin) (Dahri et al. 1994a). Não se deve excluir a possibilidade de que outros mecanismos celulares envolvidos na secreção de insulina sejam afetados permanentemente pela desnutrição.

O crescimento e a função dos chamados sítios de resistência à insulina (fígado, tecidos musculares e adiposo) também são alterados pela desnutrição intra-uterina e pós-natal. A restrição protéica modifica permanentemente a atividade das enzimas hepáticas glicogenólise e fosfoenolpiruvato carboxiquinase, envolvidas no metabolismo da glicose. Essa modificação (redução da atividade da glicogenólise e aumento da atividade da fosfoenolpiruvato carboxiquinase) indica um aumento da síntese ao invés de utilização da glicose (Desai et al. 1995). As alterações da atividade da glicogenólise e da fosfoenolpiruvato carboxiquinase são acompanhadas por mudanças paralelas nos níveis da RNA mensageiro dessas enzimas, indicando que os efeitos da desnutrição também se estendem à regulação da expressão dos genes dessas proteínas (Desai et al. 1997). A regulação hormonal da produção de glicose hepática também encontra-se alterada nesse modelo animal. Em ratos adultos recuperados da desnutrição intra-uterina e neonatal, a insulina é incapaz de inibir a produção de glicose hepática estimulada por glucagon e ao mesmo tempo esses animais apresentam resistência a esse hormônio. Os níveis de receptores de glucagon estão reduzidos enquanto que a concentração de receptores de insulina e de GLUT2 (transportador de glicose 2) encontram-se aumentados na membrana dos hepatócitos de ratos recuperados da desnutrição (Ozanne et al. 1996a). As alterações da atividade das enzimas hepáticas e o aumento dos receptores de insulina têm sido atribuídas a mudanças nas zonas metabólicas do fígado. A glicogenólise e a

As evidências encontradas na literatura mostram as influências do estado nutricional nas fases fetal e neonatal sobre os eventos envolvidos na homeostasia glicêmica. Portanto, propusemos a estudar, em ratos, os efeitos imediatos e tardios da restrição protéica durante vida intra-uterina e a lactação sobre a secreção de insulina e a sensibilidade dos tecidos periféricos a esse hormônio. Inicialmente, estudamos os efeitos da deficiência protéica sobre a homeostasia da glicose, analisando in vivo a secreção e a sensibilidade à insulina em ratos
recém-desmamados. Em seguida, investigamos o impacto da restrição protéica sobre a função pancreática, avaliando: a secreção de insulina em resposta à glicose e a aminoácidos, a dinâmica da secreção de insulina e a incorporação de cálcio induzidas por glicose em ilhotas isoladas de ratos recém-desmamados. Finalmente, investigamos a secreção e a ação da insulina em ratos adultos submetidos à restrição protéica durante a vida intra-uterina, a lactação e a fase pós-desmame, e examinamos a influência da recuperação nutricional sobre essas respostas. Nessa fase da vida, avaliamos a secreção de insulina em resposta à glicose in vivo e por ilhotas isoladas, e os passos moleculares iniciais da ação da insulina em tecido muscular esquelético.
OBJETIVOS

1- Objetivo geral:

- Testar a hipótese do “thrifty phenotype” em um modelo de desnutrição protéica fetal e neonatal, analisando a secreção de insulina e a sensibilidade à insulina em ratos recém-desmamados e adultos recuperados da desnutrição.

2- Objetivos específicos:

- Verificar a tolerância à glicose e a sensibilidade à insulina exógena in vivo em ratos recém-desmamados submetidos à desnutrição intra-uterina e durante a lactação.

- Avaliar a secreção de insulina por ilhotas isoladas de ratos recém-desmamados desnutridos durante a vida intra-uterina e a lactação em resposta a nutrientes secretagogos.

- Verificar a captação de $^{45}$Ca por ilhotas de ratos recém-desmamados desnutridos durante a vida intra-uterina e a lactação.

- Verificar a tolerância à glicose e a sensibilidade à insulina exógena in vivo em ratos adultos desnutridos e recuperados da desnutrição intra-uterina e durante a lactação.

- Avaliar a secreção de insulina em resposta à glicose por ilhotas isoladas de ratos adultos desnutridos e recuperados da desnutrição intra-uterina e durante a lactação.

- Examinar os eventos moleculares iniciais relacionados à ação da insulina em músculo de ratos adultos desnutridos e recuperados da desnutrição intra-uterina e durante a lactação.
PROTEIN DEFICIENCY DURING PREGNANCY AND LACTATION IMPAIRS GLUCOSE-INDUCED INSULIN SECRETION BUT INCREASES THE SENSITIVITY TO INSULIN IN WEANED RATS

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Running head: Glucose homeostasis in malnourished rats

Key words: protein malnutrition, insulin secretion, glucose homeostasis, rats

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ABSTRACT

We studied glucose homeostasis in rat pups from dams fed a normal protein (17%) diet (NP) or a diet containing 6% protein (LP) during fetal life and the suckling period. At birth, total serum protein, serum albumin and serum insulin levels were similar in both groups. However, body weight and serum glucose levels in LP rats were lower than NP rats. At the end of the suckling period (28 days of age), total serum protein, serum albumin and serum insulin were significantly lower and the liver glycogen and serum free fatty acid levels were significantly higher in LP rats compared to NP rats. Although the fast serum glucose level was similar in both groups, the area under the blood glucose concentration curve ($\Delta G$) after a glucose load was higher in NP rats (859 (SEM 58) mmol/l.120 min vs 607 (SEM 52) mmol/l.120 min for LP rats; $p<0.005$). The mean post-glucose increase in insulin ($\Delta I$) was higher in NP rats (30(SEM 4.7) nmol/l.120 min vs 17 (SEM 3.9) nmol/l.120 min for LP rats; $p<0.05$). The glucose disappearance rate ($K_{in}$) in NP rats (0.7 (SEM 0.1) %/min) was lower than in LP rats (1.6 (SEM 0.2) %/min) ($p<0.001$). Insulin secretion from isolated islets (1 h incubation) in response to 16.7 mmol/l glucose was augmented 14 times in NP rats but only 2.6 times in LP rats, over respective basal secretion (2.8 mmol/l) ($p<0.001$). These results indicate that in vivo as well as in vitro insulin secretion in pups from dams maintained on a LP diet is reduced. This defect may be counteracted by an increase in the sensitivity of target tissues to insulin.
INTRODUCTION

Malnutrition in humans and other mammals is associated with impaired insulin secretion and alterations in carbohydrate metabolism (Milner, 1971; Carneiro et al. 1995). In adult rats submitted to acute or chronic protein malnutrition during the growing period after weaning, a severe reduction has been observed in insulin secretion in response to glucose and other secretagogues (Carneiro et al. 1995; Okitolonda et al. 1987; Reis et al. 1997). However, glucose intolerance has been found only during the first weeks of protein deprivation (Okitolonda et al. 1987; Swenne et al. 1987). Protein malnutrition imposed during fetal life can be detrimental to the development of pancreatic B-cells, thereby leading to permanent insulin deficiency (Snoeck et al. 1990; Dahri et al. 1991). Furthermore, alterations in the activity of the key hepatic enzymes (glucokinase and phosphoenolpyruvate carboxykinase) that are differentially regulated by insulin have been reported in the offspring of mothers deprived of protein during pregnancy (Desai et al. 1995). Thus, at 21 days of age, protein-deprived pups show decreased glucokinase and increased phosphoenolpyruvate carboxykinase activities, a situation which is indicative of glucose production rather than utilization (Desai et al. 1995). Reduced insulin secretion and enzymatic changes would be predicted to predispose to diabetes.

The suckling-weaning period is associated with nutritional changes as well as with modifications in the levels of circulating insulin and the action of this hormone (Blásquez et al. 1970; Issad et al. 1987). Thus, the insulin resistance present in the suckling rats disappears after weaning and the plasma insulin levels are higher in weaned rats than in suckling rats (Issad et al. 1987).

In the present study we have examined insulin secretion and glucose homeostasis in
weaned rats from dams fed a low protein diet during pregnancy and lactation. Offspring from malnourished dams showed a severe reduction in insulin secretion in response to a glucose load, in vitro as well as in vitro. However, no alteration in glycemia was observed in these animals, indicating a compensatory increase in the sensitivity to insulin in target tissues.

MATERIAL AND METHODS

Virgin female Wistar rats (90 days old) were obtained from the State University of Campinas animal facilities. Mating was performed by housing females with adult males overnight and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained on an isocaloric diet containing 6% protein (low protein diet) or 17% protein (normal diet) from the first day of pregnancy until the end of the lactation period (Table 1). During the experimental period, the dams were fed their respective diets ad libitum and had free access to water.

The animals were kept under standard lighting conditions (12 h light/dark cycle) at a temperature of 24 °C. The food intake was monitored daily and the dams were weighed weekly. All pups were weighed at birth. Some of the pups (32 from NP mothers, 11 litters; 22 from LP mothers, 9 litters) were sacrificed for biochemical analyses. Sera from half of the NP and LP pups were used for the insulin and glucose determination while the remaining sera were used for protein and albumin measurements. The remaining animals (those not used in the above analyses; 8 litters) were weighed after birth for four consecutive weeks. At 25 days of age, the pups were weaned and maintained on their mothers’ diet. Forty-three pups from six NP litters and 45 pups from six LP litters were killed by decapitation at 28 days for the
Table 1. Composition of the normal and low protein diets (g/Kg).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Normal protein (17% protein)</th>
<th>Low protein (6% protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (84% protein)</td>
<td>202.0</td>
<td>71.5</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.0</td>
<td>480.0</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>130.5</td>
<td>159.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>121.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix (AIN-93)*</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93)*</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chlorhydrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*Detailed composition given by Reeves et al. 1993.
measurement of liver glycogen (Hassid & Abrahams, 1957) and pancreatic insulin content (Malaisse et al. 1967). Blood sample from these animals were also collected and allowed to clot; the sera were subsequently stored at -20 °C for biochemical analyses. Serum glucose (Trinder, 1969), total serum protein (Wolfson et al. 1948), serum albumin (Doumas et al. 1971), serum free fatty acids (Regouw et al. 1971), and serum insulin (Scott et al. 1981) levels were determined. Since the amount of serum obtained from each animal was not sufficient for all these biochemical parameters, the number of individual experiments varied between groups.

Six animals from each of four litters were used for the glucose tolerance test. Eleven NP and 13 LP rats from the same litters were used for the insulin tolerance test. Finally, isolated islets were obtained from rats of four LP and NP litters. All experimental procedures started between 08.00 and 09.00 a.m.

**Glucose-tolerance test (OGTT).** An oral glucose tolerance test was performed using the four-week-old male rats. After a 15 h fast, glucose (200 g/l) was administered orogastrically through a catheter at a dose of 2 g/kg body weight. Blood samples were obtained from the cut tip of the tail 0, 30, 60 and 120 min later for the determination of serum glucose and insulin concentrations. The glucose and insulin responses during the OGTT were calculated by estimating the total area under the glucose (ΔG) and insulin (ΔI) curves, respectively, using the trapezoidal method (Matthews et al. 1990).

**Insulin-tolerance test (ITT).** A subcutaneous insulin-tolerance test was performed using four-week-old male rats, after a 15 h fast. The ITT consisted of a bolus injection of insulin (30 mU/100 g body weight) beneath the dorsal skin of the animal. Blood samples were obtained from the cut tip of the tail 0, 30, 60, 120 and 180 min later for the measurement of glucose levels. The rate constant for serum glucose disappearance (K_m) was calculated using the
formula $0.693/t_{1/2}$. The serum glucose $t_{1/2}$ was calculated from the slope of a least square analysis of the serum glucose concentrations from 0-60 min after the subcutaneous injection of insulin, when the serum glucose concentrations declined linearly.

**Insulin secretion from isolated islets.** Islets were isolated by collagenase digestion. In brief, pancreas was inflated with Hanks’ balanced salt solution containing 0.7 mg collagenase/ml, excised and then maintained at 37 °C for 18 min. The digested tissue was then washed 4 times and the islets separated by hand-picking with the aid of a siliconized stretched pasteur pipet. Groups of 5 islets were first incubated for 30 min at 37 °C in 0.75 ml of a Krebs-bicarbonate buffer with the following composition (in mmol/l): NaCl 115, CaCl$_2$ 2.56, MgCl$_2$ 1, NaHCO$_3$ 24, glucose 5.6 supplemented with 3 mg of BSA/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 1 h in the presence of 2.8 or 16.7 mmol/l glucose. The insulin content of the supernatant at the end of the incubation period was measured by radioimmunoassay (Scott et al. 1981).

**Statistical analysis.** The results are presented as the mean ± SEM for the number of rats (n) indicated. When comparing NP (normal protein diet) and LP (low protein diet) groups, Student’s non-paired t-test was used. When comparing the evolution of body weight for the pups, Lavene’s test for the homogeneity of variance was initially used to check the fit of the data to the assumptions for parametric analysis of variance. A Box-Cox transformation was used to correct for variance heterogeneity or non-normality (Sokal & Rohlf, 1981). The data were subsequently analyzed by two-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons.
RESULTS

Table 2 shows that of the various parameters measured, only the total protein intake was significantly lower in dams maintained on a low protein diet compared to the control group (p<0.001). There was a significant reduction (p<0.01) in the body weight and glycemia of newborn rats from dams fed a low protein diet. However, there was no change in the insulinemia of newborn rats from protein-restricted mothers (Table 3).

The total food and protein intake during lactation were significantly greater in the NP rats than in the LP group (787 (SEM 20) g food, 134 (SEM 3.4) g protein, n=6 and 470 (SEM 32) g food, 28 (SEM 1.9) g protein, n=8, respectively, p<0.0001, in both cases). However, when the results were expressed per gram of litter body weight, the food intake was significantly greater in the LP than in the NP group (17.3 (SEM 2.0) g/100 g litter body weight/day, n=8 and 9.2 (SEM 0.3) g/100 g litter body weight/day, n=6, respectively, p<0.001) but the protein intake was significantly decreased in the LP pups than in the NP rats (1.0 (SEM 0.1) g/100 g/day and 1.6 (SEM 0.1) g/100 g/day, p<0.001).

The body weight gain of normal pups was significantly higher than that of low-protein pups during lactation (Figure 1). The two-way ANOVA revealed a significant main effect of groups (df=1; F=3345.52; p=0.00) and age (df=4; F=2405.15; p=0.00), as well as the two-way interaction group vs age (df=4; F=273.24; p=0.00).

By the 28th day of life, the liver glycogen content and serum free fatty acid levels in the fed state were significantly higher in LP pups than in the NP group, while for total serum protein, serum albumin and serum insulin the reverse was true (Table 4).

Protein restriction significantly decreased the pancreas weight (p<0.01) and the total
Table 2. Food and protein intake, weight gain during pregnancy, and litter size for dams fed a normal (NP) or low (LP) protein diet. Values are mean and standard error of the mean for eleven and nine mother from NP and LP groups, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>Food intake (g)</th>
<th>Protein intake (g)</th>
<th>Weight gain (g)</th>
<th>Number of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>NP</td>
<td>385</td>
<td>9.6</td>
<td>65.8</td>
<td>1.6</td>
</tr>
<tr>
<td>LP</td>
<td>390</td>
<td>21.3</td>
<td>23.4*</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* p<0.01 compared to the NP group.
Table 3. Body weight, serum glucose, insulin, total protein and albumin levels of newborn rats from eleven and nine mothers fed a normal (NP) or low (LP) protein diet during pregnancy. Values are mean and standard error of the mean. Number of rats in parentheses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g) Mean</th>
<th>SEM</th>
<th>Total serum protein (g/dl) Mean</th>
<th>SEM</th>
<th>Serum albumin (g/dl) Mean</th>
<th>SEM</th>
<th>Serum glucose (mmol/l) Mean</th>
<th>SEM</th>
<th>Serum insulin (nmol/l) Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>5.6 0.1</td>
<td></td>
<td>1.9 0.1</td>
<td></td>
<td>0.8 0.1</td>
<td></td>
<td>5.5 0.5</td>
<td></td>
<td>0.26 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=110)</td>
<td></td>
<td>(n=16)</td>
<td></td>
<td>(n=14)</td>
<td></td>
<td>(n=16)</td>
<td></td>
<td>(n=11)</td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>5.0* 0.1</td>
<td></td>
<td>1.8 0.1</td>
<td></td>
<td>0.8 0.1</td>
<td></td>
<td>4.1* 0.3</td>
<td></td>
<td>0.22 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=106)</td>
<td></td>
<td>(n=13)</td>
<td></td>
<td>(n=14)</td>
<td></td>
<td>(n=14)</td>
<td></td>
<td>(n=7)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.01 compared to the NP group.
Figure 1. The body weight of offspring from dams fed a normal (NP, squares) or low (LP, circles) protein diet during pregnancy and lactation. The values are the mean (SEM) of 61 and 64 pups from NP and LP groups, respectively.
Table 4. Liver glycogen, free fatty acid (FFA), total serum protein, serum albumin, serum glucose and serum insulin levels of weaned rats from dams fed a normal (NP) or low (LP) protein diet during pregnancy and lactation. Values are mean and standard error of the mean. Number of rats in parentheses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver glycogen (mg/100 mg)</th>
<th>FFA (mEq/l)</th>
<th>Total serum protein (g/dl)</th>
<th>Serum albumin (g/dl)</th>
<th>Serum glucose (nmol/l)</th>
<th>Serum insulin (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>Mean 7.4, SEM 0.4, n=43</td>
<td>Mean 0.3, SEM 0.02, n=25</td>
<td>Mean 3.6, SEM 0.1, n=28</td>
<td>Mean 2.2, SEM 0.1, n=25</td>
<td>Mean 7.1, SEM 0.2, n=28</td>
<td>Mean 0.4, SEM 0.04, n=15</td>
</tr>
<tr>
<td>LP</td>
<td>Mean 10.0*, SEM 0.5, n=45</td>
<td>Mean 0.5**, SEM 0.04, n=33</td>
<td>Mean 3.0**, SEM 0.1, n=28</td>
<td>Mean 1.7**, SEM 0.06, n=27</td>
<td>Mean 6.4, SEM 0.3, n=28</td>
<td>Mean 0.1*, SEM 0.02, n=16</td>
</tr>
</tbody>
</table>

*p<0.001, **p<0.0001 compared to the NP group.
pancreatic insulin content ($p<0.01$). However, there was no difference between the LP and NP pups when the pancreas weight and pancreatic insulin content were related to body weight or when the pancreatic insulin content was related to pancreas weight (Table 5).

Fasting serum glucose and insulin concentrations obtained before the glucose tolerance test were similar in the two groups of pups. After a glucose load the areas under the glycemia and insulinemia curves were lower in LP than NP rats ($p<0.005$, and $p<0.05$, respectively). In addition, after a subcutaneous insulin load, the glucose disappearance rate ($K_{in}$) in the LP group was significantly greater ($p<0.001$) than the NP group, thus indicating an increased sensitivity to insulin (Table 6).

Insulin secretion in the presence of low concentration of glucose (2.8 mmol/l glucose) was 0.31 (SEM 0.03) ($n=16$) and 0.43 (SEM 0.05) ($n=13$) ng/islet per h in LP and NP rats, respectively ($p<0.05$). Increasing the glucose concentration to 16.7 mmol/l glucose, insulin secretion was 0.81 (SEM 0.10) ($n=20$) and 6.39 (SEM 0.74) ($n=15$) ng/islet per h in LP and NP rats, respectively ($p<0.01$).

**DISCUSSION**

Maternal protein restriction during pregnancy and lactation can adversely affect offspring. Hales & Barker (1982) have suggested that poor maternal deprivation, mainly protein deprivation, may favor the appearance of “syndrome X”, characterized by type 2 diabetes, hypertension and hyperlipidemia. Consistent with this hypothesis, recent studies in experimental animals have shown that protein restriction, during critical periods of
Table 5. Pancreatic insulin content of weaned rats from dams fed a normal (NP) or low (LP) protein diet during pregnancy and lactation. Values are mean and standard error of the mean. Number of rats in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP ((n = 9))</td>
<td>LP ((n = 12))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of pancreas (g)</td>
<td>Mean 0.2</td>
<td>Mean 0.07*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM 0.02</td>
<td>SEM 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/kg body weight</td>
<td>3.7</td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>per pancreas 11.2</td>
<td>per pancreas 4.1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM 0.9</td>
<td>SEM 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>per g of pancreas 55.4</td>
<td>per g of pancreas 60.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM 6.8</td>
<td>SEM 5.3</td>
<td></td>
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<tr>
<td></td>
<td>per kg body weight 191.1</td>
<td>per kg body weight 201.3</td>
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<tr>
<td></td>
<td>SEM 15.1</td>
<td>SEM 15.0</td>
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</table>

*\(p<0.01\) compared to the NP group.
Table 6. Fasting serum glucose and insulin concentrations, total areas under the glucose (ΔG) and insulin (ΔI) curves obtained from the oral glucose tolerance test, and the glucose disappearance rate (K_{int}) calculated using serum samples obtained 0 to 60 min after a sub-cutaneous injection in normal (NP) and low (LP) protein groups. The values are mean and standard error of the mean. Number of the rats in parentheses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (nmol/l)</th>
<th>ΔG (mmol/l)</th>
<th>ΔI (nmol/l)</th>
<th>K_{int} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>NP</td>
<td>4.7</td>
<td>0.8</td>
<td>0.17</td>
<td>0.03</td>
<td>859</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
</tr>
<tr>
<td>LP</td>
<td>3.9</td>
<td>0.2</td>
<td>0.10</td>
<td>0.04</td>
<td>607**</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
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<td>(n = 6)</td>
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</table>

*p<0.05, **p<0.005, ***p<0.001 compared to the NP group.
development, can lead to generalized growth retardation and permanent reduction of the size of organs and tissues, including pancreas (Desai et al. 1996). Changes in the activity of some of the liver enzymes related with glucose metabolism (Desai et al. 1995), and hypertension (Langley-Evans & Jackson 1996).

Concerning mother weight, we did not observed differences between the two groups until the end of the 4th week of pregnancy. This contrast with previous result in which mother weight gain was reduced by the 10% in the LP group (Snoeck et al. 1990). However, these authors report that the reduction in the weight gain by malnourished mothers occurred only during the last two days of the pregnancy. At the end of lactation period weight gain of LP mothers was significantly reduced as compared to NP mothers (data not shown).

In the present study we verified reduction in body weight associated to low serum glucose levels in the newborns from protein malnourished dams when compared to those from normal dams. Neonatal hypoglycemia is a frequent finding in low-weight newborns (Leeun & Vries, 1976) and it has been attributed to the maternal hypoglycemia (Gruppuso et al. 1981). As previously reported (Mello et al. 1987) the low maternal glycemia due to malnutrition accounts for the reduced liver glycogen concentration and consequent low serum glucose levels in their offspring. Additionally, a long-term maternal hypoglycemia or reduced fetal glucose supply due to reduced uterine blood supply during the final of pregnancy, eventually results in fetal growth retardation (Gruppuso et al. 1981). The reduced body weight observed in neonates from dams fed a low-protein diet may also be related to a reduced supply of insulin from the mother since the insulin content of the maternal endocrine pancreas has been shown to be significantly reduced in pregnant rats fed a low-protein diet (Dahri et al. 1995). Insulin exerts a profound effect on the growth of organs and fetal metabolism as judged by the
hyperinsulinemia and macrossomia of neonates from non-compensated diabetic mothers (Van Assche & Aerts, 1979). Fetal growth induced by insulin is linked to an increase in circulating somatomedins, cross-reaction with growth factor receptors, and a lack of down-regulation of the fetal liver insulin receptors (Roth, 1979; Hill & Milner, 1980; Alvarez & Blásquez, 1984).

Our results demonstrate that the effects of maternal protein restriction were more marked in weaned than in newborn rats. Thus, malnourished pups showed a reduction in body weight while serum protein, albumin and insulin levels were preserved. In weaned, protein-deprived rats these parameters were drastically reduced in association with high levels of free fatty acids and liver glycogen concentration. Hypoalbuminemia, hypoproteinemia and elevated free fatty acid and liver glycogen levels are features commonly found in malnourished infants and in experimental animals (Milner, 1971; Carneiro et al. 1995; Okitolonda et al. 1987; Reis et al. 1997). During suckling, the growth rate of LP pups was 60% less than that of NP pups, a finding that agrees with the observations that the post-natal period is critical for overall growth (Desai et al. 1996).

In weaned rats from malnourished dams, the post-prandial serum glucose levels were similar to those of NP pups despite the significantly lower serum insulin levels in the former. These results confirm those of previous studies in fed and fasted malnourished, adult rats (Okitolonda et al. 1987; Escriva et al. 1991). Under fasting conditions, the LP weaned rats in our study had normal serum glucose and insulin levels.

A reduced insulin secretion may result from a decrease in insulin synthesis in malnourished animals. Protein restriction during pregnancy produces a variety of structural changes in the pancreas of the offspring, including a reduction in islet size, a decrease in B-cell proliferation, and a reduction of the islets vascular bed (Snoeck et al. 1990; Reusens et al.)
1997). Our data showed pups from dams protein deprived with pancreas weight and total pancreatic insulin content drastically reduced as compared to control. However, when the pancreatic insulin content was expressed relative to body or pancreas weight, no difference was noticed. Despite this observation, insulin secretion during a glucose load was severely reduced, as already related by others (Okitolonda et al. 1987; Okitolonda et al. 1988; Dahri et al. 1990; Reis et al. 1997). These observations was confirmed by our experiments with isolated islets. Glucose (16.7 mmol/l) increased insulin secretion, over basal (2.8 mmol/l glucose), by 14 times in Np but only by 2.6 times in LP islets. An adequate explanation for this phenomenon has not yet been proposed. However, it is possible that different steps in the mechanism of insulin secretion may be altered in islets from malnourished animals. Possible changes include an impaired glucose recognition by B-cells (Dixit & Kaung, 1985), reduced activity of the mitochondrial glycerophosphate dehydrogenase (Rasschaert et al. 1995), and a reduction in Ca²⁺ uptake by the islets (Carneiro et al. 1995). Moreover, the long-term exposure to high NEFA levels could also contribute to the inhibition glucose-induced insulin secretion. The detrimental effects of elevated fatty acids on B-cell function have been demonstrated in studies in vivo as well as in vitro. The mechanisms involved include the stimulation of fatty acid oxidation (Sako & Grill 1990) with consequent inhibition of glucose oxidation and ATP generation (Zhou & Grill 1994).

The paradoxical association of euglycemia with low serum insulin levels, and the low areas under the glucose and insulin curves during the oral glucose tolerance test in malnourished pups agree with previous reports (Okitolonda et al. 1987; Escriva et al. 1991). The maintenance of glucose levels in malnourished pups may reflect (1) an increased sensitivity to insulin and a consequent rise in the glucose uptake by peripheral tissues (Heard et al. 1977;
Crace et al. 1990; Ozzane et al. 1996b; Reis et al. 1997), (2) an increased ability of insulin in reduce hepatic glucose output (Escriva et al. 1991), or (3) the presence of hepatic glucagon resistance with a subsequent decrease in hepatic glucose output (Ozanne et al. 1996a). The higher liver glycogen content observed in protein-deprived pups indicates a decreased response to glucagon, perhaps as a result of low hepatic glucose-6-phosphatase activity (Heard et al. 1977) or fewer hepatic glucagon receptors (Ozanne et al. 1996a). The increased serum glucose disappearance rate ($K_{in}$) observed after an insulin load is consistent with an increase in sensitivity to insulin, with the increased phosphorylation of the insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) recently observed in hepatocytes and muscle cells (Reis et al. 1997; Latorraca et al., unpublished data).

In conclusion, protein restriction in rats during the early period of life can lead to a reduction in insulin secretion with concomitant metabolic alterations. A parallel increase in the sensitivity of target tissues to the hormone may explain the normoglycemia observed in rats maintained on a low-protein diet.

ACKNOWLEDGMENTS

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REFERENCES


REDUCED INSULIN SECRETION IN RESPONSE TO NUTRIENTS IN ISLETS FROM MALNOURISHED YOUNG RATS IS ASSOCIATED WITH A DIMINISHED CALCIUM UPTAKE

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Running title: Insulin secretion and Ca^{2+} handling in malnutrition.

Key Words: protein malnutrition, pancreatic islets, insulin secretion, Ca^{2+} fluxes, rats.

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ABSTRACT

Changes in $^{45}$Ca uptake and insulin secretion in response to glucose, leucine, and arginine were measured in isolated islets derived from four weeks old rats born from mothers maintained with normal (NP-17%) or low protein (LP-6%) diet during pregnancy and lactation. Glucose provoked a dose-dependent stimulation of insulin secretion in both groups of islets with basal (2.8 mM glucose) and maximal release (27.7 mM glucose) significantly reduced in LP as compared to NP islets. In LP group the concentration-response curve to glucose was shifted to the right compared to NP group, with the half-maximal response occurring at 16.9 and 13.3 mM glucose, respectively. In LP islets, glucose-induced first and second phases of insulin secretions was drastically reduced. In addition, insulin response to individual amino-acids, or in association with glucose were also significantly reduced in LP group as compared to NP islets. Finally, in LP islets the $^{45}$Ca uptake after 5 min or 90 min incubation (that reflects mainly the entry of Ca$^{2+}$, and the Ca$^{2+}$ retention, respectively), was lower than NP islets. These data indicate that, in malnourished rats, both initial and sustained phases of insulin secretion in response to glucose were reduced. This poor secretory response to nutrients seems to be the consequence of an altered Ca$^{2+}$ handling by malnourished islets cells.
INTRODUCTION

There are critical periods in embryonic development in which tissues and organs are established. In the endocrine pancreas, the peak pancreatic β-cell mass is determined early in life. Thus, in rats, the number of β-cells increases rapidly some days before birth whereas in humans their development occurs during intrauterine life and infancy.1

Alterations in the maternal metabolic milieu during pregnancy influence the development and functional maturation of β-cells.2 In addition, nutritional deprivation before and after birth impairs neonatal β-cell proliferation, and reduces β-cell mass, islet size, and islet vascularization.3, 4 Such structural and functional damage during these phases represents a potential hazard for the development of diabetes mellitus in adult life. Since the growth of β-cells and insulin secretion during fetal life are predominantly regulated by amino-acids, protein restriction in early life may have a major role in the appearance of type 2 diabetes.5

Insulin secretion by pancreatic β-cells is modulated by nutrients, neurotransmitters and hormones.6 Glucose, the major physiological stimulator of insulin secretion, is transported into the β-cell through GLUT2, phosphorylated by glucokinase and then undergoes glycolysis and oxidation.7, 8 This leads to an increase in the cytosolic ATP/ADP ratio which blocks the ATP-sensitive K⁺ channels (K_{ATP}) located at the plasma membrane9, and leads to the opening of voltage-dependent Ca²⁺ channels; the later results in an influx and subsequent elevation of cytosolic calcium ([Ca²⁺]).10 The increase in [Ca²⁺], triggers the exocytosis of insulin-containing granules.11 In addition, an elevation in [Ca²⁺], activates adenylate cyclase and phospholipase C, with the consequent generation of cAMP, DAG, and IP₃. These second messengers amplify the
[Ca\textsuperscript{2+}], signal through mobilization of intracellular Ca\textsuperscript{2+} stores and by the promoting phosphorylation of proteins that sensitize the secretory process to Ca\textsuperscript{2+}. Amino-acids also stimulate insulin release by increasing the [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{12,13}

Glucose insensitivity and/or alterations in the dynamics of insulin release induced by glucose have been considered as an early indicator of defective insulin secretion in diabetes.\textsuperscript{14} Disturbances in the regulation of Ca\textsuperscript{2+} fluxes and [Ca\textsuperscript{2+}]\textsubscript{i} are also common features in animal models showing alterations in insulin secretion.\textsuperscript{15} In the present study, we investigated the effect of protein malnutrition during the fetal and suckling periods on insulin release and \textsuperscript{45}Ca uptake by isolated islets. We observed that the reduced insulin secretion in islets from weaned, protein deficient rats in response to glucose and amino acids, was associated with a diminished \textsuperscript{45}Ca uptake by these islets.

METHODS AND MATERIALS

\textit{Animals}. Virgin female Wistar rats (85-90 days old) from the State University of Campinas breeding colony were housed with males until mating had occurred. Vaginal smears were examined daily and pregnancy was dated from the first day on which spermatozoa were identified. Pregnant rats were kept in individual cages at 24 °C under a 12 h light:dark cycle. The rats were separated at random and maintained on an isocaloric diet containing 6% protein (low protein diet) or 17% protein (normal diet) from the first day of pregnancy until the end of the lactation period, as described elsewhere.\textsuperscript{16} During the experimental period, the dams had access to their respective diets and to water \textit{ad libitum}. At the birth, large litters were reduced to eight pups, thus ensuring a standard litter size per mother. Two groups of male Wistar rats
28 days old were used in this study: 1) a normal protein group (NP) consisting of the offspring of rats fed a control diet during pregnancy and lactation; and 2) a low protein group (LP) consisting of the offspring of rats fed a low protein diet during pregnancy and lactation. All offspring were weaned at the 25th day after birth. The pups were weighed at birth and at the end of experimental period.

**Insulin secretion from isolated pancreatic islets.** Fed rats were killed by decapitation and the pancreas was removed. Islets were isolated by hand-picking after collagenase digestion of the pancreas, following a technique previously described. Groups of five islets were first incubated for 30 min at 37 °C in Krebs-bicarbonate solution containing 5.6 mM glucose and equilibrated with 95% O₂ / 5% CO₂, pH 7.4. The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were further incubated for 1 h with the following concentrations of glucose (in mM): 2.8, 5.6, 8.3, 11.1, 16.7 and 27.7. The incubation medium contained (in mM): NaCl 115, KCl 5, NaHCO₃ 24, CaCl₂ 1, MgCl₂ 1, and BSA 0.1% (w/v). The insulin released by each sample was measured as previously described using rat insulin as the standard. The glucose concentration producing a response that was 50% of the maximum (EC₅₀) was calculated as the mean negative logarithm (pD2). In a second series of experiments, the insulin secretion was measured from islets incubated in medium containing the following secretagogues (in mM): glucose 8.3, leucine 20, arginine 20, glucose 8.3 plus leucine 20, and glucose 8.3 plus arginine 20.

**Uptake of ⁴⁵Ca by isolated islets.** Groups of 150-200 islets, derived from the same batch of islets, were preincubated for 30 min at 37 °C, in a medium containing 5.6 mM glucose, pH 7.4.
The incubation medium contained (in mM): NaCl 115, KCl 5, CaCl₂ 1, MgCl₂ 1, buffered with 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-sodium hydroxide) and bubbled with pure O₂. The islets were then incubated for 5 or 90 min in 100 µl of the same medium containing ⁴⁵CaCl₂ (60 µCi/ml) and increasing concentrations of glucose (2.8-16.7 mM). At the end of incubation, 900 µl of ice-cold medium that containing 2 mM LaCl₃ (pH 7.4) were added to stop the reaction. After 60 min, the medium was removed and an aliquot was saved to calculate the amount of ⁴⁵Ca in the solution. The islets were subsequently washed three times with fresh, ice-cold La²⁺-containing medium. The islets were then placed in a petri dish and transferred (groups of eight islets) to counting vials containing 0.5 ml of EGTA (0.5 mM). The uptake of ⁴⁵Ca was expressed as pM Ca²⁺/islet per min.

**Islet perfusion.** Groups of 20 islets from LP or NP animals were placed on a Millipore SW 1300 filter (8 µm pore) in a perfusion chamber (4 chambers for each perfusion), with equal representation between the groups during each perfusion. The perfusion buffer consisted of Krebs-bicarbonate (see above) that was continuously gassed with a mixture of 95% O₂ / 5% CO₂. The islets were perfused at a flow rate of 1 ml/min with buffer containing 2.8 mM glucose during the first hour in order to equilibrate the system. After this period, samples were collected for 20 min. The glucose concentration was then increased to 22.2 mM and the islets were perfused for a further 40 min. Perifusate samples were collected at 2 min intervals for the determination of insulin levels as previously described.¹⁸

**Statistical analysis.** The results are presented as the mean ± SEM for the number of rats or islets (n) indicated. When comparing the NP and LP groups, a non-paired t-test was used.
When comparing the uptake of $^{45}$Ca, Lavene's test for the homogeneity of variance was initially used to check the fit of the data to the assumptions for parametric analysis of variance. These data were log-transformed to correct for variance heterogeneity or non-normality$^{19}$ and then analyzed by two-way analysis of variance (ANOVA), followed by the Tukey-Kramer test for individual differences between groups, among glucose concentrations, and to verify the interactions between these factors.

**Materials.** P Collagenase was from Boehringer Mannheim (Indianapolis, IN, USA). Antiserum against insulin was kindly provided by Dr. Leclercq-Meyer (Faculty of Medicine, Brussels Free University). Standard insulin was from Novo-Nordisk (Copenhagen). Activated charcoal was from Pfannstiehl Laboratories Inc. (Waukegan, IL, USA). Dextran T70 was from Pharmacia (Uppsala, Sweden). Arginine and leucine were from Fluka Biochemik (Switzerland). $^{45}$CaCl$_2$ and $^{125}$I-insulin were from New England Nuclear Co. (Boston, MA, USA). Scintillation fluid was from Merck (Darmstadt, FRG). Bovine serum albumin (fraction V), EGTA and the other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

**RESULTS**

At birth and at the end of the suckling period, the body weight in the LP group was significantly lower than the NP group (5.0±0.01 g and 20.4±0.1 g, n=26; 5.8±0.02 g and 61.3±0.3 g, n=26, respectively, p<0.001).

The insulin secretion by islets from the NP and LP groups over a glucose range of 2.8-27.7 mM is shown in Figure 1. In both groups, the relationship between glucose concentration
Figure 1. Glucose-stimulated release of insulin by islets from the offspring of mothers fed a normal (NP group) or low-protein (LP group) diet during pregnancy and lactation. The islets were incubated for 60 min with different concentrations of glucose. Values are the mean ± SEM of four independent experiments expressed as a percent of the maximal insulin secretion in the same experiment. The half-maximal response was obtained with 13.3 mM and 16.9 mM glucose for NP and LP islets, respectively.
and insulin secretion was sigmoidal. However, in the LP group the dose-response curve was shifted to the right compared to the NP group. The basal insulin release (2.8 mM glucose) was significantly lower in islets from the LP group compared to the NP group (0.3±0.03, n=16, and 0.4±0.1 ng/islet per h, n=13, respectively, p<0.05). Maximal insulin release, obtained at 27.7 mM glucose was 1.7±0.3 ng/islet per h (n=12) and 7.7±0.9 ng/islet per h (n=23), in the LP and NP groups, respectively (p<0.0001). The half-maximal release values were 13.3 mM and 16.9 mM glucose for the NP and LP groups, respectively (p<0.0001).

As shown in Figure 2, the insulin secretion in response to glucose, individual amino-acids, and a combination of amino-acids and glucose was significantly lower in LP islets than in NP islets. In the NP group, insulin secretion in the presence of 8.3 mM glucose was 3-fold greater than in the LP group (0.9±0.2 ng/islet per h, n=6, and 0.3±0.1 ng/islet per h, n=6, respectively, p<0.05). Leucine stimulated insulin secretion by NP islets more potently than did arginine (2.3±0.4 ng/islet per h, n=8, and 1.3±0.1 ng/islet per h, n=8, respectively, p<0.001), whereas in LP islets the insulin secretion in response to these amino acids was similar (0.3±0.1 ng/islet per h, n=8, and 0.2±0.03 ng/islet per h, n=8, respectively). Insulin secretion evoked by a combination of 8.3 mM glucose and 20 mM leucine was significantly higher in the NP group (4.9 and 3.2 times higher, for the NP and LP groups, respectively, p<0.0001). Similar results were obtained for the association glucose with arginine (3.1 times and 5.4 times in LP and NP islets, respectively, p<0.0001).

The \textsuperscript{45}Ca uptake by isolated islets after 5 min and 90 min incubation is shown in Figure 3. After 5 min incubation (Figure 3A) in a medium containing increasing concentrations of glucose, two-way ANOVA revealed a significant effect of group (df=1; F=254.8; p=0.00) and glucose concentration (df=1; F=123.7; p=0.00) as well as an interaction between groups by
Figure 2. Insulin release by islets in response to various stimuli. The mothers were fed a normal (NP group) or low-protein (LP group) diet during pregnancy and lactation. Gluc-8.3 mM, Leu-20.0 mM, Arg-20.0 mM. Gluc+Leu and Gluc+Arg same concentrations as for the individual stimuli. Values are the mean ± SEM of 6-8 experiments. * p<0.05 compared to NP islets. ** p<0.0001 compared to NP islets.
concentration (df=2; F=18.5; p=0.00). In basal conditions (2.8 mM glucose), the $^{45}$Ca uptake was higher in islets from NP offspring compared to the LP group (0.9±0.03 pM/islet per 5 min, n=24, and 0.5±0.03 pM/islet per 5 min, n=19, respectively, p<0.0001). In both groups, the incorporation of $^{45}$Ca in response to 8.3 mM glucose was higher when compared with the basal concentration (1.4±0.1 pM/islet per 5 min, n=19, and 0.9±0.1 pM/islet per 5 min, n=23, min in the NP and LP groups, respectively, p<0.0001). At 16.7 mM glucose, there was an additional increment in the $^{45}$Ca uptake (compared to 8.3 mM glucose) in islets from the NP group (2.0±0.1 pM/islet per 5 min, n=19, p<0.001), but not in those from the LP group (0.9±0.1 pM/islet per 5 min, n=32). After a 90 min incubation (Figure 3B), two-way ANOVA showed a significant effect of groups (df=1; F=6.0; p=0.015), glucose concentration (df=2; F=47.2; p=0.000) and an interaction between groups by glucose concentration (df=2; F=8.9; p=0.0002).

At 2.8 mM glucose, no difference in $^{45}$Ca uptake was observed between islets of the two groups (2.3±0.1 pM/islet per 90 min, n=26, and 2.4±0.2 pM/islet per 90 min, n=24, respectively). In the presence of 8.3 mM glucose, the $^{45}$Ca uptake by islets from the NP and LP groups was not different (3.3±0.3 pM/islet per 90 min, n=37, and 2.6±0.3 pM/islet per 90 min, n=16, respectively). In both groups, the $^{45}$Ca uptake increased only between 2.8 mM and 16.7 mM glucose (p<0.01 for the LP group, and p<0.0001 for the NP group). The $^{45}$Ca uptake at 16.7 mM glucose was significantly higher in NP islets compared to the LP group (p<0.0001).

The dynamics of insulin secretion by isolated islets is shown in Figure 4. At a low concentration of glucose (2.8 mM), the insulin secretion was similar in both groups. The mean secretion of the first twenty min of the perfusion period, was 6.6±0.5 pg/islet per min, n=4, and 7.9±0.7 pg/islet per min, n=4, for LP and NP islets, respectively). In NP islets, glucose (22.2 mM) elicited a typical biphasic insulin secretion with a rapid first-phase release reaching a peak
Figure 3. $^{45}$Ca uptake by islets from the offspring of mothers fed a normal (NP group) or low-protein (LP group) diet during pregnancy and lactation. The uptake was measured after 5 min (top) and 90 min (bottom) incubations in media containing different concentrations of glucose and $^{45}$CaCl$_2$ (60 µCi/ml). Note difference in the vertical scales. Values are the mean ± SEM of 16-26 (5 min) and 19-32 (90 min) experiments. The letters indicate significant differences between groups and conditions.
Figure 4. Kinetics of the glucose-induced insulin release by islets from the offspring of mothers fed a normal (NP group) or low-protein (LP group) diet during pregnancy and lactation. The glucose concentration from the start until the 20th min was 2.8 mM. Thereafter the concentration was raised to 22.2 mM until the end of the experiment. The inset shows the insulin secretion from LP islets. Each point represents the mean ± SEM of four experiments.
19-fold greater than basal (151.5±14.7 pg/islet per min) after 4 min of exposure to high glucose. This was followed by a nadir and a slow-rising second phase, which reached constant values 24 min after the introduction of 22.2 mM glucose to the perfusion medium. The pattern of insulin secretion by LP islets was different, with an immediate and small peak of insulin release attaining 11.4±6.5 pg/islet per min (2-fold increase over basal levels; p<0.01) that was maintained thereafter. Maximal insulin secretion was reached 18 min after the exposure to 22.2 mM glucose (13.1±3.3 pg/islet per min; n=4). Throughout exposure to high glucose concentration, the insulin secretion by LP islets (10.1±0.7 pg/islet per min; n=4) was significantly lower than for NP islets (64.5±6.5 pg/islet per min, n=4; p<0.0001).

DISCUSSION

Protein malnutrition during pregnancy is known to affect the structure of the endocrine pancreas and to impair insulin secretion by fetal islets stimulated in vitro with glucose, leucine and arginine. In the present study, the administration of a low protein diet to the mother was extended up at the end of the suckling period and resulted in pups with a reduced body weight at birth and at weaning. An elevated free fatty acid level, decreased serum insulin level in the fed state, and a poor in vivo insulin response to glucose were observed in this experimental model.

In accordance with in vivo data, islets from protein-deprived rats showed impaired insulin secretion in response to glucose, as well as an increased EC50 value, as previously described for adult rats submitted to protein malnutrition after weaning. Different, but not exclusive, explanations have been proposed for the reduced glucose-induced insulin release.
These include: 1) a smaller size and/or cell volume of β-cells;23 2) an inappropriate recognition of glucose as a stimulus due to glucoreceptor underexpression, and/or decreased metabolism of glucose (substrate site);24 3) an alteration in mitochondrial glucose oxidation due to impaired activity of the β-cell mitochondrial glycerophosphate dehydrogenase, possibly associated with other enzymatic anomalies;25 and 4) a diminished capacity of glucose to increase the Ca\(^{2+}\) uptake and/or to reduce Ca\(^{2+}\) efflux from β-cells.22

In our animal model, protein deficiency did not reduce the pancreatic insulin stores.21 This finding supports the idea that the decreased response to glucose and other stimuli is caused by a defect in the secretory mechanism. The impaired response to leucine and arginine, however, indicates that the restriction of secretion must involve some common step in the stimulus-secretion coupling. Leucine is metabolized independently of glycolysis before entering the oxidative cycle26 but is similar to glucose in its effect on K\(^+\) and Ca\(^{2+}\) permeability.12 Arginine exerts an insulinotropic action by its entry via the cationic amino acid transporter 2A 27, and by its accumulation inside β-cells, thereby provoking membrane depolarization and Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels.13 An increase in [Ca\(^{2+}\)], has been shown to play a crucial role in the regulation of insulin secretion by pancreatic β-cells in response to various stimuli, including nutrients.28 For these reason, we investigated the Ca\(^{2+}\) handling in response to glucose after 5 min and 90 min of incubation to examine the influx and retention of \(^{45}\)Ca, respectively. The concentration response curves for \(^{45}\)Ca in the presence of increasing concentrations of glucose paralleled those for insulin secretion. The \(^{45}\)Ca uptake curve for malnourished islets was shifted to the right in relation to that for normal islets. Islets from malnourished rats generally exhibited a low \(^{45}\)Ca influx at all glucose concentrations, and the
increase in \(^{45}\)Ca uptake over basal at 16.7 mM glucose was lower than in the NP group. These data confirmed previous reports\textsuperscript{22,29,30} which pointed to abnormalities in the Ca\(^{2+}\) fluxes and in [Ca\(^{2+}\)], as possible causes for a reduced insulin secretion in response to different nutrients in malnourished animals.

Since the ability of glucose to modulate K\(^{+}\) permeability in islets from malnourished rats was not affected,\textsuperscript{29} alterations of the K\(_{ATP}\) channel could be ruled out. The blockade of K\(_{ATP}\) channels, which provokes insulin secretion when Ca\(^{2+}\) is present, involves the metabolism of glucose and inhibition of fatty acid oxidation.\textsuperscript{31} We speculate, therefore, that the unresponsiveness and insensitivity of LP islets to glucose and amino acids reflects activation of the glucose-fatty acid cycle\textsuperscript{32} caused by the chronically elevated levels of serum fatty acids. A metabolic shift to fatty acid oxidation may accelerate the production of acetyl-CoA and result in inhibition of the glycolytic flux, and in a reduction of the cytosolic levels of long chain fatty acids. This may to the production or activation of second messengers (diacylglycerol) that stimulate enzymes (PKC isoforms, for example) and exocytosis.\textsuperscript{33}

The kinetics of insulin secretion exhibited by our were entirely consistent with this hypothesis. Normal islets responded to high glucose with a considerable increase in insulin secretion in a clear biphasic pattern. In malnourished islets, glucose evoked an immediate but smaller peak of insulin release that was maintained thereafter. Curiously, the kinetics of insulin release displayed by our malnourished animals were comparable to those exhibited by islets from adult rats subjected to starvation,\textsuperscript{34} and by suckling neonate islets from rats receiving a high fat diet.\textsuperscript{35} In the latter animals, alterations in the insulin release pattern were attributed to an inhibitory effect of fatty acid oxidation on glucose metabolism. The biphasic pattern of insulin secretion reflected the release of two different pools of granules and was associated with
the electrical activity of the membrane which requires changes in [Ca\(^{2+}\)], and second messengers. The first phase of secretion is produced predominantly by ion fluxes and corresponds to the release of granules located near the plasma membrane. The second phase is regulated by the cAMP and phosphoinositol pathways, and results from the mobilization of granules located in a reserve pool.\(^{35,37}\) Calcium has a role in regulating both phases through its direct effect on the exocytotic process and by modulating the cAMP and phosphoinositol pathways.\(^{36}\) Hence, the suppressed Ca\(^{2+}\) influx associated with an alteration in phosphoinositol metabolism following the activation of fatty acid oxidation would contribute to abnormal kinetics of insulin release.

In conclusion, our results indicate that in islets derived from malnourished rats, the poor secretory response to glucose and amino acids may occur through altered Ca\(^{2+}\) homeostasis. Abnormalities in Ca\(^{2+}\) handling may be a consequence of alterations in nutrients metabolism as a result to long-term exposure to fatty acids.

ACKNOWLEDGMENT

The authors wish to thank Mr. Lécio D. Teixeira and Mrs. Clarice Y. Sibuya for the technical assistance and Dr. S. Hyslop for revising the grammar. M.Q. L. is a fellow on leave from the Universidade Federal de Mato Grosso, Cuiabá, MT, Brasil.
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Protein Deficiency and Nutritional Recovery Modulate Insulin Secretion and the Early Steps of Insulin Action in Rats

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LATORRACA ET AL.: Insulin secretion and action in protein malnutrition
ABSTRACT: Epidemiological studies have shown an association between low weight birth and glucose intolerance in adult life. It has been suggested that maternal malnutrition affects early growth and determines permanent alterations in insulin secretion and sensitivity of offspring. Therefore, we investigated the insulin secretion from isolated islets and examined the early events related to insulin action in the hind-limb muscle of adult rats fed on a diet of 17% protein (control) or 6% protein (low protein) during fetal life, suckling and after weaning, and in rats receiving 6% protein during fetal life and suckling followed by a 17% protein diet after weaning (recovered). The basal and maximum insulin secretion by islets from low protein rats and the basal release by islets from recovered rats were significantly lower than for control islets. The dose-response curves to glucose of low protein and recovered islets were shifted to the right compared to control islets with the half-maximal response (EC_{50}) occurring at 16.9±1.3, 12.4±0.5 and 8.4±0.1 mmol/L, respectively. The levels of insulin receptor, as well as the insulin receptor substrate-1 phosphorylation and association between insulin receptor substrate-1 and phosphatidylinositol 3-kinase were greater in low protein-fed rats than in control rats. In the recovered rats, these parameters were not significantly different from those of the other two groups. These results suggest that glucose homeostasis is maintained in low protein and recovered rats by an increased sensitivity to insulin as a result of alterations in the early steps of the insulin signal transduction pathway.

KEY WORDS: insulin secretion, insulin receptor, insulin receptor substrate-1, nutritional recovery, rats
INTRODUCTION

Epidemiological studies have suggested that reduced growth in fetal and neonatal life are related to the development of glucose intolerance and diabetes in adult life (Hales et al. 1991, Poulsen et al. 1997). There is evidence that this association is, at least in part, a consequence of fetal and early post-natal malnutrition, possibly due to maternal deprivation. It is not clear whether the association between malnutrition during early growth and subsequent diabetes is mediated by alterations in insulin action, defects in insulin secretion or a combination of both factors. Hales and Barker (1992) have proposed that malnutrition in fetal and early infant life can result in impaired development of the pancreatic beta cells, leading to insulin deficiency in adult life. Interestingly, recent evidence suggests that the malnourished fetus undergoes metabolic adaptations from which it benefits in the short term by increasing fuel availability and that these adaptations persist throughout life, leading to insulin resistance (Phillips 1996).

Malnutrition in both humans and experimental animals is associated with reduced insulin secretion and disrupted glucose homeostasis (James and Coore 1970, Okitolonda et al. 1987). In rats fed on a low-protein diet for a limited period (4 weeks age) immediately after weaning, the glucose-stimulated secretion of insulin is impaired (Carneiro et al. 1995). In addition to a reduced insulin secretion an increased sensitivity to insulin represented by greater phosphorylation of the insulin receptor (IR)4, insulin receptor substrate-1 (IRS-1) and the association of IRS-1 with phosphatidylinositol 3-kinase (PI 3-kinase) has been observed in this same experimental model of malnutrition (Reis et al. 1997).
Fetal malnutrition in rat pups results in a reduced number of beta cells, a diminution in the proliferation of islets cells, reduced islet size and a marked decrease in islet vascularization (Snoeck et al. 1990). Following nutritional rehabilitation, the glucose tolerance test is still impaired in adult life (Dahri et al. 1991). However, the long-term consequences of intra-uterine and post-natal protein malnutrition on the early steps of the insulin signaling pathway are unknown.

In this study, we evaluated the secretion and action of insulin in adult rats made protein deficient during gestation and lactation and examined the influence of nutritional recovery on this response. We investigated the secretion of insulin by isolated pancreatic islets in response to glucose and the action of insulin by ability of insulin phosphorylate the IR and IRS-1 and to promote the association of the latter with PI 3-kinase in the muscle of protein deprived and recovered rats.

MATERIALS AND METHODS

Materials. P Collagenase was from Boehringer Mannhein (Indianapolis, IN). Antiserum against insulin was kindly provided by Dr Leclercq-Meyer (Faculty of Medicine, Brussels Free University, Belgium). Standard insulin was from Novo-Nordisk (Copenhagen, Denmark). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, bovine serum albumin (BSA, fraction V) and other chemicals were from Sigma Chemical Co. (St. Louis, MO). Antiseras against IR, PI 3-kinase (p85) and IRS-1 were from Santa Cruz (Santa Cruz,
CA). Monoclonal antiphosphotyrosine antibodies were from UBI (Lake Placid, NY). \[^{[125]I}\]Protein A was from Amersham (Amersham, UK) and protein A Sepharose 6MB was from Pharmacia (Uppsala, Sweden). Nitrocellulose membranes (BA85, 0.2 mm) were from Schleicher & Schuell (Keene, NH). Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly Co. (Indianapolis, IN).

Buffer A consisted of 100 mmol/L Tris, 10 g/L SDS, 50 mmol/L HEPES (pH 7.4), 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA and 10 mmol/L sodium vanadate. Buffer B was similar to buffer A except that 10 g/L Triton X-100 replaced 10 g/L SDS, and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg/mL aprotinin were added. Buffer C contained 100 mmol/L Tris, 10 mmol/L sodium vanadate, 10 mmol/L EDTA and 10 g/L Triton X-100.

**Animals.** All of the animal experiments were approved by the State University of Campinas Ethical Committee. Virgin female Wistar rats (85-90 d old) were obtained from the University’s own breeding colony. Mating was performed by housing males with females overnight and pregnancy was confirmed by the examination of 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 isocaloric diets containing 6% protein (low-protein diet) or 17% protein (control diet) as described previously (Reis et al. 1997). Three groups of adult male rats (90 d old) were used in this study: 1) a control group (C) consisting of rats born to and suckled by mothers fed a control diet, and subsequently fed a control diet after weaning, 2) a low-protein group (LP) consisting of the offspring of mothers fed a low-protein diet during both pregnancy and lactation and subsequently fed with the same diet after weaning, and 3) a
recovered group (R) consisting of the offspring of mothers fed a low-protein diet during pregnancy and nursing, but fed with a control diet after weaning. All of the offspring were weaned at the fourth week after birth. Throughout the experimental period, rats were given free access to food and water. They were kept under standard lighting conditions (12 h light/dark cycle), at a temperature of 24 °C. The rats were weighed at birth, and at 30 and 90 d thereafter. The food intake was recorded three times per week. At the end of the experimental period, one group of rats was killed by decapitation. Blood samples were collected, allowed to clot, and the sera stored at -20 °C for the subsequent measurement of insulin by RIA (Scott et al. 1981). The following determinations were performed immediately after decapitation: serum glucose (Trinder 1969), serum free fatty acids (Regouw et al. 1971), liver glycogen (Sjögren et al. 1938, Hassid and Abrahams 1957).

*Glucose-tolerance test.* An oral glucose tolerance test (OGTT) was performed when the rats of the three groups were 90 d old. After 15 h of food deprivation, glucose (200 g/L) was administered orogastrically through a gastric catheter at a dose of 2 g/kg body weight. Blood samples were obtained from the cut tip of the tail 0, 30, 60 and 120 min later for the determination of serum glucose and insulin concentrations (Trinder 1969, Scott et al. 1981). The glucose and insulin responses during the glucose tolerance test were calculated by estimation of the total area under the glucose (ΔG) and insulin (ΔI) curves, respectively using the trapezoidal method (Matthews et al. 1990).

*Insulin-tolerance test.* An intravenous insulin-tolerance test (ivITT) was performed with 90 d old rats, after 15 h of food deprivation. The ITT consisted of a bolus injection of regular
insulin (0.18 μmol/100 g body weight) into the dorsal penile vein. Blood samples were obtained from the cut tip of the tail 0, 5, 10, 15 and 30 min later for the measurement of glucose levels. From 5 to 30 min after the intravenous injection of insulin, the serum glucose concentration declined linearly, and the rate constant for serum glucose disappearance (Km) was calculated according to Lundbaek (1962).

*Islet isolation and insulin secretion.* The pancreas was removed from 90-d-old rats and digested with collagenase as previously described (Boschero et al. 1995). To measure insulin secretion, groups of five islets were first incubated for 30 min at 37 °C in Krebs-bicarbonate solution containing 5.6 mmol/L glucose equilibrated with a mixture of 95% O2-5% CO2, pH 7.4. The solution was then replaced with fresh buffer and the islets were further incubated for 60 min under following glucose concentrations (mmol/L): 2.8, 5.6, 8.3, 11.1, 16.7 and 27.7. The incubation medium contained 115 mmol/L NaCl, 5 mmol/L KCl, 24 mmol/L NaHCO3, 1 mmol/L CaCl2, 1 mmol/L MgCl2 and albumin (0.1%, w/v; BSA, fraction V). The insulin release was measured as previously described (Scott et al. 1981) using rat insulin as the standard. The glucose concentration producing a response that was 50% of the maximum (EC50) was calculated as the mean negative logarithm (pD2).

*Tissue extraction, immunoblotting and immunoprecipitation.* The rats were anesthetized with sodium amobarbital (15 mg/kg body weight) and used as soon as anesthesia was assured by loss of the pedal and corneal reflexes. The abdominal cavity was opened, the cava vein exposed and 0.5 mL of normal saline (9 g/L NaCl) with or without 10 μmol of insulin/L was injected. The insulin dose was chosen based on previous work in which the amount of insulin
required to achieve a significant signal was determined (Saad et al. 1995). The bolus injection of insulin leads to a transient rise in the peripheral concentration of this hormone to 5-10 times the postprandial levels at 90 s post-injection. The very high levels of insulin attained suggest that different levels of circulating insulin cannot explain some of the variations observed. Approximately 90 s after the insulin injection, the hind-limb muscle (Musculus gastrocnemius) was excised and immediately homogenized in freshly prepared boiling buffer A for immunoblotting, or freshly prepared ice-cold buffer B for immunoprecipitations. Insoluble material was removed by centrifugation for 45 min at 50,000 x g at 4 °C. The protein concentration in the supernatants was determined by the Bradford method (Bradford 1976).

For immunoprecipitation, samples containing 3 mg of total protein were incubated with 15 μL of anti-IR or anti-IRS-1 antiserum at 4 °C overnight, followed by the addition of Protein A Sepharose 6MB and mixing for 1 h. The pellets were repeatedly washed in buffer C (five times), resuspended in 50 μL of Laemmli sample buffer and boiled for 5 min prior to loading onto the gel. For immunoblotting, samples of 150 μg of total protein were suspended in 50 μL of Laemmli sample buffer and boiled for 5 min before loading onto 6% SDS-PAGE in a miniature slab gel apparatus. Electrotransfer, blotting and signal detection were performed as previously described (Saad et al. 1995).

**Statistical analysis.** The results are presented as the means ± SEM for the number of rats (n) indicated. When working with islets, n refers to the number of experiments performed (120 islets per group per experimental condition). Each experiment was performed with islets from two rats per group. When comparing the C and LP groups, non-paired t-tests were used to analyze the total food intake and total body weight gain during pregnancy, as well as the body
weight of newborn rats. When comparing the C, LP and R groups, Bartlett’s test for the homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric analysis of variance. Body weight of the weaned and adult rats, serum insulin in the fed state and liver glycogen data were log-transformed to correct for variance heterogeneity or non-normality (Sokal and Rohlf 1995). These data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey (for equal \( n \)) or Tukey-Kramer (for unequal \( n \)) test for individual differences between groups. \( P<0.05 \) was considered significant.

RESULTS

**Characteristics of the animals.** During pregnancy, the C and LP rats had a similar total food intake and total body weight gain. However, the body weight of newborn C rats was greater than that of LP rats (data not shown). At the beginning of the recovery phase, the R and LP rats had a similar body weights and in the both cases these were significantly lower than those of C rats. Although the R rats had a greater final body weight than the LP rats, at the end of experimental period, their weight was still significantly lower than that of the control rats. The total food intake during the experimental period was significantly different among the C, R and LP rats. When expressed per gram of body weight, the food intake was significantly greater in the LP rats compared to R rats, while the latter had a significantly higher food intake than C rats (17.0±0.95 g/(100 g·d), \( n=21 \), 10.4±0.54 g/(100 g·d), \( n=14 \) and 6.7±0.19 g/(100 g·d), \( n=17 \), respectively, \( P<0.05 \)). However, the feed efficiency, calculated as a ratio of grams of weight gain per gram of food intake over 60 d, was lower in LP rats compared to R rats, and the latter was similar to the C rats (0.10±0.01, 0.21±0.02, and 0.25±0.01, respectively,
Considering that the protein content is a major determinant of feed efficiency, the protein intake appeared to be limiting factor during the post-weaning growth. In addition to the impaired growth, the LP rats manifested other features typical of the protein malnutrition including hypoalbuminemia, high serum free fatty acids and greater liver glycogen content compared with the C and R groups. In the fed state, the serum glucose concentrations were similar in all three groups of rats. In contrast, the serum insulin concentration was lower in LP rats compared to R rats, and the latter was significantly lower than in C rats (Table 1).

OGTT and ITT. The basal serum glucose and insulin concentrations were significantly lower in LP rats when compared with C rats (P<0.01). In R rats, the basal insulin concentration did not differ from that of C rats and the serum glucose level was not different from the C or LP rats (Table 2). In LP and R rats, the mean total areas under the glucose curves (ΔG) in response to an oral glucose load were not significantly different from that in C rats. However, although the mean total areas under the insulin curves (ΔI) did not differ in the LP and R rats, these values were significantly lower than those of C rats (P<0.05). This finding indicates that glucose-induced insulin secretion in vivo was altered in the LP and R rats (Table 2). The glucose disappearance rate during an i.v. insulin tolerance test (K_{ir}) in the R rats was not significantly different from that in C rats. In contrast, in LP rats the K_{ir} was significantly greater (P<0.05) than in the other two groups, thus indicating an increased sensitivity to insulin (Table 2).

Glucose-induced insulin secretion. Basal insulin secretion in the presence of 2.8 mmol/L glucose was significantly lower in R and LP rats compared to the C rats (0.02±0.003
TABLE 1

Initial and final body weight, cumulative food intake, serum insulin, glucose, albumin, free fatty acids (FFA) levels and liver glycogen content of the control (C), recovered (R) and low-protein (LP) rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight</th>
<th>Food intake</th>
<th>Insulin</th>
<th>Glucose</th>
<th>Albumin</th>
<th>FFA</th>
<th>Liver glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>g</td>
<td>nmol/L</td>
<td>mmol/L</td>
<td>g/L</td>
<td>mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mg/100 mg</td>
</tr>
<tr>
<td>C</td>
<td>99 ± 5(^a)</td>
<td>389 ± 9(^a)</td>
<td>1196 ± 23(^a)</td>
<td>0.32 ± 0.03(^a)</td>
<td>7.3 ± 0.24</td>
<td>32 ± 2(^a)</td>
<td>77 ± 13(^b)</td>
</tr>
<tr>
<td>R</td>
<td>25 ± 2(^b)</td>
<td>236 ± 17(^b)</td>
<td>1021 ± 29(^b)</td>
<td>0.25 ± 0.02(^b)</td>
<td>7.3 ± 0.15</td>
<td>31 ± 3(^a)</td>
<td>90 ± 20(^b)</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td>(14)</td>
<td>(14)</td>
<td>(17)</td>
<td>(18)</td>
<td>(15)</td>
<td>(11)</td>
</tr>
<tr>
<td>LP</td>
<td>28 ± 1(^b)</td>
<td>95 ± 5(^c)</td>
<td>652 ± 28(^c)</td>
<td>0.15 ± 0.02(^c)</td>
<td>7.2 ± 0.21</td>
<td>19 ± 2(^b)</td>
<td>157 ± 17(^a)</td>
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<tr>
<td></td>
<td>(21)</td>
<td>(21)</td>
<td>(21)</td>
<td>(14)</td>
<td>(14)</td>
<td>(11)</td>
<td>(12)</td>
</tr>
</tbody>
</table>

\(^1\)Values of rats allowed free access to food.

\(^2\)Values are means ± SEM of the number of rats in parentheses. Mean in a column with different superscript letters were significantly different (\(P<0.05\)).
TABLE 2

Basal serum glucose and insulin concentration, total areas under the glucose (ΔG) and insulin (ΔI) curves obtained from the oral glucose tolerance test, and glucose disappearance rates (Km) in control (C), recovered (R) and low-protein (LP) rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Glucose(^1) mmol/L</th>
<th>Serum Insulin(^1) mmol/L</th>
<th>(\Delta G)^1 mmol/(L·120min)</th>
<th>(\Delta I)^1 mmol/(L·120min)</th>
<th>(K_m)^1 %/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.6 ± 0.6(^a)</td>
<td>0.25 ± 0.02(^a)</td>
<td>710 ± 35</td>
<td>55 ± 6.1(^a)</td>
<td>1.6 ± 0.28(^b)</td>
</tr>
<tr>
<td></td>
<td>(11)</td>
<td>(10)</td>
<td>(9)</td>
<td>(9)</td>
<td>(8)</td>
</tr>
<tr>
<td>R</td>
<td>4.3 ± 0.3(^ab)</td>
<td>0.25 ± 0.04(^a)</td>
<td>695 ± 35</td>
<td>30 ± 4.1(^b)</td>
<td>1.5 ± 0.34(^b)</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(10)</td>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td>LP</td>
<td>3.4 ± 0.3(^b)</td>
<td>0.17 ± 0.02(^b)</td>
<td>692 ± 52</td>
<td>28 ± 1.2(^b)</td>
<td>2.4 ± 0.16(^a)</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(9)</td>
<td>(9)</td>
<td>(7)</td>
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</tbody>
</table>

\(^1\)Values are means ± SEM of the number of rats in parentheses. Means in a column with different superscript letters were significantly different (\(P<0.05\)).
nmol/(islet-h), 0.03±0.005 nmol/(islet-h) and 0.1±0.009 nmol/(islet-h), respectively, \(P<0.001\). Maximal release was obtained at 27.7 mmol/L glucose in the three groups (0.61±0.09 nmol/(islet-h), 0.44±0.04 nmol/(islet-h) and 0.17±0.04 nmol/(islet-h) in C, R and LP groups, respectively). Except at the maximal dose tested (27.7 mmol/L glucose), the secretory rates were significantly different between the C and R rats \(P<0.05\). In LP rats, however, the insulin release was significantly lower than that of the C animals under all experimental conditions \(P<0.001\). In islets from all three groups of rats, the glucose-induced insulin secretion followed an S-shaped pattern, with the dose-response curve to increasing concentrations of glucose being shifted to the right in the LP and R islets (Figure 1). The half-maximal release concentration for C, R and LP islets was 8.4±0.1 mmol/L, 12.4±0.5 mmol/L and 16.9±1.3 mmol/L glucose, respectively \(P<0.001\).

**Early steps of insulin signaling in rat muscle.** Figure 2A illustrates the insulin-induced IR phosphorylation in rat muscle. Immunoprecipitation with anti-IR antibody and immunoblotting with anti-phosphotyrosine antibody, showed that there was no difference in the insulin-stimulated phosphorylation of the IR among the three groups. In contrast, the levels of IR protein detectable by immunoblotting were greater by 42.5±13.5\% \(P<0.05\) in LP rats compared to C rats, whereas in R rats the levels were not significantly different from those of the other two groups (Figure 3A).

Immunoprecipitates of IRS-1 showed 70±8.8\% greater insulin-induced phosphorylation compared with the C rats \(P<0.05\). Although R rats also showed an greater (35±18.3\%) insulin-stimulated IRS-1 phosphorylation compared to C rats, this level was not significantly different from the latter group or from LP rats (Figure 2B). The nutritional status of the rats
Figure 1. Comparative glucose dose-response curves for islets from control (C), recovered (R) and low-protein (LP) rats. Islets were incubated for 60 min with different concentrations of glucose. Values are mean ± SEM of four independent experiments were expressed as a percent of maximal insulin secretion within the same experiment. The half-maximal response was obtained with 8.4±0.1 mmol/L, 12.4±0.5 mmol/L and 16.9±1.3 mmol/L glucose, respectively for the C, R and LP rats. The data was analysed by one-way ANOVA followed by the Tukey test. Significant differences (P<0.05) were observed among the three groups.
had no effect on the muscle IRS-1 protein levels as determined by immunoblotting (Figure 3B).

To examine the association of the 85 kDa subunit of PI 3-kinase with IRS-1, the same blot was incubated with antibodies to this subunit. As expected, in the three groups of rats, an 85 kDa band was present in the IRS-1 immunoprecipitates after exposure to insulin. Greater insulin-stimulated IRS-1-p85/PI 3-kinase association was detected in the muscle of LP than of C rats (210±39.4% versus 100±23.9%, P<0.05). In R rats, the association between IRS-1 and PI 3-kinase was not significantly different from that seen in either the C or LP rats (Figure 4).

DISCUSSION

Protein malnutrition resulted in reduced serum insulin levels in food-deprived rats and rats allowed free access to food, and induced a very poor insulin response during the oral glucose tolerance test. These results agree with the reduced glucose-induced insulin secretion observed in isolated islets, and with previous reports of similar findings (Escriva et al. 1991, Okitolonda et al. 1987, Rasschaert et al. 1995, Reis et al. 1997). In recovered rats, the basal serum insulin level was similar to that of control rats. In contrast, in the fed state the serum insulin concentration in recovered rats was lower than in the control rats, but higher than in malnourished rats. During the oral glucose tolerance test, the insulin response to glucose was poor in recovered animals. In isolated islets, the insulin secretion in response to glucose was partially restored, indicating that nutritional recovery can only attenuate the damage to pancreatic beta cells produced by protein deprivation early in life.
Figure 2. Fluorographs obtained following SDS-PAGE of immunoprecipitates from hind-limb muscle from control (C), low protein (LP) and recovered (R) rats. The rats were injected with saline (not shown) or insulin and 90 s later hind-limb skeletal muscle was excised and homogenized in ice cold extraction buffer B as described in the Methods. After centrifugation, aliquots containing the same amount of protein were immunoprecipitated using anti-insulin receptor (A) or anti-IRS-1 (B) antibodies and then resolved by SDS-PAGE on 6% gels. The nitrocellulose transfers were blotted using anti-phosphotyrosine antibody in conjunction with $^{[125]}$I protein A, and then subjected to autoradiography. The graphic representations of means ± SEM of arbitrary scanning units of four experiments are depicted in the bottom. Different letters indicate significant differences ($P<0.05$) based on one-way ANOVA followed by the Tukey-Kramer test.
**Figure 3.** Fluorograph obtained following SDS-PAGE of total extracts of hind-limb muscle from control (C), low protein (LP) and recovered (R) rats. The rats were injected with saline (not shown) or insulin and 90 s later hind-limb skeletal muscle was excised and homogenized with extraction buffer A at 100 °C as described in the Methods. After centrifugation aliquots containing the same amount of protein were resolved by SDS-PAGE on 6% slab gels, transferred to nitrocellulose, and analysed using anti-insulin receptor (A) or anti-IRS-1 antibodies (B), in conjunction with $[^{125}I]$ protein A, and then subjected to autoradiography. The graphic representations of means ± SEM of arbitrary scanning units of four experiments are depicted in the bottom. Different letters indicate significant differences ($P<0.05$) based on one-way ANOVA followed by the Tukey-Kramer test.
**Figure 4.** Fluorographs obtained following SDS-PAGE of immunoprecipitates of hind-limb muscle from control (C), low protein (LP) and recovered (R) rats. The rats were injected with saline (not shown) or insulin and 90 s later hind-limb skeletal muscle was excised and homogenized in ice cold extraction buffer B as described in the Methods. After centrifugation, aliquots containing the same amount of protein were immunoprecipitated using anti-IRS-1 antibodies and then resolved by SDS-PAGE on 6% slab gels. The nitrocellulose transfers were blotted using anti-p85 (phosphatidylinositol 3-kinase) antibody in conjunction with [125I] protein A, and then subjected to autoradiography. The graphic representations of means ± SEM of arbitrary scanning units of four experiments are depicted in the bottom. Different letters indicate significant differences ($P<0.05$) based on one-way ANOVA followed by the Tukey-Kramer test.
Protein deprivation during pregnancy has been reported to cause the production of pups with reduced neonatal beta cell proliferation, islet size and islet vascularization (Snoeck et al. 1990). The maintenance of protein deprivation until adult age can lead to decreased insulin content of isolated pancreatic islet; no obvious alteration remained at adult age when protein deficiency was restricted to fetal life (Rasschaert et al. 1995). Thus, the reduced islet insulin content could explain the impairment of glucose-stimulated insulin release verified, at least in the low-protein group. However, the shift to the right in the glucose dose-response curves observed in the low protein and recovered groups suggests the involvement of one or more intrinsic defects in the mechanism of insulin secretion. Malnourished rats show reduced activities of the pancreatic glucokinase (Hales et al. 1996) and beta cell mitochondrial glycerophosphate dehydrogenase (Rasschaert et al. 1995), which could lead to impairment in glucose metabolism. The islets of the malnourished rats also have alterations in their Ca\textsuperscript{2+} handling (Carneiro et al. 1995). The possible detrimental effect of elevated free fatty acid levels (Zhou and Grill 1994) could be contributing to the defective glucose-induced insulin release observed in the low-protein group.

Protein deprived rats exhibited lower basal glucose levels but a similar total areas under the glucose curve to that observed in control rats. In addition, they showed high levels of hepatic glycogen, attributed to the inefficiency of glucagon (Okitolonda et al. 1987). This impaired glucagon action may reflect the low activity of some enzymes such as glucose-6-phosphatase and liver and muscle alanine amino transferase observed in a similar model of malnutrition (Heard et al. 1977). Apart from the enzymatic changes that suggest reduced endogenous glucose production (Desai et al. 1995), the maintenance of glucose tolerance in the face of drastically decreased serum insulin levels has been ascribed to a high hepatic and
peripheral sensitivity to insulin (Crace et al. 1990, Escriva et al. 1991, Okitolonda et al. 1987, Reis et al. 1997). This was confirmed in our study by the increased serum glucose disappearance rates ($K_{in}$) following the i.v. injection of insulin in malnourished rats.

Nutritional recovery reversed some of the metabolic alterations induced by protein malnutrition, as shown by the return to normal of albuminemia, free fatty acids and liver glycogen content. Desai et al. (1995) have shown that nutritional recovery does not fully restore the changes in liver enzyme activity (decreased glucokinase and increased phosphoenolpyruvate carboxykinase activities) produced by protein malnutrition in early life. Such enzymatic changes suggest that glucose production rather than utilization is predominant. Additionally, it has been shown in recovered rats that insulin has a delayed inhibitory effect on glucagon-stimulated hepatic glucose output (Ozanne et al. 1996a). Nevertheless, under food deprivation and fed conditions and during the oral glucose tolerance test, recovered rats succeeded in maintaining a low or near normal glycemia, despite low serum insulin levels. The maintenance of normoglycemia may result from either mechanisms that are independent of any dynamic insulin response (increased glucose effectiveness) or from mechanisms dependent on insulinemia (increased insulin sensitivity). The latter possibility was not confirmed by the $K_{in}$ value of our study.

Previous reports (Crace et al. 1990, Okitolonda et al. 1987) have suggested that nutritional recovery only partially reverses the high tissue sensitivity to insulin. We therefore investigated the changes in insulin sensitivity in both groups by evaluating the early molecular steps of insulin signaling in skeletal muscle, since this tissue is one of the main target of insulin action and comprises about 40% total body weight (Crace et al. 1990). The increased insulin sensitivity of liver and skeletal muscle of protein-deprived rats has been demonstrated (Crace
et al. 1990). This in vivo insulin sensitivity in peripheral tissue and liver has been confirmed using the euglycemic-hyperinsulinemic clamp technique (Escriva et al. 1991) and, more recently by the insulin signaling system (Reis et al. 1997). In contrast to a study that showed no significant effect on the levels of insulin receptor in muscle of malnourished rats (Reis et al. 1997), we found that protein deprivation up to 12th week of age significantly increased the insulin receptor levels in skeletal muscle. No change was observed in the IRS-1 protein levels nor in insulin receptor autophosphorylation in these animals. However, the phosphorylation of IRS-1 that reflects the kinase activity of the insulin receptor towards its endogenous substrate was increased, and was accompanied by an increase in the association of IRS-1 with the lipid metabolizing enzyme PI 3-kinase. Nutritional recovery partially restored the changes in the early steps of insulin action, as demonstrated by an intermediary increase in the insulin receptor level as well as in the phosphorylation of IRS-1 and its association with PI 3-kinase.

The increases in these three early steps of insulin action following hormone binding may have an important role in the increased insulin sensitivity observed in both groups. Insulin increases glucose uptake into cells, partially through the translocation of GLUT4 from intracellular compartments to the plasma membrane in muscle and adipose tissues (Stephens and Pilch 1995). Several studies have shown that PI 3-kinase is necessary for insulin-stimulated GLUT4 translocation (Okada et al. 1994, Sanches-Margalet et al. 1994). Hence, the pathway involving IRS-1/PI 3-kinase and the increased receptor level, may be linked to insulin sensitivity in the muscle of malnourished and recovered rats. This correlates with a previous study (Ozanne et al. 1996b) which showed in adult rats subject to the identical nutritional manipulation (protein restriction during pregnancy and lactation, and nutritional recovery after weaning) an increase in insulin receptor number on skeletal muscle as well as a higher GLUT4
translocation to the plasma membrane. The mechanisms whereby protein restriction induces these alterations are unknown but it is probable that at least in part the reduced insulin secretion observed in these animals may play a role. Hypoinsulinemia itself can induce alterations in the early steps of insulin action, as demonstrated by prolonged fasting in rats and by streptozotocin-induced diabetes rats (Saad et al. 1992). The persistently high tissue sensitivity to insulin verified in recovered animals may have resulted from an incomplete resturation of body weight. A lower body weight associated with decreased serum insulin levels apparently has the opposite, compensatory effect.

In conclusion, our results are consistent with the hypothesis that early protein deprivation produces pancreatic beta cell dysfunction, that is not completely restored by nutritional recovery. Furthermore, these data provide direct evidence that in low protein and recovered animals glucose homeostasis is maintained, at least in part, at the expense of increased insulin sensitivity. The initial steps in the insulin signal-transduction pathways contribute to the increase in insulin-stimulated glucose uptake in the skeletal muscle of protein-deprived and recovered rats. Further studies are necessary to elucidate the mechanisms involved in the secretory defect and the mechanisms by which malnutrition alters these three early steps of insulin action.

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LITERATURE CITED


TEXT FOOTNOTES

Footnotes to the title:

1 This work was supported, in part, by the Brazilian foundations: FAPESP, CAPES, CNPq and FINEP/PRONEX.

2 Márcia Q. Latorraca is a fellow on leave from Universidade Federal de Mato Grosso, Cuiabá, MT, Brasil.

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Footnotes to the text

4 Abbreviations used: BSA, bovine serum albumin; C, control group; EC50, glucose concentration producing half-maximal response; IR, insulin receptor; IRS-1, insulin receptor substrate-1; IV ITT, intravenous insulin-tolerance test; KIR, rate constant for serum glucose disappearance during insulin-tolerance test; LP, low protein group; OGGT, oral glucose tolerance test; PI 3-kinase, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonylfluoride; R, recovered group.
CONCLUSÕES

Os resultados obtidos mostram que:

- A restrição protéica durante a gravidez e a lactação comprometeu o crescimento da prole e produziu alterações bioquímicas típicas da desnutrição tais como hipoalbuminemia, hipoproteinemia, aumento da concentração de ácidos graxos livres e acúmulo de glicogênio no figado.

- Ratos desnutridos recém-desmamados apresentaram glicemia normal ou reduzida, apesar das baixas concentrações de insulina séricas. Esses ratos também exibiram um aumento da sensibilidade à insulina exógena.

- Ilhotas isoladas de ratos desnutridos recém-desmamados apresentaram secreção de insulina reduzida em resposta à glicose e a aminoaídos, e padrão secretório alterado.

- A incorporação de $^{45}$Ca frente à estimulação com glicose foi menor em ilhotas de ratos desnutridos do que ilhotas de ratos controles.

- Nos ratos recuperados, a ingestão de dieta normoprotéica corrigiu parcialmente o déficit de peso corporal e reverteu as alterações bioquímicas características da desnutrição.

- Ratos adultos recuperados e desnutridos apresentaram glicemia normal ou reduzida a despeito da hipoinsulinemia. A sensibilidade à insulina exógena foi normal em ratos recuperados e aumentada em ratos desnutridos quando comparados com os ratos controles.

- Em ilhotas isoladas a secreção de insulina em resposta à glicose foi diferente entre os grupos testados, sendo maior no grupo controle e menor no grupo desnutrido.
• A restrição protéica aumentou os níveis de receptor de insulina, a fosforilação do substrato-1 do receptor de insulina (IRS-1) e a associação do substrato-1 do receptor de insulina com fosfatidilinositol 3-quinase (PI 3-quinase) no tecido muscular. Nos ratos recuperados essas alterações foram parcialmente restauradas.

Esses resultados tomados em conjunto sugerem que a desnutrição durante a vida intrauterina e a lactação altera permanentemente a secreção de insulina. A resposta insulínica deficiente frente à estimulação com glicose e aminoácidos pode estar associada à incapacidade das células beta em “manejar” adequadamente os íons cálcio. Em ratos desnutridos e recuperados, a homeostasia glicêmica é mantida, pelo menos em parte, pelo aumento da sensibilidade à insulina, resultante das alterações nos passos iniciais da via de sinalização do referido hormônio.
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ABSTRACT

Epidemiological studies have suggested that fetal and infantile malnutrition are major factors determining susceptibility to type 2 diabetes and the insulin-resistance syndrome. It has been suggested that maternal malnutrition affect early growth and determine permanent alterations in insulin secretion and sensitivity of offspring. Therefore, we investigated the influence of protein deprivation during intrauterine and lactation phases on insulin secretion and action in weaned rats and in adult rats recovered from malnutrition. At weaning (28 days of age) glucose homeostasis was examined in offspring from dams fed a normal protein (17%) diet (NP group) or a diet containing 6% protein (LP group). The oral glucose tolerance test revealed less blood glucose and insulin concentration in the offspring from the LP group when compared to the NP group. The glucose disappearance rate (K_m) was higher in the LP group than in the NP group, indicating increased sensitivity to insulin in target tissues. Isolated islets derived from LP rats displayed reduced insulin secretion in response to glucose, arginine and leucine. In LP islets, the first and second phases of glucose-induced insulin secretion were drastically reduced. Finally, in LP islets the uptake of Ca after 5 or 90 min incubation in medium containing glucose (that reflects mainly the entry of Ca^2+, and the retention of Ca^2+, respectively) was lower than in NP islets. Insulin secretion and sensitivity were also evaluated in adult rats (90 days of age) maintained on a diet of 17% protein (C group) or 6% protein (LP group) during fetal life, suckling and after weaning, and in rats receiving 6% protein during fetal life and suckling followed by a 17% protein diet after weaning (R group). In LP and R rats, the mean total areas under the glucose curves in response to an oral glucose load were not significantly different from that in C rats. The mean total areas under the insulin curves were similar in the LP and R
rats, and these values were lower than those of C rats. The glucose disappearance rate during an i.v. insulin tolerance test ($K_{in}$) was greater in the LP group than in the C and R groups. In isolated islets glucose-induced insulin secretion was significantly different among the three groups, LP islets showing the highest values and C islets the lowest. When hind-limb muscles were analyzed, LP rats showed greater insulin receptor levels, higher insulin receptor substrate-1 phosphorylation and greater association between insulin receptor substrate-1 and phosphatidilinositol 3-kinase. In the R group, the changes in the early steps of insulin action were partially restored. In conclusion, these results suggest that malnutrition during early life produces pancreatic beta cell dysfunction, that is not completely restored by nutritional recovery. The poor secretory response to glucose and amino acids may occur through altered Ca^{2+} handling. Furthermore, in LP and R rats glucose homeostasis is maintained, at least in part, at the expense of increased insulin sensitivity that results from alterations in the early steps of the insulin signal transduction pathway.