UNIVERSIDADE FEDERAL DE MATO GROSSO
FACULDADE DE NUTRIÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS

Consequências tardias da restrição protéica na vida intra-uterina e na lactação sobre o mecanismo de acoplamento estímulo-secreção de insulina em ilhotas isoladas de ratas prenhes

Letícia Martins Ignácio de Souza

Cuiabá-MT,
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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biociências da Universidade Federal de Mato Grosso para a obtenção do título de Mestre.
Área de Concentração: Nutrição
Linha de Pesquisa: Metabolismo e Transdução de Sinais

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Cuiabá-MT,
Fevereiro/2010

Obrigada Senhor.
"Por todas as vezes que me conduziu para os lugares em que eu deveria estar,
Por todas as vezes que me apresentou às pessoas com as quais eu deveria conviver,
E por todas as vezes que me aproximou das lições que deveria ter por meio de oportunidades imperdíveis. "

A minha mãe e colega de profissão, Denise Souza. Por ter compartilhado comigo sua jornada universitária e me apresentado, tão cedo, à pesquisa básica em Nutrição.

A professora Márcia Latorraca pelo exemplo de dedicação, profissionalismo e pela oportunidade precoce que tornou tudo isso possível. Pela sabedoria, discernimento e bom senso com que conduz seus alunos.

Dedico

AGRADECIMENTOS

Ao meu pai *Célio*, pela simplicidade e integridade ímpares e que junto à minha mãe, são exemplos diários de dedicação e esforço. Aos dois, pelo amor e cuidado em todos os momentos da minha vida, pelo incentivo acima de tudo. Por me mostrarem todos os dias o quanto sou abençoada. Pela educação, que é o maior legado que poderiam ter me deixado. Pelo exemplo do que é justo.

As minhas irmãs *Celina e Camila, meus tios, avós, primos e afilhado*, pelo amor incondicional. Um dia vocês entenderão a minha ausência e os caminhos distantes, mas necessários, em busca desses trabalhos.

A família *Mendonça*, por representarem um segundo lar. Especialmente à minha grande amiga *Carolina Mendonça* pelos muitos anos de amizade incondicional. Por participar de toda e cada etapa da minha vida. Por conhecer o que há de pior e me mostrar, todos os dias, o que há de melhor em mim.

A Professora *Márcia Latorraca*, pela orientação e dedicação materna, por ter confiado em mim desde o princípio e pelo exemplo diário de sabedoria e generosidade. Obrigada pelas inúmeras horas, mesmo as de descanso, cedidas gentilmente ao meu aprendizado e crescimento pessoal e profissional. Sem o seu exemplo, talvez o caminho fosse diferente e isso me ajuda a crescer.

A professora *Marise Reis*, que co-orientou todas as etapas desse trabalho e do meu crescimento. A professora *Vanessa Arantes* que me acolheu e sempre ofereceu horas de atenção sincera e clara. Vocês que me programaram “desde a vida pós-natal”! Por terem acompanhado de perto toda minha caminhada, pelas horas de bancada dedicadas ao meu aprendizado, pelo sorriso sincero e pelas broncas saudáveis. Por me tornarem uma *Lelê* e por tudo que isto significa para mim. Obrigada!

Ao Professor *Roberto Veloso* que me acompanha desde a vida escolar e a professora *Maria Salete*. Pela alegria contagiante, disposição para sanar qualquer dúvida e pela atenção acolhedora.

A vocês, agradeço, sobretudo, pela oportunidade de crescimento intelectual, profissional e pessoal, pelo exemplo que representam e pelos laços criados que são, sem dúvida, alguns dos melhores resultados dessa dissertação.

Ao Professor *Luiz Fabrizio Stoppiglia*, pela disponibilidade e pelo espírito sempre questionador.

Aos Professores do Núcleo de Estudos e Pesquisas em Nutrição e Metabolismo, em especial à *Maria Helena Gaíva* e *Nair Kawashita*, pela convivência agradável, carinho e acompanhamento desde a graduação.

Ao Professor *Carlos Henrique Fregadolli*, pelo auxílio nas análises estatísticas e presteza sempre que solicitado.

As nutricionistas *Graciane* e *Keyla*, e professoras *Nauale* e *Nilma*, pela paciência e acompanhamento de parte do meu estágio docência.

As minhas companheiras de qualquer hora *Hellen Barbosa* e *Lunara Campos*, pelas risadas com e sem motivos, por acreditarem na amizade e me mostrarem isso mesmo quando não estamos juntas. Por confiarem em mim mais do que eu mesma e pelo crescimento que isso me proporcionou.

A *Carolina Mendonça, Daniela Farias, Camila Soares, Ludmila Araújo, Talita Schimidt, Bárbara Moreira* e *João Luis Chainça*, pelo simples fato de saber que "quando há amizade qualquer reencontro retoma a relação e o diálogo no exato ponto em que foram interrompidos".

Por tudo que aprendi com vocês e por todas as cores que eu reconheci na amizade inestimável.

A *Sílvia Regina Reis*, pela amizade que é uma das grandes conquistas dos últimos seis anos e que supera gerações. Pelo trabalho competente e conjunto. Uma das melhores profissionais que eu conheço.

A *Nauale Fares, Maristela Milanski* e *Cristiana Salvatierra*, pelo tempo inesquecível que convivemos e que crescemos juntas no laboratório, mas principalmente pela amizade cultivada e mantida.

Aos amigos do Mestrado em Biociências, principalmente *Paulo, Adriene, Ana Amélia, Paula e Leonardo*, pelos dias de trabalho e aprendizado conjunto, pelas discussões enriquecedoras, pelas (muitas) risadas e pelo poder de transformarem o ambiente à nossa volta. Especialmente ao Paulo que me acompanha desde a graduação e que junto comigo fez os planos para os quais nos dedicaríamos no futuro, que hoje é presente.
A *Simone, Albina e Bianca* por terem tornado o último semestre desse trabalho mais leve, alegre e agradável, pelo auxílio nos experimentos e a sincera amizade cultivada.

Aos *amigos do LABA (Renata, Grazielle, Ilkilene, Jorge, Walter),* especialmente ao *Celso Afonso,* pela amizade agradável, cumplicidade, acolhida e paciência desde a época em que eu era apenas uma criança curiosa no laboratório.
As eternas *Lelezinhas (Bárbara, Rafaela, Gabriela Dalcin, Gabriela Damin, Hellen e Lunara)*, pela confiança que me fez buscar e aprender e, acima de tudo, pelo carinho e amizade com que crescemos durante os últimos quatro anos.
Aos alunos de iniciação científica do LABA (*Jaline, Faena, Thaís, André, Laila, Felipe, Marina e Carlos*), pelo trabalho alegre e conjunto. Especialmente as minhas companheiras *Talitta e Suzana*, pelo compromisso com o nosso projeto.

Ao *Rodrigo Moura*, pela parceria junto ao Laboratório de Nutrição, Exercício Físico e Metabolismo da UNESP - Rio Claro, pela prontidão e incentivo.

Aos melhores anfitriões e os mais lindinhos de Campinas: *Marciane Milanski e Hamilton Ferreira*, especialmente à Marciane pela acolhida, companhia, pelos cafés e cantorias diários, pela amizade sincera, por todo o incentivo e por suas várias formas de arrancar sorrisos.

Aos Professores *Antônio Carlos Boschero, Everardo Carneiro, Helena Oliveira e Lício Velloso*, por terem disponibilizado os seus laboratórios e me acolhido em seus grupos de pesquisa durante os seis meses do "*mestrado sandwich*" na UNICAMP.

Aos colegas do *Laboratório de Pâncreas Endócrino e Metabolismo e Laboratório de Sinalização Celular da UNICAMP*, pelos encontros diários, o riso fácil, a compreensão e acolhida durante o tempo em que estive em Campinas.

Aos Professores *Fabiano Ferreira e Marciane Milanski*, pela avaliação do trabalho e contribuições no exame de qualificação.

À Professora *Maria Alice Mello*, pela prontidão em aceitar participar da banca de defesa da dissertação e por ser um exemplo desde a minha iniciação científica.

As melhores supervisoras de trabalho (e de vida social) que alguém pode ter: *Ana Paula Arruda, Andressa Coope e Marciane Milanski*. Agradeço, sobretudo, pela confiança e por tudo o que eu pude aprender simplesmente por poder conviver com vocês.

A *Camila Oliveira*, por ter dividido comigo as gavetas, os armários, os cafés entre os experimentos no laboratório e todo conhecimento que ela pôde transmitir. Agradeço principalmente, pela amizade nascida durante a minha estada em Campinas.

A *Faculdade de Nutrição da Universidade Federal de Mato Grosso e todos os seus membros*, pela minha formação profissional e por todo apoio dedicado à minha carreira.

As *Indústrias Corn Products do Brasil Ltda*, pela doação de ingredientes empregados no preparo das dietas.

Aos *animais de experimentação*, pela vida doada inocentemente à pesquisa.

Ao *CNPq, FAPEMAT e CAPES*, pelo apoio financeiro para a realização deste estudo e concessão da bolsa que possibilitou dedicação integral às atividades do curso.

A *Todos* que de alguma forma contribuíram para a realização deste trabalho.

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LISTA DE ABREVIATURAS

CNP	Grupo controle não prenhe
CP	Grupo controle prenhe
LPNP	Grupo hipoprotéico não prenhe
LPP	Grupo hipoprotéico prenhe
RNP	Grupo recuperado não prenhe
RP	Grupo recuperado prenhe
GLUT2	Transportador de glicose tipo 2
Gck	Glicoquinase
Hxk	Hexoquinase
AC	Adenilato Ciclase
PKA	Proteína Quinase A
PKC	Proteína Quinase C
PLC	Fosfolipase C
IRS-1	Primeiro substrato do receptor de insulina
PI3	Inositol trifosfato
ATP	Trifosfato de adenosina
ADP	Difosfato de adenosina
AMPc	Adenosina monofosfato cíclica
IP_3	Inositol-1,4,5-trifosfato
DAG	Diacilglicerol
VDCCs	Canais de cálcio voltagem dependente
KATP	Canais de potássio sensíveis a ATP
NADH	Nicotinamida adenina di-nucleotídeo reduzido
ΔG	Área sob a curva de glicose

ΔI	Área sob a curva de insulina
K_{itt}	Constante de desaparecimento de glicose
HEPES	Ácido N-2-Hidroxietilpiperazina-N'-2'-Etanossulfônico
EC_{50}	Concentração efetiva que proporciona metade da resposta máxima
pD_2	Logarítmo negativo
kDa	Quilodalton
DTT	Ditiotreitol
IBMX	Isobutilmetilxantina
PMA	Forbol-12-miristato-13-acetato
μCi	Micro Curie
EIA	Ensaio Imunoenzimático
EDTA	Ácido etileno diamino tetracético
EGTA	Ácido etileno glicol tetracético
PMSF	Fluoreto de fenilmetil sulfonila
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
BSA	Soro de Albumina Bovina
Tris	Tri(hidroximetil)-aminometano
TTBS	Tampão salino tris-tween20
SD	Desvio padrão
ANOVA	Análise de variância
LSD	Diferença mínima significativa

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and RP). The bars are means ± SD. Mean values within a column with unlike superscript letters were statistically different (P <0.05, LSD test)

Figure 6. Adenylate cyclase 3 (A), protein kinase A alpha (B), phospholipase C beta 1(C) and protein kinase C alpha (D) concentrations detected by Western Blot in isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. § presents difference between nutritional status. Mean values within a column with unlike superscript letters were statistically different (P <0.05, LSD test)

Figure 7. Increment in glucose stimulated insulin secretion (2.8mmol/L or 8.3mmol/L glucose) by IBMX (A and B), and PMA (C and D) in isolated islets from non pregnant or pregnant rats submitted to control diet (CNP and CP), to low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. Mean values within a column with unlike superscript letters were statistically different (P <0.05, LSD test)

RESUMO

A restrição protéica em fases críticas do desenvolvimento prejudica a secreção de insulina na vida adulta e a prenhez é uma condição que requer um grande aumento na secreção de insulina em resposta a normoglicemia. Para atender a esse aumento de demanda por insulina, as ilhotas pancreáticas sofrem modificações adaptativas funcionais. O dano permanente no processo secretório resultante da má nutrição pode prejudicar essas mudanças adaptativas na célula β durante a prenhez e contribuir para o aparecimento do diabetes. Assim, avaliou-se a homeostase glicêmica e alguns passos do processo de acoplamento estímulo-secreção de insulina em ilhotas isoladas de ratas prenhes submetidas à restrição protéica em fases críticas do desenvolvimento. Ratas adultas, prenhes e não prenhes submetidas à dieta normoprotéica (17% de proteína) ou hipoprotéica (6% de proteína) desde a vida fetal até a vida adulta, ou submetidas à restrição protéica na vida fetal e neonatal e recuperadas após o desmame foram avaliadas. Durante a prenhez o ganho de peso corporal e as concentrações séricas de albumina não foram prejudicados em ratas recuperadas, mas foram reduzidos em ratas submetidas à prolongada restrição protéica. A prenhez não produziu a típica resistência à insulina nem intolerância a glicose ou diabetes gestacional em ratas recuperadas. Ratas prenhes submetidas à prolongada restrição protéica exibiram déficit no conteúdo total de insulina nas ilhotas e esse déficit foi parcialmente revertido em ilhotas de ratas prenhes recuperadas. A curva dose-resposta de secreção de insulina estimulada por glicose foi deslocada para a esquerda em resposta à prenhez, exceto no grupo recuperado. Ilhotas de ratas recuperadas prenhes exibiram conteúdo de GLUT2 e taxa de captação de glicose similar em relação às ilhotas de ratas controles prenhes. O conteúdo de glicoquinase e hexoquinase não foi alterado em todos os grupos prenhes, entretanto em ilhotas de ratas recuperadas e desnutridas as taxas de utilização de glicose em concentrações estimulatórias de glicose foram reduzidas comparadas com aquelas exibidas por ilhotas de ratas controles. A produção de $^{14}CO_2$ foi similar em todos os grupos prenhes. O perfil de geração de AMPc em ilhotas de ratas recuperadas e desnutridas prenhes indicaram aumento da atividade de AC3. A prenhez não aumentou o conteúdo de PKAα, mas sua atividade foi reduzida em ilhotas de ratas desnutridas e recuperadas em concentrações basais de glicose, a julgar pelo grau de potencialização da secreção de insulina na presença de isobutilmetilxantina. O conteúdo de PLCβ1 foi

similar em todas as ilhotas de ratas prenhes e a expressão de PKCα foi reduzida em ilhotas de ratas recuperadas prenhes. Forbol 12-miristato 13-acetato foi menos eficiente em potencializar a secreção de insulina em ilhotas desse grupo de ratas. Assim, a má nutrição em fases críticas do desenvolvimento prejudicou as mudanças adaptativas clássicas durante a prenhez, aparentemente devido a um desacoplamento entre metabolismo de glicose e sinais amplificadores do processo secretório bem como por uma severa diminuição na via PLC/PKC. As alterações observadas em ilhotas de ratas desnutridas parecem ter resultado mais de reduzida massa e/ou biosíntese de insulina do que de prejuízos no processo de acoplamento estímulo secreção.

Palavras-chave: restrição protéica, recuperação nutricional, prenhez, homeostase glicêmica, função da célula β.

1.0 INTRODUÇÃO

Em períodos críticos do desenvolvimento, o organismo é capaz de responder a situações ambientais que são estranhas ao seu desenvolvimento normal, através de adaptações moleculares, celulares e bioquímicas. Adaptações precoces a um estímulo, como deficiências nutricionais, produzem modificações permanentes na fisiologia e no metabolismo. Assim, mesmo na ausência do estímulo que as iniciam, o organismo continua a expressar essas adaptações, e esse processo é denominado "programação metabólica" (Lucas 1991).

Vinte anos antes, o conceito de "programação metabólica" foi discutido e recebeu a denominação de "diferenciação metabólica" (Greengard 1969). Segundo Greengard (1969), a "diferenciação metabólica" resultaria de alterações não só enzimáticas, mas também de fatores de transcrição, hormônios, receptores de hormônios, transportadores transmembrana e outros elementos. Nesse processo, as células, individualmente, desenvolveriam uma quantidade estável e padrão de expressão gênica basal e induzível. Entretanto, a capacidade adaptativa de "diferenciação metabólica" frente a alterações na fisiologia e no ambiente de desenvolvimento seria tão importante quanto a programação de expressão gênica basal.

A natureza integrada dos organismos superiores dificulta estabelecer se um efeito específico representa uma programação primária ou secundária. A regulação de muitas variáveis fisiológicas resulta em uma homeostase adaptativa coordenada e mútua, em diferentes órgãos (Latini et al. 2009).

Estudos epidemiológicos e experimentais têm confirmado a hipótese da "programação metabólica" em resposta a deficiências nutricionais durante períodos críticos do desenvolvimento com conseqüências na vida adulta. É amplamente conhecida a associação entre crescimento fetal e neonatal reduzido e a presença, na vida adulta, de doenças crônicas.

Hales e Barker na década de 1990 postularam a hipótese de que o baixo peso ao nascer e no primeiro ano de vida, resultante da deficiência nutricional materna, está associado à maior risco de desenvolvimento do diabetes tipo 2 e da síndrome metabólica na vida adulta – "hipótese do fenótipo econômico" (Hales & Barker 1992;

Hales et al. 1996; Barker et al. 1995) - e alguns autores têm proposto que essa relação se deve à adaptações morfológicas e funcionais da célula β pancreática (Wills et al. 1996).

Em roedores, a má nutrição em fases críticas do desenvolvimento causa déficit permanente no crescimento somático (McCance 1962), malformações nos núcleos hipotalâmicos envolvidos no controle do balanço energético (Bennis-Taleb et al. 1999), alterações morfológicas e funcionais do pâncreas endócrino (especialmente das células beta) (Desai et al. 1996; Latorraca et al. 1998) e dos sítios de ação da insulina (fígado, tecidos muscular e adiposo) (Desai et al. 1995; Desai et al. 1997; Latorraca et al. 1998).

Existem algumas situações normais do desenvolvimento fisiológico que aumentam a demanda metabólica e exigem adaptações nos sistemas para responder a esse estímulo, como por exemplo, a gestação. O terceiro trimestre é caracterizado por aparecimento de discreta resistência à insulina para que ocorra disponibilização de suprimento nutricional para o feto (Leturque et al. 1987). Os mecanismos celulares que envolvem os hormônios gestacionais e a resistência à insulina foram primeiramente investigados por Ryan e Enns (1988) que mostraram que progesterona, lactogênio placentário e prolactina em concentrações similares àquelas encontradas no soro de mulheres grávidas são capazes de induzir resistência à insulina em cultura de adipócitos. Nessas células a ligação da insulina ao seu receptor e o transporte de glicose estão prejudicados. No fígado ocorre redução da fosforilação em tirosina do receptor de insulina (Marínez et al. 1989) e no músculo esquelético, soma-se o mesmo efeito no IRS-1 e inibição da ativação da PI3-kinase (Sivan & Boden 2003).

No curso da gravidez normal, a homeostase glicêmica é mantida à custa do aumento gradual da secreção de insulina pelas ilhotas pancreáticas (Costrini & Kalkhoff 1971), devido a adaptações em vários passos do processo de acoplamento estímulo-secreção de insulina.

Assim, o presente estudo relata os efeitos da restrição protéica em fases críticas do desenvolvimento (vida intra-uterina e lactação) sobre o mecanismo de acoplamento estímulo-secreção de insulina durante a prenhez.

2.0 REVISÃO DE LITERATURA

2.1 Mecanismos de acoplamento estímulo-secreção de insulina induzido por glicose

A concentração da glicose no plasma deve ser mantida numa estreita faixa durante todo o dia (4,0 a 9,0mM) apesar de amplas oscilações na produção e remoção da circulação e das mudanças episódicas de ingestão de nutrientes e jejum. A manutenção da glicemia constante dentro desta estreita faixa de variação é chamada homeostase glicêmica (Gerich 2000).

A manutenção da glicemia requer equilíbrio entre a taxa de produção e de utilização da glicose. Desse modo, a quantidade de glicose removida da circulação é determinada pela demanda dos tecidos, efeito da ação da concentração da glicose por si mesma e pelo número e característica dos transportadores de glicose em tecidos específicos. Insulina, glucagon e catecolaminas são os mais importantes hormônios glicorreguladores agudos (Gerich 1988, 2000).

A glicose é o agente estimulador mais importante da secreção de insulina, pelo menos nos mamíferos onívoros, porém o mecanismo preciso e os fatores que estão envolvidos no processo secretório não estão completamente entendidos (Rutter 2001).

Propõe-se que a secreção de insulina estimulada por glicose dependa de seu reconhecimento via transportador de glicose GLUT2 e conseqüente entrada na célula β. O passo seguinte é a fosforilação dessa hexose pela hexoquinase IV (glicoquinase), essencial para a entrada desse substrato na glicólise. Na via glicolítica, a glicose fosforilada será clivada e o produto final, piruvato, encaminhado à mitocôndria onde será utilizado pelo ciclo de Krebs e formação de ATP tanto pelas reações citosólicas quanto pela fosforilação oxidativa. A relação ATP/ADP leva ao bloqueio dos canais de K^+ ATP-sensíveis (Green et al. 1973) e esse evento promove acúmulo desse cátion com conseqüente despolarização da membrana e abertura dos canais de Ca^{2+} voltagem-sensíveis. O aumento do influxo de Ca^{2+} ativa a maquinaria exocitótica finalizando com a migração e extrusão dos grânulos de insulina para o interstício (Hadescov 1980; Wollhiem & Sharp 1981; Petersen & Findlay 1987; Grodsky 1989; Hiriart & Aguilar-Bryan 2008).

O total do número de grânulos de insulina presentes na célula β, excede o número secretado durante o estímulo logo após uma refeição, mas somente uma proporção relativamente pequena (~10%) desses grânulos está ancorada à membrana

celular em uma forma “pronta para a liberação” (Straub & Charp 2002). O canal de K^+ ATP sensível parece ser particularmente importante para gerar a exocitose deste pequeno número de “grânulos de liberação rápida” que são responsáveis pela primeira e aguda fase de secreção de insulina, que ocorre dentro dos 10 primeiros minutos após um estímulo de glicose (Aschoft et al. 1984; Cook & Hales 1984; Straub & Charp 2002). Entretanto, a amplificação dos sinais intracelulares que culminam na extrusão dos grânulos responde por mais de 70% do total de secreção de insulina estimulada por glicose (Henquin et al. 2000). A segunda e sustentada fase de secreção de insulina envolve então, tanto os grânulos ancorados para liberação rápida, quanto os recém recrutados do *pool* de estocagem intracelular.

Dos sinais amplificadores, a homeostase dos íons cálcio é um dos mais importantes. O acúmulo de cálcio intracelular estimula ainda as enzimas adenilato ciclase (AC) e fosfolipase C (PLC) que gerarão adenosina monofosfato cíclica (AMPc) e inositol-1,4,5-trifosfato (IP_3) e diacilglicerol (DAG) respectivamente (Zawalish & Zawalish 2001). O IP_3 mobiliza estoques intracelulares de Ca^{2+} e o AMPc e DAG amplificam o sinal deste íon facilitando seu influxo por ativação das proteínas quinases dependente de AMPc (PKA) (Dyachok et al. 2008) e dependente de cálcio (PKC), o que potencializa a secreção de insulina (Berridge et al. 2003, Tengholm & Gylfe 2009).

A PKA e a PKC parecem agir na maquinaria secretória das células β via mecanismos distintos. Estudos simultâneos avaliando a capacitância da membrana e a concentração de Ca^{2+} intracelular revelam que a ativação da PKA aumenta a secreção de insulina em célula β de camundongos e aumenta a corrente de Ca^{2+}, enquanto que PKC provoca aumentos similares na secreção, sem mudanças nos eventos elétricos (Yu et al. 2000).

A PKA modula a atividade de vários canais de cátions na membrana plasmática da célula β, conduzindo um aumento transitório no influxo de Ca^{2+} estimulado por glicose e elevação da concentração de Ca^{2+} livre no citosol (MacDonald et al. 2002). Em adição, o AMPc pode modular o Ca^{2+} citosólico através da estimulação da mobilização de estoques intracelulares por mecanismos dependentes ou independentes da PKA (Tsuboi et al. 2003). Uma vez que essas ações da via AMPc/PKA são conhecidas por ser glicose-dependentes, o AMPc surge como um modulador do sistema de sinalização da célula β pancreática, o qual em sinergismo com a glicose pode atuar

para regular a atividade dos canais iônicos e para sensibilizar a maquinaria exocitótica por Ca^{2+} (Thams et al. 2005).

Múltiplas isoformas de PKC estão presentes nas células β pancreáticas (Knutson & Hoenig 1994; Yedovitzky et al. 1997), sendo a isoforma PKCα sensível à Ca^{2+} e DAG mais dominante (Zawalich & Zawalich 2001; Carpenter et al. 2004). Embora o papel desta quinase na secreção de insulina estimulada por nutrientes seja controverso, na secreção de insulina estimulada por glicose, sua função está clara, uma vez que foi reportada sua translocação do citosol para a membrana plasmática em ilhotas pancreáticas de roedores estimuladas com glicose (Deeney et al. 1996). É possível que a PKC regule o recrutamento dos grânulos secretórios para a membrana plasmática suprindo a maquinaria exocitótica (Zhang et al. 2004).

2.2 Restrição protéica e alterações no mecanismo de acoplamento estímulo-secreção de insulina

Os efeitos da restrição protéica sobre o metabolismo de carboidratos têm sido extensivamente avaliados em estudos clínicos e experimentais.

É consenso que a restrição protéica causa redução da insulina basal e da secreção de insulina em resposta à glicose e a estímulos como aminoácidos e glucagon (Becker et al. 1971,1975; Brun et al. 1978; Carneiro et al. 1995; Milner 1971; Okitolonda et al. 1987; Smith et al. 1975). Quando imposta durante a vida intra-uterina e a lactação, produz: 1) número reduzido de células β e aumento das células α pancreáticas (Berney et al. 1997) ; 2) redução do tamanho, conteúdo de insulina, da densidade dos vasos sanguíneos das ilhotas pancreáticas (Snoek et al. 1990), bem como da densidade, massa absoluta e conteúdo de insulina das células β (Garofano et al. 1997).

Além das alterações morfológicas, esses animais exibem ilhotas menos sensíveis e responsivas à glicose, com deslocamento da curva dose-resposta para a direita, o que não é totalmente revertido pela recuperação nutricional após o desmame (Latorraca et al. 1998). Essas alterações na funcionalidade da célula β pancreática se devem a modificações dos processos de acoplamento estímulo-secreção de insulina por: 1) alterações no metabolismo da glicose na via glicolítica (Wilson & Hugnes 1997); 2) alteração da oxidação mitocondrial da glicose devido a prejuízo na atividade da glicerofosfato desidrogensase mitocondrial na célula β, possivelmente associadas a

outras anormalidades enzimáticas (Rasschaert et al. 1995); 3) diminuição da capacidade da glicose em aumentar a captação e/ou reduzir o efluxo de cálcio na célula β (Latorraca et al. 1999); 4) alteração nas vias do AMPc e PLC (Dahri et al. 1994). Em modelo de restrição protéica em fase de crescimento pós-desmame verificaram-se quantidades reduzidas de PKCα e PKAα em ilhotas pancreáticas (Ferreira et al. 2003, 2004).

Heywood et al (2004) relataram ainda uma diminuição na V*max* da Gck e Hxk e no conteúdo protéico de Gck em ilhotas de ratos jovens (3 semanas de vida até a idade adulta) submetidos à restrição protéica durante todo o período experimental e naqueles recuperados com dieta normoprotéica após o nascimento. Além disso, o pico de secreção foi minimizado no grupo desnutrido e o padrão pulsátil de secreção no grupo recuperado foi totalmente perdido.

2.3 Prenhez e alterações no mecanismo de acoplamento estímulo-secreção de insulina

No estado alimentado, a mais importante adaptação metabólica materna é o desenvolvimento da resistência à insulina. Essa resistência foi primeiramente demonstrada pela redução no grau de hipoglicemia produzido por injeções de insulina (Burt 1956) e por uma hiperinsulinemia em resposta a mudanças no ambiente de glicose (Spellacy & Goetz 1963). Mais tarde, novos e diferentes testes, como as análises computacionais de testes intravenosos de tolerância à glicose (Buchanan et al. 1990), os estudos de clamp euglicêmico-hiperinsulinêmico (Holness et al. 1996) e testes de tolerância à insulina (Saad et al. 1997), confirmaram e quantificaram essa resistência. A resistência à insulina diminui a utilização da glicose em até 50% (Freemark 2006) e de outros nutrientes pela mãe, com a finalidade de aumentar a disponibilidade desses substratos para o feto, garantindo seu crescimento.

Acredita-se que o aumento na concentração de hormônios circulantes como estrógenos, progesterona, prolactina e lactogênio placentário que atuam como antagonistas da insulina tenham uma importância no mecanismo de resistência a esse hormônio. Em roedores, a segunda metade da prenhez é caracterizada por um aumento dos níveis de lactogênio placentário e prolactina, bem como de outros hormônios como progesterona e 17β-estradiol (Parsons et al. 1992; Soares 2004). Também os níveis sanguíneos de corticosterona encontram-se aumentados dos 18 dias de gravidez até o parto (Rushakoff & Kalkhoff 1983) e poderia estar contribuindo para o quadro de resistência à insulina (Saad et al. 1997). Essa condição, associada à hiperfagia

(Nieuwenhuizen et al. 1999), contribui para o aumento da demanda de insulina na gravidez garantindo a manutenção da glicemia normal ou ligeiramente diminuída.

Para atender ao aumento da demanda de insulina, há um aumento da massa total das ilhotas pancreáticas, resultante da hiperplasia e hipertrofia das células β e α, mas não das células δ (Green et al. 1981; Hellman 1960; Van Assche 1974; Van Assche et al. 1980). Em roedores, o aumento da proliferação das células β é primeiramente observado ao redor do décimo dia de prenhez, com picos por volta do décimo quarto dia e retorno aos valores encontrados na fase pré-gestacional aos vinte e um dias (Sorenson & Brelje 1997; Kawai & Kishi 1999). Outras mudanças estruturais verificadas nessa fase incluem: aumento das "gap junction" entre as células β (Sheridan et al. 1988), aumento do número de receptores de prolactina (Moldrup et al. 1993; Sorenson et al. 1995), aumento do conteúdo de proteína (Weinhaus et al. 1996) e de insulina nas ilhotas (Marcoff et al. 1990).

Contudo essas alterações não são suficientes para explicar o mecanismo pelo qual as ilhotas pancreáticas exibam no curso da gravidez, aumento gradual da secreção de insulina estimulada por glicose (Costrini & Kalkhoff 1971). Parsons et al (1992) verificaram uma liberação total de insulina aumentada 4,0 vezes no décimo quinto dia associada a um limiar de estimulação pela glicose consideravelmente diminuído (3,25mM).

Além das modificações nos processos iniciais de acoplamento estímulo-secreção de insulina, outras alterações têm sido encontradas. Os achados sobre aumento do metabolismo do AMPc e da PLC encontram reforço nas observações que em ilhotas de ratas prenhe a elevação da secreção de insulina em resposta à glicose, está associada ao aumento da atividade da adenilato ciclase, e conseqüente elevação da concentração do AMPc (Green et al. 1978, Weinhaus et al. 1998), e de produtos da quebra de lipídeos de membrana pela PLC (Brelje et al. 1988).

Considerando que restrição protéica e prenhez levam a modificações antagônicas na morfologia e função da célula β pancreática é importante avaliar o efeito da restrição protéica sobre o acoplamento estímulo-secreção de insulina durante a prenhez.

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4.0 OBJETIVOS

4.1 Objetivo Geral

Avaliar o efeito tardio da restrição protéica na vida intra-uterina e na lactação sobre alguns passos do mecanismo de acoplamento estímulo-secreção de insulina em ratas prenhes.

4.2 Objetivos Específicos:

- ✓ Determinar o efeito tardio da restrição protéica nas vidas fetal e neonatal sobre o perfil nutricional de ratas prenhes;
- ✓ Avaliar a tolerância à glicose e a resposta insulinêmica *in vivo* em ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Avaliar a sensibilidade periférica à insulina em ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Verificar o peso corporal da prole de ratas submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Determinar o conteúdo total de insulina em ilhotas isoladas de ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Determinar a sensibilidade à glicose em ilhotas pancreáticas de ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Examinar o conteúdo protéico de GLUT2, Gck, Hxk, AC3, PKAα, PLCβ1 e PKCα em ilhotas pancreáticas de ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Avaliar o metabolismo de glicose em ilhotas isoladas de ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Quantificar as concentrações intracelulares de AMPc em ilhotas isoladas de ratas prenhes submetidas à restrição protéica nas vidas fetal e neonatal;
- ✓ Verificar o efeito da restrição protéica na vida intra-uterina e na lactação sobre a secreção de insulina em resposta a estimuladores das vias AMPc/PKA (IBMX) e PLC/PKC (PMA) durante a prenhez.

5.0 ARTIGO

Submedido em 18 de Fevereiro de 2010 para "American Journal of Physiology – Cell Physiology"

Manuscript #	C-00052-2010
Current Revision #	0
Submission Date	18th Feb 10 14:25:17
Current Stage	Manuscript Under Review
Title	Protein restriction in early life is associated with changes in insulin sensitivity and pancreatic β-cell function during pregnancy
Running Title	Low protein diet and β-cell function at pregnancy
Manuscript Type	Research Article
Special Section	N/A
Category	Cellular and Mitochondrial Metabolism
Corresponding Author	Dr. Márcia Latorraca (Universidade Federal de Mato Grosso)
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Protein restriction in early life is associated with changes in insulin sensitivity and pancreatic β-cell function during pregnancy

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ABSTRACT

Malnutrition in early life impairs glucose stimulated insulin secretion (GSIS) in adulthood. Conversely, pregnancy requires a very large increase in GSIS at normoglycemia. A failure in β-cell adaptative changes may contribute to onset diabetes. Thus, glucose homeostasis and β-cell function were evaluated in pregnant and non-pregnant normal nourished (CP; CNP) or protein-restricted (LPP; LPNP) rats from fetal to adult life or protein-restricted rats during early life and recovered after weaning (RP; RNP). Somatic parameters were impaired in LPP rats. Pregnancy did not produce the typical insulin resistance in RP rats and not augmented the insulin content/islet in LPP group. Dose response curve of GSIS in islets from pregnant rats was shifted to the left compared to non-pregnancy, except in the recovered group. GLUT2, glucokinase and hexokinase content and glucose uptake did not differ between RP and CP islets. However, in islets from RP and LPP rats, glucose utilization but not oxidation was reduced compared to islets from CP at 8.3mmol/L glucose. cAMP content and the degree of potentiation of the GSIS by isobutilmetilxantine at 2.8mmol/L glucose indicated augmented AC3 and PKAα activities in RP and LPP islets. PKCα but not PLCβ1 expression was reduced in RP islets. Phorbol-12-myristate-13-acetate potentiated less the GSIS in this group. Thus, while in LPP islets the failure appears to result from reduced islet mass and/or insulin biosynthesis, in the RP group occurred an uncoupling between glucose metabolism and the amplifying signals of secretory process as well as the severe attenuation of PLC/PKC pathway.

Keywords: Malnutrition, nutritional recovery, glucose homeostasis, insulin secretion, insulin resistance.

INTRODUCTION

The basic mechanism of insulin secretion involves coupling between glucose metabolism and secondary signals to maintain the insulin release in the course of raised blood glucose. First, glucose can balance rapidly across the β-cell membrane due to the expression of the high capacity and low affinity glucose transporter-2 (GLUT2) (25). After, glucose is phosphorylated to glucose-6-phospate by a high Km glucokinase (Gck, hexokinase IV), which is considered as a 'glucose sensor' in the pancreatic β-cells (22). This reaction is the main pathway for glycolysis (16, 25, 22) and the products are cytosolic pyruvate and NADH, that in the mitochondria are metabolized to generate ATP (26). ATP production and membrane conductance reduction by glucose stimulus leads to membrane depolarization and electrical activity initiation (5, 27, 5). Thus, ATP-sensitive channels (K_{ATP}) set the β-cell membrane potential and closure of these channels lead to membrane depolarization. Membrane depolarization triggers action potential firing and opening of voltage-dependent Ca^{2+} channels (VDCCs), leading to Ca^{2+} influx which triggers exocytosis of insulin granules (21). Thus, granule movement can be stimulated by glucose (28) or ATP (40) and other agents (14). The accumulation of intracellular calcium also activates several enzymes including adenylate cyclase (AC) and phospholipase C (PLC), leading to increase levels of cyclic adenosine monophosphate (cAMP), and inositol-1 ,4,5-triphosphate (IP3) and diacylglycerol (DAG), respectively (47). IP3 mobilizes intracellular Ca^{2+} ($[Ca^{2+}]_i$) mainly from endoplasmic reticulum, in another instance cAMP and DAG amplify the Ca^{2+}signal, facilitating its influx by activation of cAMP-dependent kinase (PKA) and calcium-dependent kinase (PKC) (1). The PKA and PKC are responsible to increase insulin release on a synergistic manner, because the PKA is involved on movement of insulin granules to periphery by altering the Ca^{2+} micro domains. PKC is responsible to the extrusion of insulin granules from beta cells but does not change electrical events (46).

In rodents, malnutrition in critical stages of development causes alterations in the stimulus-secretion coupling of insulin. It has been showed in pancreatic islets from rats fed a low protein diet during intrauterine life and/or lactation reduced Gck, Hexokinase (Hxk) V*max* and Gck protein content (13). Islets from these rats also exhibited decreased glycolytic pathway (44) and change the mitochondrial glucose oxidation due to reduced mitochondrial glycerophosphate desidrogensase activity (30). Finally, low

protein diet fed rats reduced ability of glucose to increase the Ca^{2+} uptake and/or to reduce Ca^{2+} efflux in pancreatic islets (19) and showed altered levels of cAMP and PLC pathways (4). These alterations resulted in permanent loss of glucose sensitivity and secretory capacity in pancreatic islets (18).

During pregnancy occur several, but reversible β-cell adaptations, in order to attend the typical increased demand for insulin. Such adaptations include enhanced β-cell proliferation, increase in insulin biosynthesis and secretion, and lowered threshold for glucose-stimulated insulin secretion (41, 43). Adaptations in the insulin secretion and in the stimulation threshold are mediated, at least in part, by changes in the activity/content of key enzymes of glucose metabolism (Glut2, Gck, Hxk) and modulators of the insulin secretory process (PLC/PKC, cAMP/PKA) (24, 43).

The permanent damage of the secretory process imprinted by the early protein restriction could impair the classical β-cell adaptative changes during pregnancy and contribute to onset of diabetes. Thus, in this work we evaluated the glucose homeostasis and investigated some steps of insulin stimulus-secretion coupling in isolated islets from pregnant rats submitted a low protein diet during intrauterine and postnatal period recovering after weaning.

METHODS AND MATERIALS

Animals and diets

The experimental procedures involving rats were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the Ethics Committee at the Federal University of Mato Grosso (protocol number 23108.002104/08-5). Male and virgin female Wistar rats (85-90 days old) were obtained from the University's own breeding colony. Paring was performed by housing males with females overnight (1 male to 4 female), 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% of protein (low protein or LP diet) or 17% of protein (control or C diet) protein. Spontaneous delivery took place at day 22 of pregnancy after which, at 3 days of age, large litters were reduced to eight pups to

ensure a standard litter size per mother. At weaning (4th wk after birth) female were divided into three groups: C, consisting of offspring born to and suckled by mothers fed a C diet and subsequently fed the same diet after weaning; LP, consisting of the offspring of mothers fed a LP diet and subsequently fed the same diet after weaning; and R, consisting of the offspring of mothers fed a LP diet, but fed a C diet after weaning. At 90 days old paring was performed and control nonpregnant (CNP) and pregnant (CP) groups, recovered non-pregnant (RNP) and pregnant (RP) were fed a C diet, whereas the low-protein non-pregnant (LPNP) and pregnant (LPP) groups were maintained a LP diet from day 1 to 19 or 1 to 15 day of pregnancy. The diets were isocaloric, as described by Milanski et al (24). During the experimental period, the rats had access to their respective diets and to water *ad libitum* and were housed at 22°C with a 12h light/dark cycle. One group of rats was maintained at 15 days of pregnancy for the islet study. The second group of rats was maintained at 19 days of pregnancy for evaluation of responses to glucose and insulin tolerance test. After insulin tolerance test, rats were anesthetized and sacrificed by decapitation and the body weight of offspring was recorded. The remaining rats were weighed, sacrificed and the blood collected to biochemical analysis.

Glucose-tolerance test

After 12h fast, glucose (200g/L) was administered intra peritoneal at a dose of 2g/kg of body weight. Blood samples were obtained from the cut tip of the tail 0, 30, 60 and 120 minutes later for the determination of serum glucose and insulin concentrations. The glucose and insulin responses during the glucose-tolerance test were calculated by estimating the total area under the glucose (ΔG) and insulin (ΔI) curves, using the trapezoidal method (23).

Insulin-tolerance test

After 12h fast, insulin (regular) was administered intra peritoneal at a dose of 1,5U/kg of body weight. Blood samples were obtained from the cut tip of the tail 0, 5, 10 and 15 minutes later for the determination of serum glucose concentrations. The glucose responses during the insulin-tolerance test was evaluated by the constant of disappearance of plasma glucose (K_{itt}) that was calculated from the slope of the fall in

log transformed plasma glucose between 0 and 15 minutes (20) after the insulin administration, when the glucose concentration declined linearly.

Serum parameters

Blood samples were collected and allowed to clot. Sera were stored at -20°C for the subsequent measurement of serum glucose by oxidase-peroxidase method (39) and serum albumin (8) concentrations by colorimetric method. Serum insulin level was determinate by RIA (33).

Total insulin content in islets

Twenty islets per group were sonicated in 1mL of distillated water, submitted to spin in a centrifuge and maintained at -20ºC to total insulin content determination by RIA (33).

Glucose stimulated insulin secretion (GSIS)

Islets were isolated by collagenase digestion of the pancreas, as described (2). Groups of five islets were first incubated for 45 minutes at 37°C in Krebs-bicarbonate buffer with the following composition (mmol/L): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES and 5.6 glucose, supplemented with 3g of bovine serum albumin/L and equilibrated with a mixture of 95% O_2:5% CO_2 to give a pH of 7.4. This medium was replaced with fresh buffer and then islets were further incubated for 1,5h with the glucose at 2.8 mmol/L, 5.6 mmol/L, 8.3 mmol/L, 11.1 mmol/L and 16.7 mmol/L. The glucose concentration producing a response that was 50% of the maximum (EC_{50}) was expressed as the mean negative logarithm (pD_2).

In the second series of experiments, the insulin secretion was measured in response to: (1) glucose (2.8 or 8.3 mmol/L) in the absence and presence of isobutilmetilxantine (IBMX 1 mmol/L; Sigma) and (2) glucose (2.8 or 8.3 mmol/L) in the absence or presence of phorbol 12-myristate 13-acetate (PMA; 100 nmol/L; Sigma). The insulin released was measured by radioimmunoassay using rat insulin as the standard (33).

Glucose metabolism

The rate of glucose metabolism was measured in islets freshly isolated by collagenase digestion.

2-Deoxy-D-glucose uptake

Groups of 20 islets were incubated in 100µL of buffered Krebs-bicarbonate solution supplemented with 3µCi of [^{14}C] 2-deoxy D-Glucose (Amersham Biosciences, USA) with enough unlabeled glucose (Sigma-Aldrich, USA) to achieve a final concentration of 2.8 or 8.3 mmol/L. Tubes were incubated in a shaking water bath at 37ºC and transport was stopped after 2h with 0.5 ml of TRIZOL reagent (Invitrogen Life Science, USA). The vials were added of 3 ml of scintillation fluid. The specific radioactivity was determined by liquid scintillation spectrometry. Uptake was expressed as pmol/islet/h.

D-Glucose utilization and oxidation

Glucose utilization and glucose oxidation were measured in batches of 20 islets in final incubation volumes of 100µl buffered Krebs-bicarbonate solution supplemented with trace amounts of both [5-^{3}H]glucose and D-[U-^{14}C] glucose (3µCi/mL) (Amersham Biosciences, USA) plus non-radioactive glucose at a final concentration of 2.8 mmol/L or 8.3 mmol/L. The wells were suspended in 20 ml scintillation vials that were gassed with 95% O_2 and 5% CO_2 and capped airtight with rubber membranes. The vials were then shaken continuously for 2h at 37°C in a water bath. After incubation, 0.1 mL of 0.2 N HCl and 0.2 mL of sodium hydroxide were injected through the rubber cap into the glass cup containing the incubation medium and into the counting vial, respectively. After 1h at 4°C, 10mL of scintillation fluid was added to the sodium hydroxide and the radioactivity was counted. To determine glucose utilization, cups were transferred to a fresh set of scintillation vials, each containing 500µL distilled H_2O. The vials containing the islets were then incubated for 18 h to allow the [^{3}H]water in the incubation solution to equilibrate with the water at the base of the vial before cups and islets were removed and 10 mL scintillation fluid were added to the distilled H_2O. The specific radioactivity was determined by liquid scintillation spectrometry. Rates of glucose utilization and glucose oxidation were expressed as pmol/islet/h.

Measurement of intracellular cAMP content

The cAMP content of islets was determined according to the manufacturer's instructions cAMP EIA system (Amersham cAMP Biotrack Enzymeimmunoassay – EIA – Sistem #RPN225. Amersham Life Sciences). Groups of 20 islets were incubated in Krebs

solution supplemented with 2.8 or 8.3mmol/L glucose in the presence of IBMX 250µmol/L. The content of cAMP was measured by enzyme immunoassay.

Western blotting

After isolation by collagenase digestion of pancreas and subsequent separation by hand-picking, a pool of at least 500 clean islets from each experimental group was homogenized by sonication (15s) in an anti-protease cocktail (10 mmol/L imidazole, pH 8.0, 4 mmol/L EDTA, 1 mmol/L EGTA, 0,5 g/L pepstatin A, 2 g/L aprotinin, 2.5 mg/L leupeptin, 30 mg/L trypsin inhibitor, 200 μmol/L DL-dithiothreitol and 200 μmol/L phenylmethylsulfonyl fluoride). After sonication, an aliquot of extract was collected and the total protein content was determined by the dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples containing 50 μg of protein from each experimental group were incubated for 5 minutes at 80°C with 4x concentrated Laemmli sample buffer (1 mmol sodium phosphate/L, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% mercaptoethanol) (4:1, v/v) and then run on 10% polyacrylamide gels at 120 V for 30 min. Electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was done for 1h at 120V (constant) in buffer containing methanol and SDS. After checking the efficiency of transfer by staining with Ponceau S, the membranes were blocked with 5% skimmed milk in TTBS (10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) overnight at 4°C. GLUT2, Gck, HxkI, AC3, PKAα, PKCα, PLCβ1 were detected in the membranes after 2-h incubation at room temperature with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:500 in TTBS containing 3% dry skimmed milk). The membranes were then incubated with a secondary specific immunoglobulin G antibody (diluted 1:5000 in TTBS containing 3% dry skimmed milk) for 2h at room temperature. Enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody was used for detection by autoradiography. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

Statistical analysis

The results are presented as the mean ± SD. When working with islets, *n* refers to the number of experiments performed. To evaluate the effect of PMA or IBMX, were considered values obtained from differences between insulin secretion in presence and

absence of each potentiator. Levene's test for the homogeneity of variances was initially used to check the fit of data to the assumptions for parametric analysis of variance. To correct for variance heterogeneity or non-normality, data were log-transformed (36). Except to offspring body weight that was analyzed by one-way ANOVA, all results were analyzed by 2-way ANOVA (nutritional status and physiological status). When necessary, these analyses were complemented by the LSD test to determine the significance of individual differences. The level of significance was set at $P < 0.05$. The data were analyzed using the Statistic Software package (Statsoft).

RESULTS

At the end of recovery period and before pregnancy, body weight from R groups was significantly lower than C groups, but was higher than in LP groups ($F_{2,29}$=106.5, P<0.0001). During pregnancy the body weight gain was higher in the pregnant groups in relation to non pregnant groups, independently of the nutritional status ($F_{1,29}$=202.4, P<0.0001). However, in pregnant rats the body weight gain was lower in LPP group than in CP group (P<0.05). In RP group body weight gain did not differ from that of CP and LPP groups. Thus, pregnant rats had final body weight higher than in non pregnant rats ($F_{1,29}$=49.2, P<0.0001), but RP rats exhibited higher final body weight than in LPP rats and lower than CP rats (P<0.05). The serum albumin level was lower in low protein groups than in recovered and control groups, independently of the physiological status ($F_{2,19}$=10.4, P<0.001) and was not observed interaction between nutritional status and physiological status (Table 1).

The basal serum glucose levels as well as the mean total areas under the ΔG curves in response to a glucose load were lower in the pregnant than in non pregnant groups ($F_{1,28}$=5.2, P<0.03 and $F_{1,28}$=39.2, P<0.0001, respectively), independently of the nutritional status. Pregnancy did not enhance the basal serum insulin concentration just in the recovered group, which had lower value than CP and LPP groups (P<0.05). In contrast, the mean total areas under the ΔI curves were significantly raised by pregnancy only in the low protein diet fed group. The ΔI curve from the RP group was lower than the CP group (P< 0.05) and similar to the LPP group. The ΔG:ΔI ratios were significantly affected by nutritional status, physiological status and by interaction between them ($F_{2,28}$=4.4, P<0.05, $F_{1,28}$=12.1, P<0.001, $F_{2,28}$=4.8, P<0.05, respectively).

Thus, ΔG:ΔI ratio from LPNP was higher in relation to others groups ($P<0.01$). Pregnancy reduced ΔG:ΔI ratio just in LPP group ($P<0.01$).

To measure the insulin sensitivity, we used the glucose disappearance rate through insulin tolerance test. Insulin sensitivity was higher in LPNP group than in other groups ($F_{2,18}=15.3$, $P<0.001$). Moreover, K_{itt} from RNP group was smaller than in CNP ($P<0.05$). Pregnancy induced insulin resistance in CP ($P<0.05$) and LPP ($P<0.001$) but not in the RP group that had K_{itt} similar to the RNP group (Table 2). Offspring for mothers fed with low protein diet during all life had lower body weight than other groups (LPP= 1.5±0.2g, RP= 4.0±0.4g, CP= 3.0±0.2g). Recovered rats had heavier offspring, 30% more than control group ($F_{2,30} = 187.20$, $P=0.000$).

LPNP and RNP islets exhibited similar total insulin content and significantly lower than in CNP islets ($P<0.01$ and $P<0.05$, respectively). Pregnancy increased the total insulin content only in control and recovered groups, but islets from RP showed lower total insulin content than in islets from the CP group ($P<0.001$) (Fig. 1).

The dose response curve to glucose from the LPNP group was shifted to the right in relation to those from RNP and CNP groups ($P<0.01$ and $P<0.05$, respectively) and these groups had similar dose response curve to glucose. Pregnancy shifted the dose response curve to the left in relation to non pregnancy ($F_{1.2}=16.9$, $P<0.01$), except in the RP group compared to the RNP group. The half-maximal release concentration of glucose for CNP, CP, LPNP, LPP, RNP and RPP islets was 9.4±1.5mmol/L, 7.7±0.15mmol/L, 11.2±0.1mmol/L, 7.1±0.5mmol/L,8.8±0.62mmol/L and 10.4±0.15mmol/L of glucose, respectively (Fig. 2).

The GLUT2 content was similar in RNP and LPNP islets and both groups exhibited GLUT2 content significantly higher than in CNP ($P<0.001$) and CP islets ($P<0.001$). Pregnancy did not modify the GLUT2 content in LPP and CP islets in relation to LPNP and CNP islets, respectively, but decreased the GLUT2 expression in RP compared to RNP islets ($P<0.001$) (Fig. 3A). The Gck and Hxk contents were affected by the nutritional status ($F_{2,12}=9.9$, $P<0.05$ and $F_{2,16}=4.1$, $P<0.05$, respectively). Thus, LPP and LPNP islets expressed higher Gck content than in RP, RNP, CP and CNP islets (Fig. 3B). In contrast, the Hxk content was lower in RP and RNP islets, as compared to other groups (Fig. 3C).

Glucose uptake rate in presence of glucose 2.8 mmol/L was similar in RNP and CNP islets and in both glucose uptake rates were lower in relation to that exhibited by LPNP

islets ($P<0.001$) (Fig. 4A). In presence of 8.3 mmol/L glucose RNP islets exhibited lower glucose uptake rate than in islets from the CNP group ($P<0.05$), that was lower compared to islets from the LPNP group ($P<0.001$) (Fig. 4B). In both glucose concentration pregnancy increased the glucose uptake rates in RP ($P<0.001$) and CP ($P<0.001$ and $P<0.05$, respectively) islets and reduced in LPP islets ($P<0.001$) (Fig. 4A and 4B). The $^{3}H_2O$ production in presence of 2.8 mmol/L glucose (Fig. 4C) as well as in 8.3mmol/L glucose (Fig. 4D) was similar in all non pregnant groups. In 2.8mmol/L glucose pregnancy decreased glucose utilization rate in CP and LPP islets in relation to CNP ($P<0.001$) and LPNP ($P<0.05$), respectively, but enhanced in RP compared to RNP ($P<0.001$) islets (Fig. 4C). Raising the glucose concentration to 8.3mmol/L, the glucose utilization ratio increased in islets from CP and RP islets compared to CNP ($P<0.001$) and RNP ($P<0.05$), respectively, but not in LPP compared to LPNP islets (Fig. 4D). As the next step in glucose metabolism, we evaluated the glucose oxidation rate in presence to glucose 2.8 (Fig. 4E) and 8.3mmol/L (Fig. 4F). In basal conditions, the $^{14}CO_2$ production was decreased in islets from LNP and RNP groups compared to CNP ($P<0.05$). Pregnancy improved this parameter in all groups ($F_{1.29}=260$, $P<0.001$) but the magnitude of this increment was higher in islets from the LPP group than in CP and RNP groups. Thus, LPP islets exhibited equal oxidation rate to CP islets and higher than RP ($P<0.05$) islets. However, the $^{14}CO_2$ production from the last group was not significantly different from that in the CP group (Fig. 4E). Under stimulatory concentrations the glucose oxidation rate only was altered by physiological status ($F_{1,26}=1398.0$, $P<0.01$). Islets from pregnant rats had higher glucose oxidation rate, as compared to islets from non pregnant rats (Fig. 4F).

The measurement of intracellular cAMP content was evaluated in presence of glucose 2.8 or 8.3mmol/L with IBMX (0.5mmol/L) to inhibit the degradation of this second messenger. In the first condition, this content was lower in islets from the LPNP group compared to RNP and CNP islets ($P<0.001$). Pregnancy increased the intracellular cAMP content in all groups ($F_{1,17}=366.5$, $P<0.001$). However, RP islets showed higher cAMP content than in LPP ($P<0.001$), and the latter islets had significantly higher cAMP content than in CP ($P<0.001$) islets (Fig. 5A). In presence of glucose 8.3mmol/L the intracellular cAMP content in islets from RNP and LPNP groups was similar and significantly lower, as compared to islets from the CNP group ($P<0.001$). Pregnancy

enhanced the cAMP content in all groups ($F_{1,17}$=254.7, P<0.001) and no significant difference was observed among LPP, RP and CP groups (Fig. 5B).

The protein content of AC3 in islets from RNP and LPNP groups was higher, compared to islets from the CNP group (P<0.001). Pregnancy increased the AC3 expression around 23% and 124% in LPP and CP, in relation to LPNP (P<0.01) and CNP (P<0.001), respectively, and reduced 21% in RP islets compared to RNP islets (P<0.01) (Fig. 6A).

The content of PKAα was modified by the nutritional status ($F_{2,12}$=16.60; P<0.001). Hence, islets from recovered (RNP and RP) and low-protein (LPNP and LPP) rats had a content of PKAα 156% and 118% higher than in islets from control rats (CNP and CP), respectively (Fig. 6B).

The PLCβ1 content was higher in islets from RNP and LPNP groups than in islets from the CNP group (P<0.01 and P<0.05, respectively). LPP and CP islets had more PLCβ1 content than LPNP (P<0.05) and CNP islets (P<0.001), respectively, whereas RP islets exhibited similar PLCβ1content to RNP islets. The PLCβ1 concentrations did not differ among the three pregnant groups (Fig. 6C). The protein expression of PKCα in islets from RP rats was lower than in RNP islets (P<0.01), whereas in islets from CP, LPP, CNP and LPNP its content was similar (Fig. 6D).

At nonstimulatory (Fig. 7A) and physiological (Fig. 7B) glucose concentrations, in the absence of pregnancy, the increase in the insulin secretion provoked by IBMX in islets from LPNP and CNP groups was similar, and this increment was higher in relation to that exhibited by islets from the RNP group (P<0.001). In presence of pregnancy, the magnitude of the increase of insulin secretion evoked by IBMX did not differ among RP, LPP and CP groups in both glucose concentrations. In 2.8 mmol/L glucose, the potentiator effect of IBMX was higher in RP than in RNP islets (P<0.001), was lower in LPP than in LPNP islets (P<0.01) and equal in CP and CNP islets. Raising glucose concentration to 8.3mmol/L the magnitude of potentiation of IBMX was higher in islets from all pregnant groups, compared to islets from non-pregnant groups ($F_{1,22}$=101.2, P<0.001).

At 2.8mmol/L glucose and in non-pregnant islets the increase in the insulin secretion by PMA was higher in the RNP group than in the LPNP group (P<0.01), but the first group was significantly lower, as compared to the CNP group (P<0.001). Pregnancy enhanced

the potentiator effect of PMA in all groups ($F_{1,23}$=157.4, P<0.001), but this effect was lower in islets from the RP group than in islets from LPP and CP groups (P<0.001), that exhibited similar magnitude of increment of insulin secretion (Fig. 7C). At 8.3 mmol/L, glucose potentiation of insulin secretion by PMA in LPNP islets was lower in relation to those exhibited by CNP islets (P<0.05). The potentiator effect of PMA in islets from the RNP group did not differ from that exhibited by islets from LPNP and CNP groups. The magnitude of potentiation of PMA was higher in islets from all pregnant groups in relation to islets from non-pregnant groups ($F_{1,19}$=92.2, P<0.001). However, in presence of pregnancy, PMA evoked similar increment the insulin secretion in LPP and CP islets and both increases were higher than those exhibited by RP islets (P<0.05 and P<0.01, respectively) (Fig. 7D).

DISCUSSION

In this work, body weight gain during pregnancy was impaired in rats fed a low protein diet, confirming the studies that show that low protein diet during pregnancy reduces the body weight gain, even when food intake is increased (35, 9). The reduced weight gain in rats fed a low protein diet may be associated to hyperactivity of the brown adipose tissue, an adaptive response to nutritional stress, that lead to increase on thermogenesis and consequently high energy expenditure (31). Rats fed a low protein diet showed hypoalbuminemia, a typical feature showed by malnourished animals and humans and nutritional recovery reversed this alteration.

Interestingly, in this study non-pregnant recovered rats were insulin resistant and in a previous study male recovered rats were insulin sensitive, at the judge by the K_{itt} significantly decreased and increased, respectively, in relation to control rats (18). The indicators of *in vivo* (fasting serum insulin concentration, ΔI and ΔG:ΔI ratio) and *in vitro* (dose-response curve) insulin secretion from non pregnant control and non pregnant recovered rats were similar, whereas in male recovered rats these parameters signalized impairment in the insulin secretion (18). Differences between genders may be related to diverse hormonal milieu that could be modulating insulin secretion and sensitivity. Despite these alterations, glucose tolerance (accessed by ΔG and fasting glucose concentration) was similar in all non pregnant groups. Thus, our previous (18) and present results are contrary to the observation that females fed a low protein diet during early life become relatively more insulin deficient while males are more insulin

resistant (3). However, alterations in the metabolic function that contribute to glucose intolerance may be related to the age of animals and the studies by Chamson-Reig et al. (3) measured animals at 130 days of age; on the other hand, our present study was carried out with animals aged 90 days.

Curiously, the typical insulin resistance (32, 11) and functional changes of the endocrine pancreas to adapt to increased demand for insulin in the pregnancy (41, 43) were observed in low protein pregnant but not in recovered pregnant rats. Even so, recovered pregnant rats did not develop gestational diabetes or glucose intolerance, possibly because insulin sensitivity and insulin secretion were yet reciprocally related. However, fetus from recovered pregnant rats exhibited higher body weight than those from low protein and control rats, an indication of disrupted maternal metabolism.

To a proper response to increased insulin demand, the islets should combine mechanisms that enhance its secretory capacity and reduces the threshold for glucose stimulated insulin secretion. Total insulin content per islets that reflects the insulin biosynthesis and/or β cell mass must be amplified during pregnancy. In our study, protein restriction in the critical phase of development and prolonged protein restriction produced deficit in the total insulin content per islet. The amplification of total insulin content seen during pregnancy was verified only in recovered pregnant islets, but this increase was not enough to reach the absolute value from the control pregnant group. However, reduced total insulin content does not totally explain alterations of islets functionality, because islets from recovered pregnant rats exhibited higher total insulin content than islets from a low protein fed pregnant rats, but showed lower glucose sensitivity. These results suggest alterations on intrinsic mechanisms of insulin secretion, including metabolic-secretion coupling and exocytotic pathways.

Glucose uptake is the initial step on glucose stimulated insulin secretion by pancreatic β-cell. In rodents, GLUT2 is the mainly glucose transporter founded in pancreatic β–cells and presents high K_m to glucose (37, 17). Glucose is phosphorylated to glucose-6-phosphate by two enzymes: Hxk I-III, that determine the amount of insulin secreted at basal glucose concentrations because its low K_m (15), and the Gck (Hxk IV), that has a high K_m and is considered the “glucose sensor” in the pancreatic β cell (22). Reduction of insulin secretion by islets of rats fed a low protein diet has been attributed to problems with the recognition and utilization of glucose (7) as well an impaired activity

of pancreatic β-cell mitochondrial glycerophosphate dehydrogenase (30) that alters glucose metabolism. Contrary to these assumptions, in the present study islets from rats fed a low protein diet up to the 12th week of age had increased GLUT2 content and glucose uptake, unaltered Hxk content and unaltered glucose utilization in basal glucose concentration. Moreover, these islets had increased Gck content but unchanged glucose utilization on stimulatory glucose concentration. These results are very interesting because these islets had higher glucose uptake but similar glucose utilization, as compared to control islets. To explain these results, we hypothesized that increased glucose uptake was caused by a shift from glucose metabolism to other pathways, for instance pentose shunt, providing β-cells NADH for the insulin secretion or ribose substrates for the synthesis of nucleotides. Finally we observed in islets from non pregnant rats fed a low protein diet a decreased glucose oxidation in basal glucose concentration and unaltered glucose oxidation in physiological glucose concentration. These results contrast with the report that this animal model D-[3,4-^{14}C] glucose oxidation rate is unaltered in presence of basal glucose and decreased in stimulatory glucose (34).

Our islets from non pregnant recovered rats showed increased GLUT2 levels associated to decreased glucose uptake, reduced Hxk content with similar glucose utilization in basal glucose concentration. In addition, we found in islets from non pregnant recovered rats unaltered Gck content and unchanged glucose utilization on physiological glucose concentration, compared to non pregnant control islets. Thus, these results are in disagreement to the report that islets from rats recovered of early malnutrition after weaning show reduced Hxk activity and Gck expression and activity (13).

The pregnancy adaptive changes have been associated with increase in the glucose utilization due to enhanced activity and/or content of GLUT2, Gck and Hxk as well as augment in the glucose oxidation (41). In this study, GLUT2 content in islets from recovered pregnant islets was reduced at the value of control pregnant islets and resulted in similar glucose uptake. Gck and Hxk contents were not altered in all pregnant groups. However, in recovered and low protein pregnant islets the glucose utilization rates in presence of stimulatory glucose concentration were reduced, as compared to control pregnant islets. These results suggest lower Gck activity in islets from pregnant recovered and pregnant low protein rats compared to islets from the control pregnant group. At the judge by the EC_{50} values, the relationship between Gck activity and

glucose-sensitivity was observed in islets from control groups but it was lost in islets from non pregnant and pregnant recovered and low protein groups. Also, results of glucose oxidation did not correlate with the changes observed in the glucose sensitivity, since $^{14}CO_2$ production was similar in all groups. Thus, we investigated other pathways of stimulus-secretion coupling that could explain the secretory pattern exhibited by low protein and recovered islets.

The ability of the second messenger cAMP to potentiate insulin secretion stimulated by glucose through the activation of PKA is well documented (45). Protein restriction in critical phases of development and prolonged protein restriction produced increase AC3 content that appeared to have been compensated by the decreasing in the AC activity, since those groups exhibited reduced cAMP content. This supposition is reinforced by the observation that the regulation of cAMP generation dependent of AC3 isoform is modulated by Ca^{2+}/calmodulin complex (38) and, at least in low protein islets, Ca^{2+} handling is compromised (19). PKA expression was increased in islets from low protein and from recovered rats and its activity was reduced in both groups, but in higher degree in the recovered islets in the face of the lower potentialization magnitude elicited by IBMX on insulin secretion.

The expected rise in cAMP generation previously reported on pregnancy (42) was observed in this study, even in islets from recovered pregnant rats with decreased AC3 content. The cAMP generation profile showed by islets from pregnant recovered and pregnant low protein rats indicated increased AC3 activity. As previously showed (24), pregnancy did not increase the PKA content. However, taking account of the potentiator effect of IBMX in low glucose concentration, the PKA activity was reduced by pregnancy in islets from rats submitted to prolonged protein restriction and increased in islets from rats recovered of early protein restriction. In physiological glucose concentration the PKA activity became equal in all pregnant groups. Thus, the blunted secretory capacity observed in recovered pregnant islets cannot be attributed to the attenuation in the cAMP/PKA pathway.

PLC/PKC system is involved in the reduction of stimulatory threshold glucose concentration and in the potentiation of insulin secretion during pregnancy (42) and this system is impaired in a model of protein restriction after weaning (10). We observed that in the absence of pregnancy, the PLC content was increased in islets from protein restricted rats and in islets from recovered rats, possibly in the attempt to raise IP3 and

DAG, whose actions result in increased intracellular Ca^{2+} levels. PKC content was not modified but its activity was reduced, especially in islets from rats submitted to prolonged protein restriction. PLC content was similar in all pregnant islets and PKC expression was reduced in recovered pregnant islets. However, PMA was less efficient in potentiating insulin secretion in recovered pregnant rats, indicating lower PKC activity in that group. Hence, the PLC/PKC system in islets from recovered pregnant appears to be severely attenuated.

CONCLUSIONS

These results showed that protein restriction in early life impaired the classical β-cell adaptative changes during pregnancy and disrupted the maternal metabolism, but not enough to promote the onset of gestational diabetes. Loss adaptive capacity by islets from recovered pregnant rats apparently resulted from uncoupling between glucose metabolism and the amplifying signals of secretory process as well as the severe attenuation of PLC/PKC pathway. Alterations exhibited by islets from pregnant rats undergoing sustained protein deficiency up to the 12th week of age did appear to result more from reduced islet mass and/or insulin biosynthesis than from impairment in the stimulus-secretion coupling process.

ACKNOWLEDGMENTS

The authors are grateful to Celso Roberto Afonso, for the excellent technical assistance and to Dr Maria Alice Rostom de Mello for kindly provide the [^{14}C] 2-deoxy D-Glucose for glucose metabolism assays in isolated islets.

GRANTS

This work was supported by the Brazilian foundations FAPEMAT, CAPES and CNPq, by the financial support. This work is part of a dissertation presented by Letícia Martins Ignácio de Souza as a partial requirement for the Master's degree in Biosciences at the School of Nutrition, UFMT.

We declare that we have no conflict of interest.

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TABLES

Table 1. Initial and final body weight, body weight gain and serum albumin concentrations from non-pregnant or pregnant rats maintained on control diet (CNP and CP), low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP)

Parameters	*Groups*					
	CNP	CP	LPNP	LPP	RNP	RP
Initial body weight (g)	284±23[a]	286±16[a]	162±19[c]	162±28[c]	222±19[b]	218±14[b]
Final body weight (g)	290±17[b]	363±30[a]	174±28[d]	219±44[c]	215±18[c]	288±13[b]
Body weight gain (g)	6±7[ab]	76±19[c]	12±10[b]	57±19[d]	-8±7[a]	71±7[cd]
Albumin (g/L)	29±0.2	26±0.4	21±0.2[§]	23±0.3[§]	28±0.1	25±0.2

Values are the mean ± SD for 5-6 rats per group. Mean values with unlike superscript letters were statistically different ($P < 0.05$, LSD test) [§]indicate difference between nutritional status.

Table 2. Fasting serum glucose and insulin concentrations, total areas under the glucose (ΔG) and insulin (ΔI) curves, ΔG:ΔI ratio obtained from the intraperitoneal glucose tolerance test and glucose disappearance ratio (K_{itt}) obtained from the intraperitoneal insulin tolerance test from non-pregnant or pregnant rats maintained on control diet (CNP and CP), low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP)

Parameters	*Groups*					
	CNP	CP	LPNP	LPP	RNP	RP
Serum glucose (mmol/L)	4.5±0.9	3.7±0.4	4.0±0.9	3.6±0.7	4.7±0.5	4.0±1.1
Serum insulin (nmol/L)	$0.16±0.02^{a}$	$0.56±0.45^{b}$	$0.03±0.02^{a}$	$0.5±0.30^{b}$	$0.23±0.31^{a}$	$0.20±0.20^{a}$
ΔG (mmol/L.120min)	844±246	580±45	772±78	502±44	765±132	574±105
ΔI (nmol/L.120min)	$65±14^{cd}$	$83±20^{d}$	$15±25^{ab}$	$59±18^{cd}$	$62±46^{cd}$	$44±25^{bc}$
ΔG:ΔI (mmol/nmol)	$13±4^{b}$	$7±2^{b}$	$152±89^{a}$	$9±2^{b}$	$61±105^{b}$	$24±24^{b}$
K_{itt} (%/min)	$4.5±1.0^{b}$	$2.6±1.5^{cd}$	$6.3±0.9^{a}$	$1.1±0.6^{c}$	$2.4±1.2^{cd}$	$3.6±0.8^{bd}$

Values are the mean ± SD for 5-6 rats per group. Mean values with unlike superscript letters were statistically different ($P < 0.05$, LSD test).

FIGURES

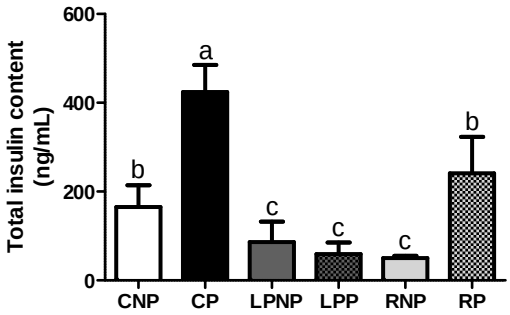

Figure 1. Total insulin content in isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. Mean values within a column with unlike superscript letters were statistically different ($P < 0.05$, LSD test)

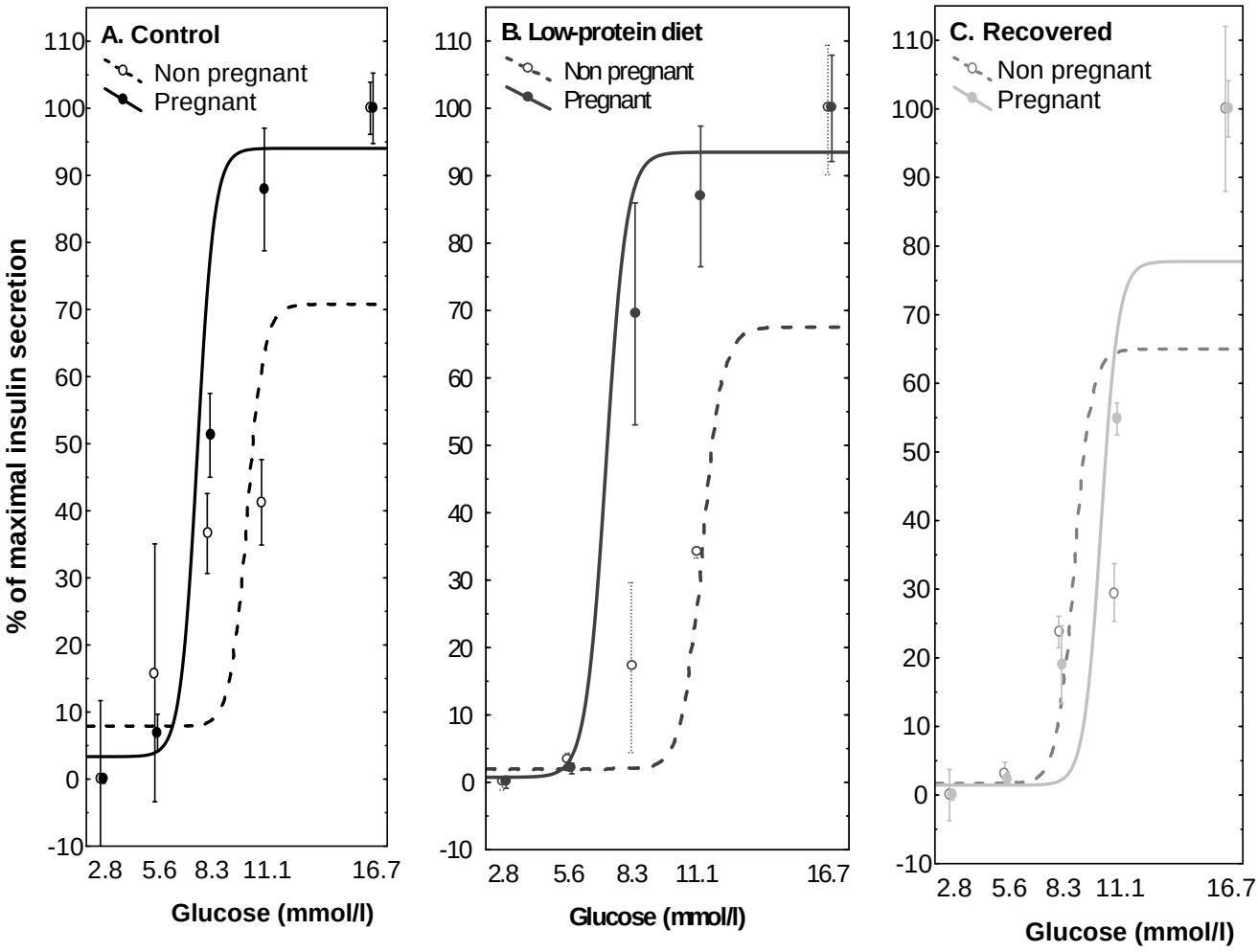

Figure 2. Comparative glucose dose-response curves for isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP).The values are the mean ± SD of three independent experiments expressed as a percentage of the maximal insulin secretion within the same experiment. The half-maximal response was obtained with 9.4±1.5mmol/L, 7.7±0.15mmol/L, 11.2±0.10mmol/L, 7.1±0.50mmol/L, 8.8±0.62mmol/L and 10.4±0.15mmol/L of glucose, respectively, for the CNP, CP, LPNP, LPP, RNP, RP islets

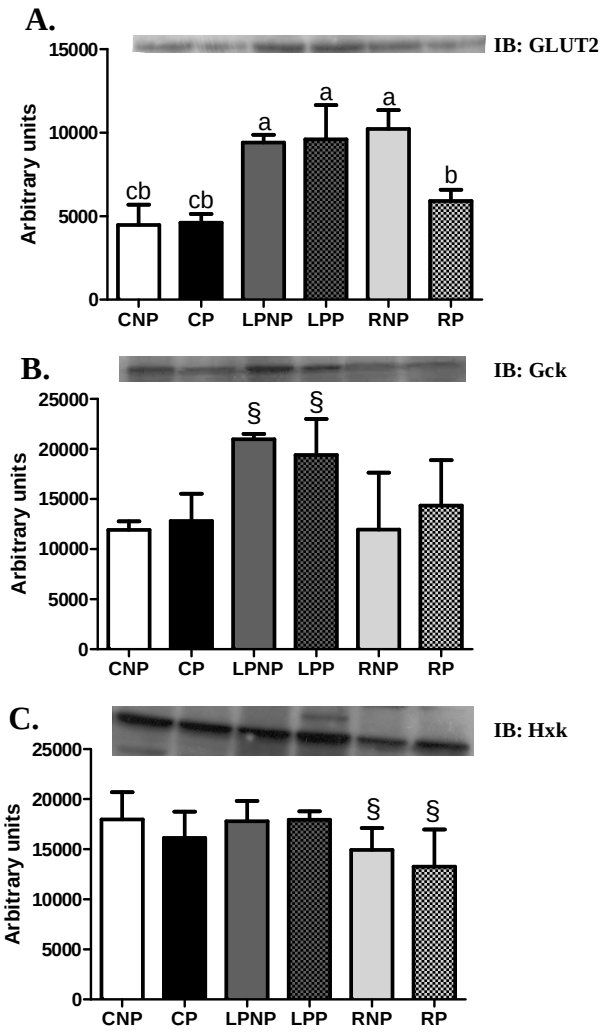

Figure 3. GLUT2 (A), glucokinase (B) and hexokinase (C) concentrations detected by Western Blot in isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. [§]indicate difference between nutritional status. Mean values within a column with unlike superscript letters were statistically different ($P < 0.05$, LSD test)

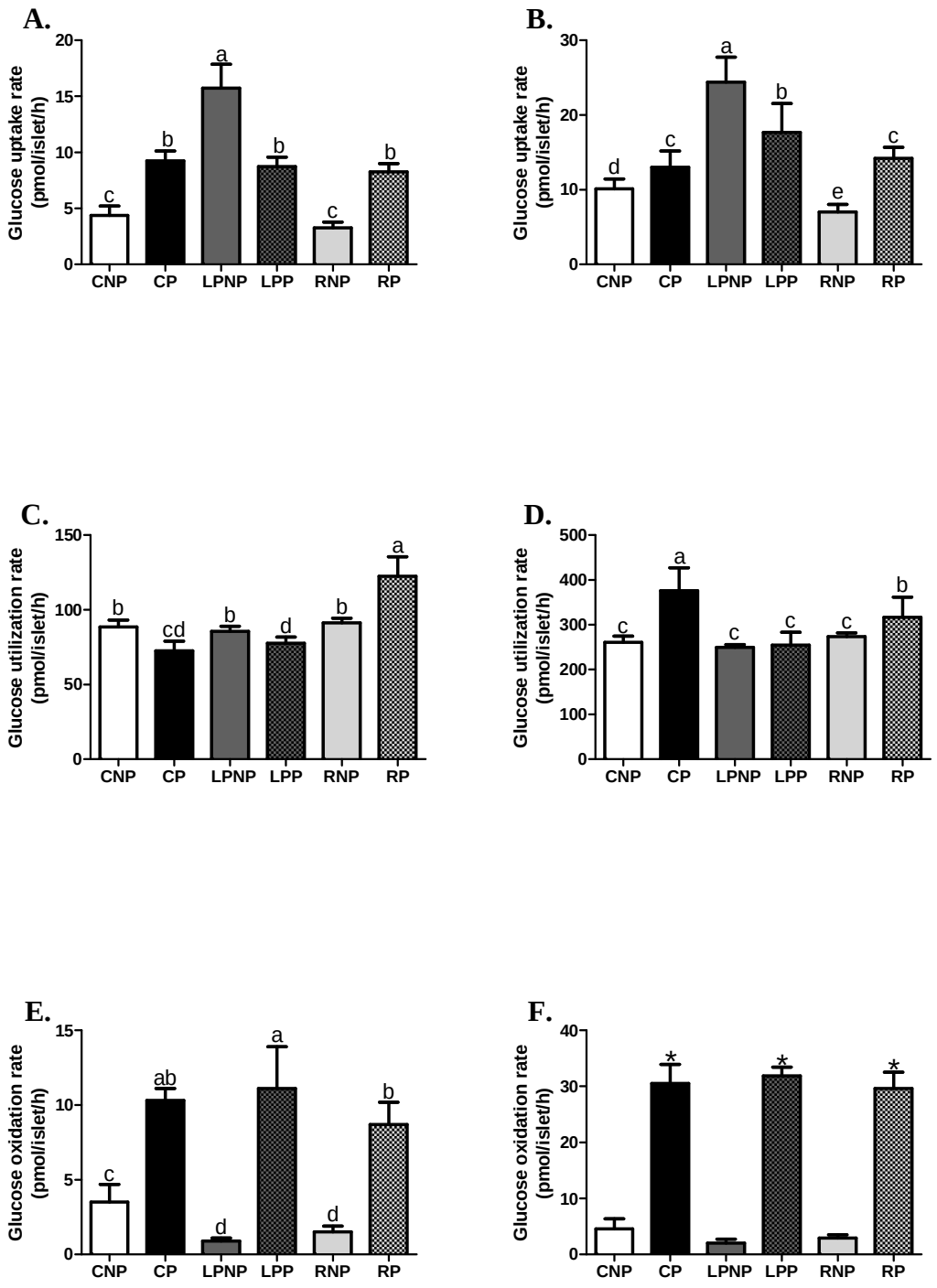

Figure 4. Glucose uptake (A and B), glucose utilization (C and D) and glucose oxidation (E and F) rates in 2.8 or 8.3mmol/L glucose (respectively) by isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP) . The bars are means ± SD. * presents difference between physiological status. Mean values within a column with unlike superscript letters were statistically different (P <0.05, LSD test)

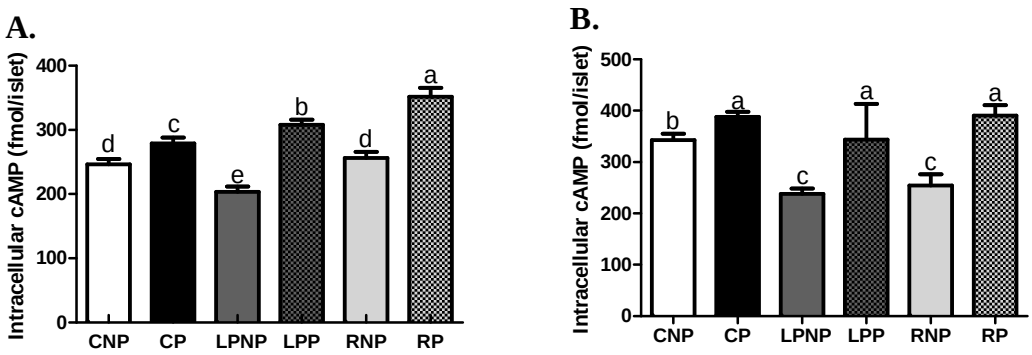

Figure 5. Intracellular cyclic AMP content in presence of 2.8 (A) or 8.3 (B) mmol/L glucose in isolated islets from non pregnant or pregnant rats submitted to control diet (CNP and CP), to low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. Mean values within a column with unlike superscript letters were statistically different ($P < 0.05$, LSD test)

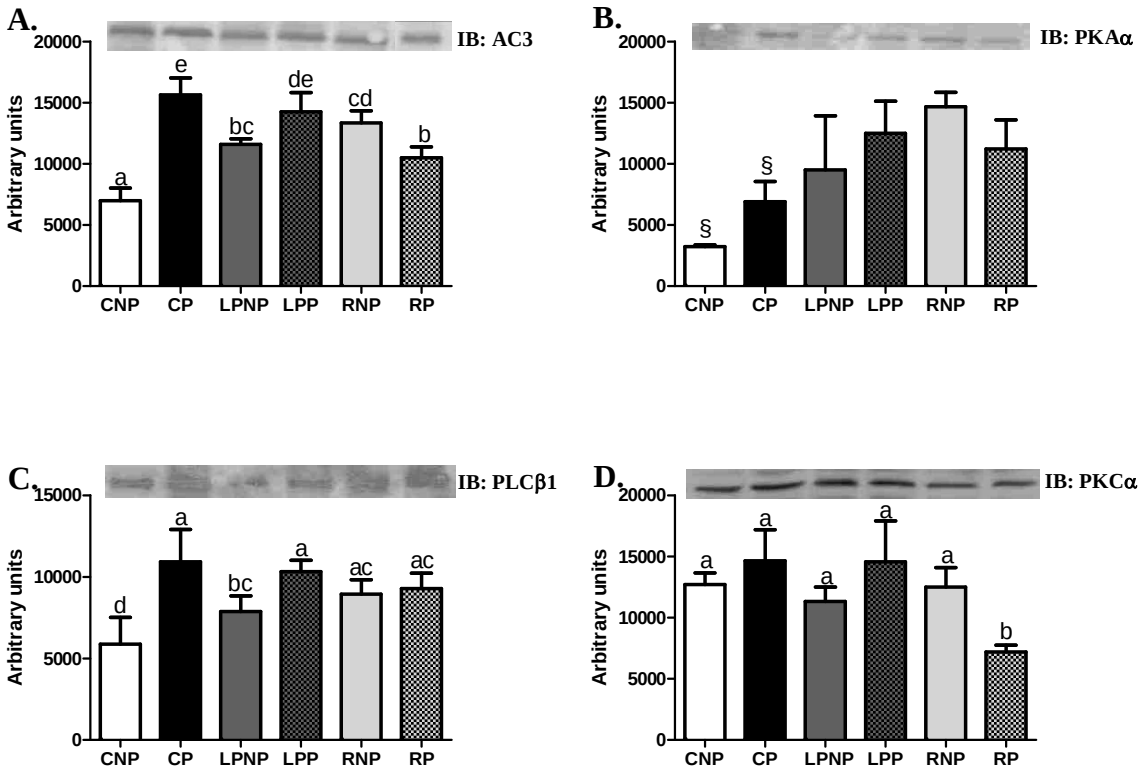

Figure 6. Adenylate cyclase 3 (A), protein kinase A alpha (B), phospholipase C beta 1(C) and protein kinase C alpha (D) concentrations detected by Western Blot in isolated islets from non pregnant or pregnant rats maintained on control diet (CNP and CP), on low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. [§]presents difference between nutritional status. Mean values within a column with unlike superscript letters were statistically different (P <0.05, LSD test)

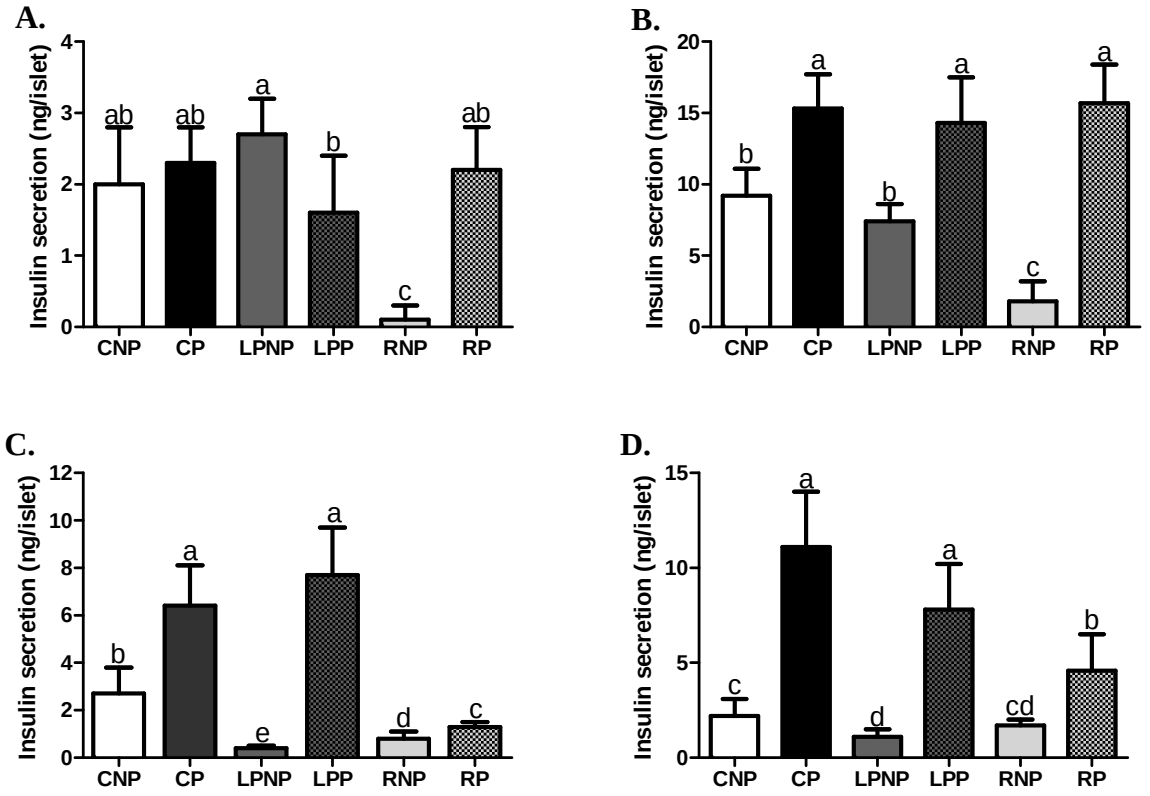

Figure 7. Increment in glucose stimulated insulin secretion (2.8mmol/L or 8.3mmol/L glucose) by IBMX (A and B), and PMA (C and D) in isolated islets from non pregnant or pregnant rats submitted to control diet (CNP and CP), to low-protein diet (LPNP and LPP) or recovered after weaning (RNP and RP). The bars are means ± SD. Mean values within a column with unlike superscript letters were statistically different ($P < 0.05$, LSD test)

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