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Fabiano Ferreira

PARTICIPAÇÃO DAS PROTEÍNAS QUINASES *C* E *A* NA MODULAÇÃO DA SECREÇÃO DE INSULINA EM ILHOTAS DE LANGERHANS DE RATOS SUBMETIDOS À RESTRIÇÃO PROTÉICA.

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BANCA EXAMINADORA:

Prof. Dr. Antonio Carlos Boschero (Orientador) _____

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Profa. Dra. Helena Coutinho Franco de Oliveira _____

Prof. Dr. Ângelo Rafael Carpinelli _____

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Profa. Dra. Márcia Queiróz Latorraca _____

A todos os que me conhecem

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SUMÁRIO

RESUMO

A regulação da secreção de insulina em resposta à alimentação é determinada pela ação direta de nutrientes sobre as células B pancreáticas e pelos efeitos indiretos mediados pela ativação hormonal e neural. Sabe-se que 50% da insulina secretada no período pós-prandial imediato depende da estimulação colinérgica e que no diabetes tipo 2, a fase rápida da secreção de insulina é a primeira a desaparecer. Ilhotas de ratos desnutridos também apresentam perfil de secreção de insulina sem o padrão bifásico. Sabe-se que a regulação da secreção de insulina estimulada por glicose pode ser modulada pelas vias que envolvem a ativação das proteínas quinase C e A (PKC e PKA).

Assim, o presente trabalho teve como objetivo estudar a participação das proteínas PKA e PKC na modulação do processo secretório de insulina em ilhotas isoladas de ratos submetidos à restrição protéica.

Os animais foram divididos em dois grupos experimentais, um grupo controle tratado com dieta normoprotéica (NP) (17%) e outro grupo desnutrido tratado com dieta hipoprotéica (LP) (6%). Após dois meses de desnutrição os animais foram sacrificados para o início dos experimentos.

Ilhotas isoladas do pâncreas destes animais foram utilizadas para protocolos de secreção estática e dinâmica de insulina, imunohistoquímica para as enzimas PKA e PKC e análise da expressão gênica para as proteínas em questão.

Nossos resultados demonstraram que a dieta LP reduziu a massa corpórea e a albuminemia dos animais. O grupo de animais submetidos a dieta LP mostrou níveis elevados de ácidos graxos livres no plasma e redução dos níveis de proteínas totais em

relação ao grupo NP, caracterizando o quadro de desnutrição. A insulinemia no período alimentado apresentou-se reduzida nos animais LP e a glicemia mantida, sugerindo uma maior sensibilidade dos animais LP ao hormônio insulina..

A resposta secretória de insulina pelas ilhotas LP foi menor que a resposta das ilhotas NP, quando estimuladas com o forbol éster (PMA), um potente estimulador da PKC e Forskolin, um potente estimulador da PKA.

Quando ilhotas foram estimuladas com PMA (400 ηM) o aumento da secreção de insulina foi significativamente maior no grupo NP quando comparado ao grupo LP. A técnica de imunohistoquímica, revelou uma diminuição na quantidade das enzimas PKA e PKC, estes resultados foram confirmados utilizando-se a técnica de Western Blotting, onde foram encontrados níveis reduzidos da PKC e PKA em aproximadamente 30% no grupo LP ($p<0.05$).

RT-PCR mostrou uma diminuição também na quantidade de mRNA para ambas as enzimas em estudo, o que nos sugeriu uma possível relação entre a restrição protéica, a qual o animal foi submetido, e alterações na transcrição de genes que codificam enzimas relacionadas com o processo secretório.

Até o momento podemos concluir que, a alteração no conteúdo destas enzimas neste modelo experimental permite explicar, pelo menos em parte, a diminuição na resposta secretória encontrada na estimulação com glicose, PMA e Forskolin. Mais ainda, a restrição protéica no período pós-desmame induziu modificações transcricionais para enzimas chave no processo secretório de insulina.

ABSTRACT

The effects of carbamylcholine (CCh) and phorbol 12-myristate 13-acetate (PMA) on insulin secretion were studied in pancreatic islets from rats maintained on a normal (17%; NP) or low (6%; LP) protein diet. Isolated islets were incubated for 1 h in Krebs-bicarbonate solution containing 8.3 mmol/L glucose, with or without PMA (400 μmol/L) and CCh. Increasing concentrations of CCh (0.1-1000 μmol/L) dose-dependently increased the insulin secretion by islets from both groups of rats. However, the dose-response curve to CCh was shifted to the right in LP islets with an EC_{50} of 2.15 ± 0.7 and 4.64 ± 0.1 μmol CCh/L in NP and LP islets, respectively ($P < 0.05$). The PMA-induced insulin secretion was three-fold higher in NP compared to LP islets. Western bloting revealed that the PKCα and PLCβ1 contents of LP islets were 30% lower than in NP islets ($P < 0.05$). In addition, PKCα mRNA expression was reduced by 50% in islets from LP rats. In conclusion, a reduced expression of PKCα and PLCβ1 may be involved in the decreased insulin secretion by islets from LP rats following stimulation with CCh and PMA.

We also investigated the effects of forskolin, a stimulator of adenylyl cyclase, on insulin secretion by pancreatic islets from rats fed a normal (17%; NP) or low (6%; LP) protein diet for 8 weeks. Isolated islets were incubated for 1 h in Krebs-bicarbonate solution containing 8.3 mmol glucose/L, with or without 10 μmol forskolin/L. The forskolin-induced insulin secretion was higher in NP than in LP islets ($P < 0.05$). Western blotting revealed that the amount of the alpha catalytic subunit of protein kinase A (PKAα)

was 35% lower in LP compared with NP islets ($P < 0.05$). Moreover, PKAα mRNA expression was reduced by 30% in islets from LP rats ($P < 0.05$). Our results indicated a possible relationship between a low protein diet and a reduction of PKAα expression. These alterations in PKAα may be partly responsible for the decreased insulin secretion by islets from rats fed a low protein diet.

Keywords: PKA; PKC; Islets; Insulin; Low protein diet

1. INTRODUÇÃO

1. Introdução

1.1 O Pâncreas Endócrino e a Secreção de Insulina

É sabido que todos os tipos celulares enviam e recebem mensagens químicas, sendo esta troca de sinais, muito importante para manter o bom funcionamento do organismo. O sistema endócrino tem um papel primordial no controle destas atividades celulares e portanto na manutenção da homeostasia.

Em se tratando de pâncreas, além da sua importante função exócrina (digestão) este órgão possui também uma função endócrina e, tal como as outras glândulas que constituem o sistema endócrino (ex. hipófise, tiróide, glândula adrenal e gônadas), secreta hormônios (dentre os mais conhecidos, Insulina, Glucagon e Somatostatina) na corrente sanguínea que vão atuar em órgãos ou tecidos distantes.

Uma das funções essenciais do sistema endócrino é a manutenção de uma concentração razoavelmente constante de glicose no sangue. Esta manutenção estrita é controlada pelo balanço entre a absorção de glicose pelo intestino, sua produção no fígado e rim, e sua entrada e metabolização pelos tecidos periféricos.

A insulina é o único hormônio capaz de reduzir a concentração de glicose no sangue, pois é capaz de aumentar a captação de glicose pelas células do músculo e tecido adiposo. Mais ainda a insulina é capaz de inibir a produção hepática de glicose e manter a homeostase glicêmcia. A insulina estimula também o crescimento e diferenciação celular, promovendo a lipogênese, síntese de proteínas e glicogênio. Deficiências na produção de insulina ou resistência dos tecidos alvos à sua ação, podem resultar em uma profunda alteração destes processos citados, com elevações dos níveis pós-prandiais de glicose e lipídios (GOODMAN & GILLMAN, 1996).

Ilhotas de Langerhans

A porção endócrina do pâncreas representa apenas, 1% do peso total deste órgão (ORCI et al., 1988). É constituída por pequenas estruturas com formas variáveis denominadas ilhotas de Langerhans, que devem o seu nome ao autor que primeiro as

identificou em 1869, Paul Langerhans. Estas unidades celulares encontram-se rodeadas por tecido exócrino e por algumas células isoladas espalhadas pelos ductos pancreáticos.

As ilhotas de Langerhans, verdadeiros micro-órgãos com um diâmetro entre 40 e 400 μm, contêm entre 500 e 5000 células, das quais cerca de 80% são células B produtoras de insulina. Estas células ocupam a parte central homogênea da ilhota, sendo a zona periférica da ilhota mais heterogênea, constituída por outros três tipos celulares: células α produtoras de glucagon, células δ produtoras de somatostatina e células PP secretoras de polipeptídeo pancreático.

As ilhotas de Langerhans encontram-se entre os órgãos mais vascularizados do organismo, possuindo uma microcirculação diferenciada. O sangue flui pela ilhota e percorre uma rede de pequenos capilares que irriga em primeiro lugar as células B centrais e só depois as outras células periféricas da ilhota. A existência deste sistema de irrigação sanguínea intra-ilhota tem conseqüências no controle hormonal entre as diferentes células da ilhota (interações parácrinas entre as células, podem regular as secreções umas das outras através do fluido intersticial que todas compartilham). De fato, a secreção de glucagon pelas células α parece ter um papel importante na determinação da amplitude e da velocidade da resposta insulinêmica a certos fármacos e nutrientes (MARKS et al., 1992).

Além deste controle hormonal endógeno, a atividade secretora da ilhota é ainda modulada por estímulos de origem nervosa e hormonal externos à ilhota que provêm da extensa rede de fibras do sistema nervoso autônomo que inerva as ilhotas (BONNER-WEIR, 1991). Exemplos destes estímulos incluem os neurotransmissores clássicos do sistema nervoso simpático (noradrenalina) e parassimpático (acetilcolina (ACh)), bem como os neuropeptídeos co-secretados (peptídeo liberador de gastrina, galanina, neuropeptídeo Y, colecistoquinina (CCK) e substância P) e hormônios do sistema endócrino gastrointestinal (Peptídeo inibidor gástrico e glucagon like peptide-1). A relevância fisiológica de algumas destas moléculas para a secreção de insulina está ainda por ser estudada. No entanto, no caso da acetilcolina, a importância da inervação colinérgica para a potencialização da resposta secretora às necessidades do organismo já foi amplamente estudada. A estimulação parassimpática é responsável pela fase cefálica da secreção de insulina (a resposta inicial de secreção de insulina anterior à ingestão de alimentos) e encontra-se diminuída durante situações de hipoglicemia, de modo que ocorre

um aumento dos níveis de glucagon a fim de estimular a secreção hepática de glicose e restaurar a euglicemia (DUNNING et al., 1994).

Dinâmica da secreção de insulina

O controle dos níveis plasmáticos de glicose é exercido em uma escala temporal de minutos através de uma resposta rápida de secreção de insulina, que apresenta um padrão pulsátil complexo. O perfil temporal da secreção de insulina em resposta a um pulso de glicose aplicado por via intravenosa consiste num pico de secreção, que surge durante o terceiro e o quinto minuto, podendo durar até dez minutos, seguido de uma segunda fase mais lenta (LEAHY et al., 1992). Durante esta última fase ocorrem pulsos de pequena amplitude em cada 5-10 minutos, que estão sobrepostos a oscilações mais lentas e de maior amplitude que ocorrem a cada 1-2 horas (BELL & POLONSKY, 2001).

Flutuações plasmáticas de insulina foram detectadas em várias espécies de mamíferos, incluindo o homem (LANG et al., 1982), macaco (GOODNER et al., 1977), cão (JASPAN et al., 1986) e rato (CHOU et al., 1991). Esta secreção oscilatória foi também encontrada em preparações de pâncreas isolado (STAGNER & SALMOLS, 1985), em grupos de ilhotas isoladas (CHOU & IPP, 1990; LONGO et al., 1991) e, posteriormente, em uma única ilhota isolada (ROSARIO et al.,1986; BERGSTEN & HELLMAN, 1993a; BERGSTEN et al., 1994; BERGSTEN, 1995), sugerindo que a pulsatilidade da secreção de insulina é uma propriedade intrínseca de cada ilhota. Esta hipótese é comprovada pelo fato de, no homem, a freqüência das oscilações de insulina medida a partir de grupos de ilhotas isolados (cerca de 0,1 min^{-1}) ser idêntica à observada no plasma *in vivo* (MARCHETTI et al., 1994). Em modelos animais de roedores, no entanto, a periodicidade das oscilações de insulina diminui a medida que a complexidade celular aumenta, apresentando valores de freqüência que vão de cerca de 0,07-0,1 min^{-1} no plasma a 0,3-0,5 min^{-1} (ou mesmo 2-3 min^{-1}) em ilhotas isoladas (BERGSTEN & HELLMAN, 1993b). Não se pode, portanto, excluir um papel modulador por parte de fatores intra e extra-pancreáticos, tais como os sinais provenientes dos gânglios pancreáticos ou do nervo vago.

A sincronização existente entre as células da ilhota, que faz com que a ilhota de Langerhans se comporte como um sincício, depende criticamente da comunicação e

acoplamento entre as células. Está bem estabelecido que as células B isoladas não exibem oscilações. À medida que o número de células nas preparações experimentais aumenta, favorecendo o aparecimento de agregados celulares e mesmo de pseudo-ilhotas, parece também aumentar a funcionalidade das células e a regularidade dos eventos oscilatórios (GYLFE et al., 1991; JONKERS et al., 1999; HAUGE-EVANS et al., 1999). Esta sincronização é possível devido a comunicação entre as células através de junções comunicantes (*gap junctions*), que permitem a transferência de íons e pequenos metabólitos entre as células (ORCI et al., 1973; MEDA et al., 1984). Para além do acoplamento através destas junções, a propagação dos sinais intracelulares também pode ser mediada por um componente de sinalização extracelular (BERTUZZI et al., 1999). De fato, a existência de oscilações extracelulares de K^+, medidas com um microelétrodo sensível a K^+ colocado no espaço extracelular da ilhota (PEREZ-ARMENDARIZ et al., 1985), levantou a hipótese de que aquelas oscilações pudessem contribuir, juntamente com as junções comunicantes, para a sincronização da atividade das células no ilhota intacto (STOKES & RINZEL, 1993).

Desnutrição e Diabetes.

A desnutrição é um problema em todas as populações do mundo, onde crianças com baixo peso ao nascer correspondem a 15% do total da taxa de nascimento (DAHRI, SNOECK et al., 1991).

Em nove países da América Latina, onde foi efetuado um levantamento populacional, a desnutrição foi um dos fatores responsáveis pela elevada mortalidade em crianças abaixo de 5 anos de idade (ANGELIS, 1977).

Deficiências protéicas e energéticas normalmente ocorrem juntas, embora possa haver a predominância de uma sobre a outra. Quando se manifestam de forma severa, podem desencadear síndromes clínicas denominadas " Kwashiorkor " e " Marasmo ". Kwashiorkor resultante de ingestão insuficiente de proteínas e o marasmo resultante de deficiência energética (SHILS et al., 1994).

Desde a antiguidade reconhece-se a importância do hábito alimentar no desenvolvimento do diabetes mellitus. Redução do peso e a baixa ingestão de energia foram associados a uma menor incidência e prevalência do diabetes. Nos países desenvolvidos, o

sedentarismo associado à alimentação hipercalórica, pobre em fibras e rica em proteínas, gorduras saturadas e carboidratos simples, têm contribuído para o rápido aumento do desenvolvimento do diabetes mellitus do tipo 2 (RAO, 1988). Esta é, geralmente, acompanhada de obesidade, hipertensão arterial sistêmica, dislipidemia e resistência à insulina. O conjunto dessas anomalias foi designada de Síndrome X" (ZAVARONI et al., 1994) e sua etiopatogenia ainda não está totalmente elucidada..

HALES & BARKER (1992) formularam a hipótese do *"thrifty phenotype"* para explicar a etiopatogenia do diabetes mellitus tipo 2 e da "Síndrome X". De acordo com essa hipótese, estas duas patologias são determinadas por fatores ambientais, inclusive nutricionais, e são originadas *in utero* ou durante a infância. Presume-se que a desnutrição intra-uterina e durante o primeiro ano de vida produz mudanças morfológicas e funcionais no pâncreas, fígado, e no tecidos muscular e adiposo, traduzidas em insulinopenia e resistência à insulina. Quando a resistência à insulina é agravada pela obesidade, inatividade física ou idade, o pâncreas não consegue suprir a demanda de insulina e o diabetes se instala. O padrão de anormalidades metabólicas e funcionais depende da fase da vida em que a desnutrição ocorre, do tipo e da intensidade da carência nutricional.

Antigamente, havia evidência de que a desnutrição poderia ser diabetogênica, pois foram realizados estudos que mostraram intolerância à glicose, insulinopenia e resistência à insulina em animais (HEARD & TURNER, 1967; SWENNE et al. 1987) e em seres humanos desnutridos (COOK 1967, BECKER et al. 1971, SMITH et al., 1975). Duas formas de diabetes foram associadas à desnutrição crônica: 1) diabetes do tipo J, semelhante ao diabetes tipo 1 pelo seu início abrupto e em uma idade bastante precoce, caracterizado por deficiência de insulina, resistência periférica a esse hormônio e ausência de cetose; 2) diabetes pancreático tropical, presente em indivíduos severamente desnutridos, acompanhado por sinais de pancreatite, com macrocalcificações pancreáticas e falência na produção de insulina (RAO, 1988).

Mais recentemente a Organização Mundial de Saúde (OMS) apresentou sua nova classificação para o diabetes mellitus. Nesta nova classificação a desnutrição não é indicada como um fator diabetogênico (The expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). É possível, baseado em resultados do nosso grupo de pesquisa e

da literatura, verificar alterações no padrão secretório de insulina em ilhotas isoladas de animais submetidos à desnutrição.

Como no diabetes tipo 2, o comprometimento da secreção de insulina parece desempenhar uma função central na etiopatogenia do diabetes. Avaliando a insulinopenia no organismo desnutrido, pelo menos quatro possibilidades devem ser consideradas: 1) a redução do número de células B pancreáticas; 2) o defeito funcional das células B pancreáticas; 3) a combinação da redução do número de células B com alterações funcionais das células B remanescentes; 4) Alteração no sistema autônomo que inerva as ilhotas.

Nos últimos anos temos estudado as alterações morfológicas e funcionais das ilhotas pancreáticas em modelos de desnutrição pré e pós-natal (CARNEIRO et al., 1995; LATORRACA et al., 1998a, b). Observou-se que a restrição protéica determina a redução do peso absoluto, mas não do peso relativo do pâncreas (LATORRACA et al., 1998a). Quanto à morfologia, verificou-se células B com volume reduzido e com número menor de grânulos de insulina vários dos quais imaturos. Observou-se ainda, células B com degenerações mitocôndriais e com indícios de permeabilidade celular diminuída (CARNEIRO, 1996). Avaliando a capacidade funcional das ilhotas de ratos desnutridos, observou-se comprometimento da secreção de insulina estimulada por aminoácidos; perda do padrão bifásico de secreção, sensibilidade à glicose diminuída (CARNERO et al., 1995). Além de apresentarem captação de ^{45}Ca menor do que as ilhotas controle, quando em presença de diferentes concentrações de glicose. Portanto, defeito na mobilização do íon Ca^{2+} parece contribuir para as alterações secretórias verificadas em ilhotas de ratos desnutridos (CARNEIRO et al., 1995; LATORRACA et al., 1998b).

Curiosamente, algumas anormalidades observadas no organismo desnutrido, tais como, redução do volume das ilhotas, atrofia e perda de granulação das células B, insensibilidade das ilhotas à glicose, alteração da cinética secretória, são similares àquelas observadas no diabético do tipo 2 (GEPTS & LECOMPT 1981, GRODSKY 1996, LEAHY, 1996).

É provável que, a exemplo do que ocorre em modelos animais de diabetes tipo 2, alterações ultraestruturais na inervação da célula B (DIANI et al., 1983), nos níveis de neuropeptídeos (BAILEY & FLATT, 1988), ou na ação de neurotransmissores, como a

acetilcolina (HOLST, 1992), contribuam para a redução da secreção de insulina nas ilhotas de animais desnutridos.

Há relatos de modificações no sistema nervoso autônomo com alteração da modulação simpática e parassimpática em ratos Wistar que receberam dieta hipoprotéica (LEON-QUINTO et al., 1998). Animais submetidos a este tipo de dieta, apresentaram impulsos parassimpáticos diminuídos e simpáticos aumentados, acarretando uma elevação nos níveis de catecolaminas na circulação e possivelmente uma diminuição na estimulação colinérgica envolvida na potencialização da secreção de insulina.

Com a estimulação simpática exacerbada em ratos desnutridos, altos níveis de catecolaminas estariam circulantes atuando nos receptores encontrados na célula B. O resultado desta ligação seria a inibição da secreção de insulina (LEON-QUINTO et al., 1998). Esta característica pode ser também observada em pacientes com diabetes tipo 2 (CHRISTENSEN, 1979).

Mecanismo de Secreção de Insulina .

As células B respondem positivamente, secretando insulina, a mudanças na glicose extracelular, ativadores da fosfolipase-C (PLC) (acetilcolina (ACh) e CCK) e ativadores da adenilato ciclase (AC) (Glucagon e GIP) (HADESCOV, 1980; PRENTKI & MATSCHINSKY, 1987; TURK et al., 1993).

A secreção de insulina estimulada por glicose inicia-se com o reconhecimento e subseqüente fosforilação e metabolização deste nutriente pelas células B, gerando diversos sinais que levam à redução da condutância ao K^+ **(Fig. A)** A diminuição da condutância ao K^+ provoca um acúmulo relativo do referido cátion nas células B, com conseqüente despolarização da membrana e abertura dos canais de Ca^{2+} (L) sensíveis a voltagem. O influxo de Ca^{2+} e o aumento do Ca^{2+} intracelular ($[Ca^{2+}]_i$) ativa a maquinária exocitótica, finalizando com a migração das vesículas armazenadoras de insulina em direção à membrana e subseqüente extrusão do conteúdo granular para o líquido intersticial (HADESCOV, 1980; SUTTER, 1982; PETERSEN & FINDLAY, 1987; GRODSKY, 1989).

Um efeito secundário da elevação do $[Ca^{2+}]_i$ é a estimulação da adenilato ciclase (AC) e da fosfolipase C (PLC) que podem ser diretamente ativadas por receptores de membrana específicos para hormônios (FLATT, 1996), e gerando AMP_c e DAG ativadores da proteína quinase A (PKA) e proteína quinase C (PKC), respectivamente.

Vários estudos têm demonstrado que a acetilcolina, na presença de concentrações sublimiares de glicose, pode despolarizar a membrana da célula B e iniciar a atividade elétrica que culmina com a secreção de insulina. Porém, esse efeito sobre a atividade elétrica foi observado mediante a despolarização prévia não só pela glicose como também por uma variedade de agentes estimulatórios (GAGERMAN et al., 1978; COOK et al., 1981; HERMANS et al., 1986; SANTOS & ROJAS, 1989). Três mecanismos iônicos foram propostos para explicar a despolarização induzida pela estimulação muscarínica: 1) aumento da permeabilidade ao Na^+ (HENQUIN et al., 1988); 2) aumento da permeabilidade ao Ca^{2+}; 3) redução da permeabilidade ao K^+ (SANTOS & ROJAS, 1989).

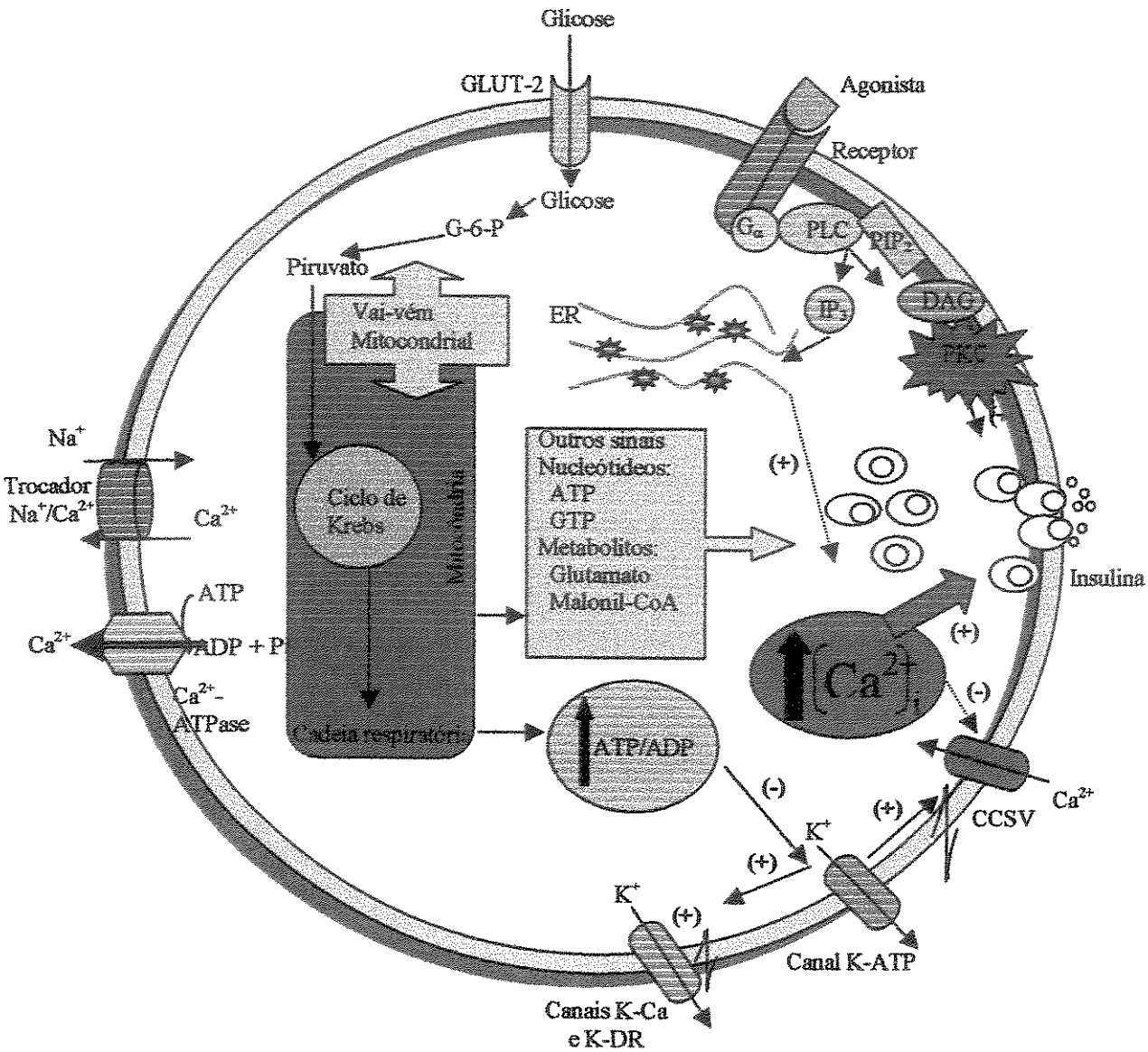

Figura A - Representação esquemática do mecanismo de acoplamento estímulo-secreção na célula B pancreática, onde estão ilustrados os principais canais iónicos existentes na membrana plasmática e receptores que estimulam a hidrólise de fosfolipídios membranares, bem como a sequência de eventos que conduz à secreção de insulina. Sumariamente, o metabolismo da glicose produz vários fatores de acoplamento, entre os quais se destaca o aumento da razão ATP/ADP. Este aumento conduz à inibição de canais de potássio sensíveis a ATP (canais K-ATP), com consequente despolarização da membrana e ativação de canais de Ca^{2+} sensíveis à voltagem (CCSV). O aumento da $[Ca^{2+}]_i$ constitui o fator determinante da exocitose dos grânulos de insulina. Canal K-Ca: Canal de K^+ sensível a Ca^{2+}; Canal K-DR: canal de K^+ rectificador tardio; DAG: diacilglicerol; G-6-P: glicose-6-fosfato; GLUT-2: transportador de glicose tipo 2; G_α: subunidade α da proteína G; ER: retículo endoplasmático; IP_3: inositol trisfosfato; PIP_2: fosfatidilinositol bisfosfato; PLC: fosfolipase C; PKC: proteína quinase C.

Proteína quinase C (PKC)

A PKC foi inicialmente descrita como uma quinase dependente de Ca^{2+} e fosfolipídios (TAKAI ET AL., 1979), que apresenta uma vasta distribuição em diversos tecidos. A posterior descoberta de que o DAG estimula fortemente a atividade da PKC (KISHIMOTO ET AL., 1980) despertou grande interesse pela quinase por parte de investigadores interessados na elucidação dos mecanismos através dos quais os sinais externos são transmitidos para o interior da célula. A identificação da PKC como ligante dos ésteres de forbol (TPA e PMA), promotores de tumores em diferentes tecidos (CASTAGNA ET AL., 1982, NIEDEL ET AL., 1983), veio também implicar esta quinase nos processos de gênese tumoral.

Atualmente é claro que a PKC constitui uma família de quinases de serina/treonina, com um número de membros ainda crescente. As isoenzimas da PKC podem ser classificadas em subfamílias, que apresentam diferentes mecanismos de regulação. As isoformas 'convencionais', cPKC-α, βI, βII e γ, requerem fosfolipídios carregados negativamente (ex. fosfatidilserina), DAG e Ca^{2+} para a sua ativação ótima (**Fig. B**), sendo também ativadas por ésteres de forbol, de forma independente de DAG e com uma menor necessidade de Ca^{2+} (CASTAGNA ET AL., 1982). As isoformas 'novas', nPKC-δ, ε, θ e η/L, são insensíveis a Ca^{2+}, mas dependem de DAG e fosfatidilserina para a sua ativação (ONO ET AL., 1988; BAIER ET AL., 1993), funcionando também como ligantes para os ésteres de forbol (KONNO ET AL., 1989). As isoformas 'atípicas', aPKC-ζ e λ/ι, são insensíveis a Ca^{2+} e não respondem nem a DAG nem aos ésteres de forbol (ONO ET AL., 1989).

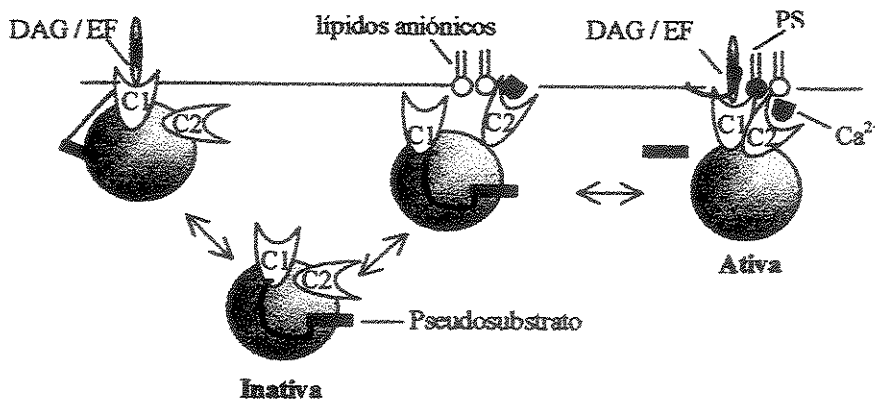

Figura B - Representação esquemática da PKC na sua forma inativa (no citossol), da sua translocação para a membrana através do domínio C1 (esquerda) ou do domínio C2 (centro), e da sua ativação resultante da ligação dos domínios C1 e C2 à membrana (direita). DAG: diacilglicerol; EF: ésteres de forbol; PS: fosfatidilserina.

Uma das características mais marcadas da regulação da PKC é a extrema especificidade para os seus ativadores lipídicos. A enzima é maximamente ativada por sn-1,2-diacilglicerol, tanto em micelas mistas de detergente/lipídeos como em modelos membranares, sendo o sn-1,3-DAG e o sn-2,3-DAG inativos (BELL & BRUNS, 1991). Tem-se admitido que a natureza dos grupos acilo do DAG não parece ser crítica para a sua interação com a PKC. No entanto, a presença de ácidos graxos poli-insaturados na posição sn-2 do DAG parece ser importante para a ativação da quinase (PETTITT et al., 1997). Foi ainda demonstrado num estudo recente, através da síntese de DAGs com grupos acilo poli-insaturados ω-6 e ω-3 na posição sn-2, que a ativação *in vitro* de diferentes isoformas da PKC varia em resposta a diferentes espécies de DAGs (MADANI et al., 2001).

O aumento do nível de DAG, produzido durante a ativação celular tem normalmente um caráter bifásico. A produção inicial de DAG é assegurada pela hidrólise de fosfatidilinositol-4,5-bisfosfato, catalisada pela fosfolipase C (PLC). O DAG assim produzido é rapidamente convertido em ácido fosfatídico por ação da DAG quinase. A segunda fase prolongada resulta da hidrólise de fosfatidilcolina, tendo o DAG resultante uma composição diferente em ácidos graxos, que o tornam um substrato mais fraco para a DAG quinase. O DAG também pode ser formado por hidrólise de ácido fosfatídico,

catalisada pela fosfolipase D (NISHIZUKA, 1995). Inicialmente foi proposto que só o DAG gerado pela hidrólise de fosfatidilinositois, e não o gerado a partir da fosfatidilcolina, era capaz de ativar a PKC (LEACH et al., 1991). Posteriormente esta idéia foi refutada, defendendo alguns autores que os diferentes DAGs produzidos pela célula ativam diferentes subfamílias da PKCs (HA & EXTON, 1993).

A ativação da PKC pela fosfatidilserina também revela uma elevada especificidade em relação à sn-1,2-fosfatidil-L-serina. Pensa-se que esta especificidade deriva do reconhecimento estereoespecífico do fosfolípidio por determinantes da quinase (JOHNSON et al., 1998).

A PKC é vista atualmente como um componente crítico de certas vias de transdução de sinais que muitas células utilizam para reconhecer e responder a uma variedade de agentes extracelulares (NISHIZUKA, 1986). Estes estímulos externos causam o aumento dos níveis de DAG na membrana, que funciona como um segundo mensageiro e ativa a PKC. As respostas celulares induzidas pela ativação da PKC resultam da fosforilação de proteínas alvo em resíduos de serina e treonina, envolvendo processos tão variados como a modulação de condutâncias iónicas e de transportadores (SHEARMAN et al., 1989; MOVSESIAN et al., 1984; LIANG, 1998), secreção de neurotransmissores e hormônios (COORSSEN et al., 1990; BITTNER & HOLZ, 1990), dessensibilização de receptores (BOEHM et al., 1996; LIANG et al., 1998), mobilidade e contractibilidade celular (FAN & BYRON, 2000; KOBAYASKI et al., 2001) e crescimento e diferenciação celular (BUTEAU et al., 2001, NISHIZUKA, 1986; 1992).

As primeiras evidências da existência da PKC na célula β pancreática datam do início da década de oitenta (LORD et al., 1984), com a descrição de uma atividade de quinase dependente de Ca^{2+} e de fosfolípidios. A regulação da PKC na célula B é já razoavelmente bem conhecida, sugerindo que a quinase apresenta características semelhantes às reportadas para outros tecidos no que se refere aos seus requisitos para ativação por DAG, Ca^{2+} (LORD ET AL., 1984), ácido araquidónico (BAND et al., 1992) e outros derivados dos ácidos graxos (BASUDEV et al., 1993). Mais ainda a enzima apresenta uma redistribuição intracelular após sua ativação (PERSAUD et al., 1989; EASOM et al., 1989).

Não há dúvida de que vários reguladores fisiológicos da secreção de insulina podem ativar a PKC na célula β. Vários secretagogos não nutrientes que atuam em receptores

membranares (como sejam a Ach, CCK ou vasopressina) ativam a PLC da célula B, levando à hidrólise de fosfatidilinositol bisfosfato e à produção de DAG (BERRIDGE, 1987). Os nutrientes também aumentam o conteúdo de DAG na célula B, seja por síntese *de novo* (sendo esta via a mais relevante no caso da glicose (PETER-RIESCH et al., 1988)), quer por ativação da PLC dependente de Ca^{2+} (BIDEN et al., 1987).

Está também bem documentado que a ativação das isoformas da PKC sensíveis a DAG e ésteres de forbol potencia fortemente a secreção de insulina induzida por glicose (ARKHAMMAR et al., 1989; GAO et al., 1994; DEENEY et al., 1996; HARRIS et al., 1996), sem no entanto exercer efeitos de potência comparável na dinâmica da $[Ca^{2+}]_i$ (ARKHAMMAR et al., 1989; KINDMARK et al., 1992; ZAITSEV et al., 1995). Esta dissociação entre os efeitos da ativação da PKC na secreção e em outros parâmetros fisiológicos da célula β, assim como a observação de que a aplicação de um éster de forbol a células permeabilizadas, em que a $[Ca^{2+}]_o$ é mantida constante, potencializa a secreção de insulina (JONES et al., 1989; 1992), sugerem que a PKC tem um efeito de sensibilização da maquinaria exocitótica à $[Ca^{2+}]_i$.

Tendo em conta estas evidências, é pertinente levantar a hipótese de que a ativação da PKC pode estar envolvida na resposta secretora da célula B à glicose, ou a outros secretagogos que estimulem a hidrólise de fosfolipídios membranares (ex. acetilcolina). Neste campo, os resultados não são conclusivos, e muitas vezes confusos e controversos, com alguns autores a considerarem que a ativação da PKC é essencial para a resposta fisiológica à glicose (THAMS et al., 1990; STUTCHFIELD et al., 1986; ZAWALICH et al., 1991) e outros a considerarem que o envolvimento da quinase só é relevante para a resposta a agonistas muscarínicos (ARKHAMMAR ET AL., 1989; PERSAUD et al., 1989a; GAO et al., 1994).

Uma outra abordagem usada para estudar a ativação da PKC em resposta a secretagogos de insulina consiste em avaliar a translocação da enzima do compartimento citossólico para o compartimento membranar (KRAFT & ANDERSON, 1983), quer através da medição da atividade das isoformas sensíveis a DAG nas frações membranar e citossólica, quer através da imunodetecção de isoformas particulares da PKC. Embora os resultados obtidos com ésteres de forbol ou com agonistas muscarínicos não deixem dúvidas de que ocorre de fato uma redistribuição da PKC entre frações subcelulares

(EASOM et al., 1989; GANESAN et al., 1990; REGAZZI et al., 1990), os efeitos da glicose não são tão claros. Enquanto que em alguns trabalhos se observa um aumento da atividade da PKC associada à membrana induzido pela glicose (GANESAN et al., 1990; CALLE et al., 1992; ZAITSEV et al., 1995; YEDOVITZKY et al., 1997; MIURA et al., 1998), vários outros estudos indicam o contrário, não se observando qualquer translocação da PKC para a membrana em resposta a nutrientes secretagogos (EASOM et al., 1989; MARTIN et al., 1995; DEENEY et al., 1996).

Estudos mais recentes, que envolvem a utilização de inibidores da PKC mais seletivos e que permitem distinguir entre várias isoformas da enzima (HARRIS et al., 1995; HARRIS et al., 1996), assim como de peptídeos inibidores de isoformas específicas que permeiam a membrana (HARRIS et al., 1996), apontam para um modelo em que a secreção de insulina induzida pela glicose e por outros nutrientes, em ilhotas de rato, envolveria a ativação de isoformas atípicas da PKC, enquanto que a potenciação da secreção por secretagogos não nutrientes que ativam a PLC seria mediada pela ativação de uma ou mais isoformas convencionais e novas da PKC (JONES & PERSAUD, 1998b). O mecanismo subjacente à ativação das isoformas atípicas da PKC não é claro, mas um candidato possível é o fosfatidilinositol 3,4,5-trisfosfato, que ativa a PKC-ζ *in vitro* (NAKANISHI et al., 1993) e pode ser gerado nos ilhotas por ativação da fosfatidilinositol 3-quinase em resposta a secreção de insulina estimulada por nutrientes (glicose e aminoácidos) (ALTER & WOLF, 1995).

Os elementos do citoesqueleto estão envolvidos no movimento dos grânulos secretores dentro da célula β (HOWELL, 1984), e podem ser também locais susceptíveis de sofrer regulação por fosforilação mediada pela PKC, como acontece em células cromafins (VITALE et al., 1992). A proteína MARCKS de 80-KDa, aliás a primeira proteína a ser identificada como substrato da PKC em células Swiss 3T3 (ROZENGURT et al., 1983), é uma proteína que liga actina, tendo sido observada a sua fosforilação em ilhotas intactos de rato e camundongo em resposta a ésteres de forbol, agentes colinérgicos e glicose (ARKHAMMAR et al., 1989; CALLE et al., 1992; EASOM et al., 1990).

A fosforilação da MARCKS pela PKC faz com que a proteína perca a sua capacidade de estabelecer ligações cruzadas com os filamentos de actina, destabilizando-os e levando à disrupção da rede do citoesqueleto (VITALE et al., 1995). Um outro potencial local de

modulação pela fosforilação mediada pela PKC é a reação final de exocitose, incluindo os processo de ancoragem, preparação dos grânulos e sua fusão com a membrana plasmática. Todo este mecanismo é regulado por proteínas da membrana granular, da membrana plasmática e também proteínas citossólicas, podendo algumas delas servir de substratos para a PKC (VAUGHAN et al., 1998). Na célula β foram já encontrados vários substratos para a PKC que se localizam nos grânulos secretores (BROCKLEHURST & HUTTON, 1984; HUTTON et al., 1984), mas a sua identidade ainda permanece desconhecida. Uma exceção é a anexina I, uma proteína granular implicada nos eventos de fusão membranar, cuja fosforilação mediada pela PKC ocorre após estimulação com glicose (OHNISHI et al., 1995).

Proteína Quinase A

As proteínas quinases dependente de AMPc (PKAs) são importantes para a transdução de sinais hormonais e estão envolvidas em diversas cascatas enzimáticas que culminam com a fosforilação de substratos chaves no metabolismo celular (KREBS & BEAVO, 1979), proliferação (BOYNTON & WITHFIELD, 1983), diferenciação (CHO-CHUNG, 1990) e transcrição gênica (MAURER, 1981).

Podemos encontrar principalmente duas isoformas de PKA, denominadas PKAI e PKAII as quais são identificadas de acordo com a sua separação em colunas de DEAE-celulose (REINMANN et al., 1971; CORBIN et al., 1975). A clonagem de cDNAs para as subunidades regulatórias da PKA identificaram as seguintes subunidades: RIα (LEE et al., 1983; SANDBERG et al., 1987) e RIβ (CLEGG et al., 1988; SOLBERG et al., 1991) e outras duas subunidades denominadas RIIα (SCOTT et al., 1987; OYEN et al., 1989) e RIIβ (JAHNSEN et al., 1986; LEVY et al., 1988). Mais ainda é possível encontrar diferentes domínios catalíticos denominados Cα (UHLER et al., 1986) e Cβ (UHLER et al., 1986; SHOWERS et al., 1986). As subunidades RI e RII possuem um domínio amino-terminal responsável pela interação com as subunidades C e um domínio carboxi-terminal responsável pela ligação ao AMPc, estes sítios de ligação para o AMPc denominam-se A e B(CORBIN et al., 1978; DOSKELAND et al., 1978).

A associação das subunidades R e C depende dos resíduos ácidos entre os aminoácidos 15-258 na subunidade R, estes resíduos geram interações eletrostáticas com domínios específicos na subunidade C (LEON et al., 1997). Quando o AMPc liga-se aos seus respectivos sítios A e B ocorre uma alteração conformacional na proteína que culmina com a liberação das subunidades catalíticas (DOSKELAND et al., 1993) estas por sua vez podem fosforilar os substratos alvo.

A PKA pode ser encontrada em diversos tecidos, mas a expressão de suas subunidades R e C pode ser diferenciada em diferentes tecidos (CADD & MCKNIGHT, 1989). Foi demonstrado que a subunidade RIα é expressa em coração e sistema nervoso central, por outro lado a RIβ é especialmente encontrada em tecidos nervosos tais como: medula e cérebro. Além disso é possível encontrar as subunidades RIIα e RIIβ em cérebro, mas podemos encontrar RIIα predominantemente no coração e RIIβ predominantemente no fígado e tecido adiposo (CUMMINGS et al., 1996).

No pâncreas endócrino quando temos aumento dos níveis de AMPc em resposta a estímulos externos, o AMPc liga-se as subunidades regulatórias (R) da PKA e esta última libera suas subunidades catalíticas (C). Estas subunidades (C) fosforilam e ativam proteínas alvos no citoplasma e núcleo, ativando o processo secretório **(Figs. C e D).**

A expressão da PKA é finamente regulada por hormônios os quais atuam através de proteínas G acopladas a receptores (JAHNSEN et al., 1985; LANDMARK et al., 1993; OYEN et al., 1988), sinais mitógenos via receptores associados a proteínas tirosina quinase, bem como hormônios esteróides (LEVY ET AL., 1989). A regulação da expressão da PKA por hormônios ocorre via AMPc, pois o AMPc é capaz de alterar a expressão gênica das subunidades da PKA alterando a transcrição gênica (TASKEN et al., 1991; TASKEN et al., 1993), estabilidade do RNAm (KNUTSEN et al., 1991) e também a estabilidade das subunidades R e C após a dissociação da holoenzima por AMPc (TASKEN et al., 1993; HOUGE et al., 1990).

As subunidades C estão associadas a dimeros de subunidades R, mais ainda as isoformas PKAI são capazes de se dissociar mais rapidamente em presença de AMPc que as isoformas PKAII (DOSTMANN et al., 1990). Podem ocorrer diferentes combinações entre as subunidades R e C com formações de diferentes holoenzimas (Ex. $RI\alpha_2C_2$ e $RI\beta_2C_2$) estas diferentes holoenzimas formadas podem exercer diferentes funções em uma

mesma célula (HOUGE et al., 1990). A PKA pode se encontrar compartimentalizada dentro da célula, esta compartimentalização é dependente da ligação da sua subunidade R com substratos celulares (SCOTT et al., 1994). Estudos identificaram que as holoenzimas $RI\alpha_2C_2$ e $RI\beta_2C_2$ são solúveis e preferencialmente estão localizadas no citosol (MEINKOTH et al., 1990).

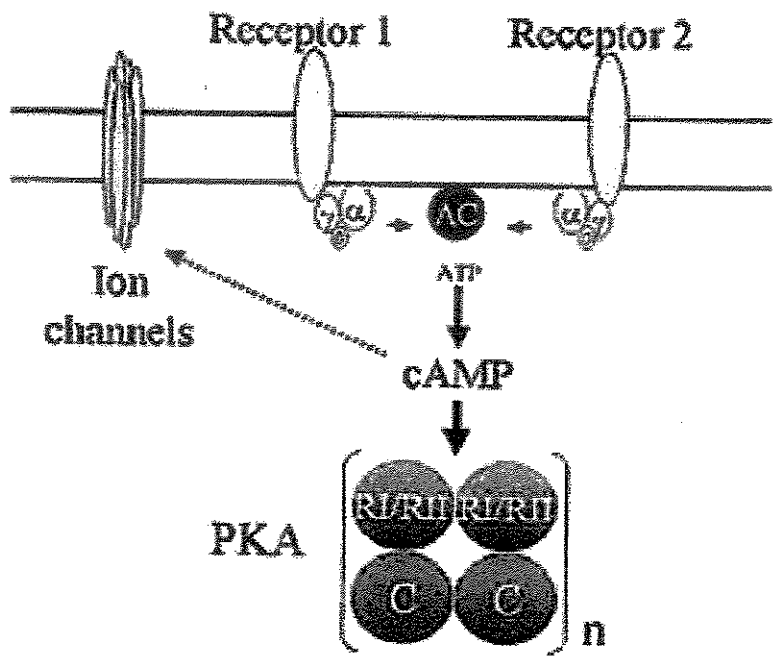

Figura C – AMP_c é gerado a partir do ATP quando uma proteína G acoplada ao receptor é ativada e estimula a adenilato ciclase (AC). AMP_c livre estimula e altera a atividade de três moléculas capazes de responder ao AMP_c (canais iônicos, Epac e PKA) RI ou RII e C são subunidades da PKA.

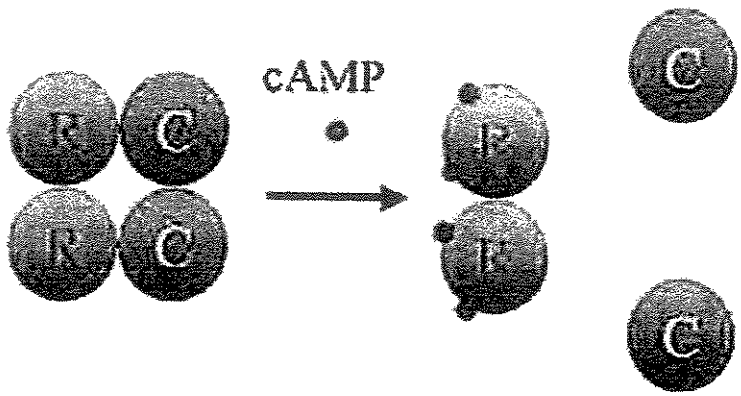

Figura D – PKA é uma holoenzima que possui duas subunidades regulatórias (R) e duas subunidades catalíticas (C). Ativação da PKA ocorre quando quatro moléculas de AMP_c se ligam às subunidades regulatórias da PKA (duas em cada subunidade).

Sinergismo entre PKC e PKA

Dentre as proteínas fosforiladas pela PKC está a PKA (NEWTON, 1995) o que indica uma possível interação entre estas duas quinases.

A fosforilação de substratos pela PKC também pode alterar a produção de segundos mensageiros na célula B, como parece ser o caso da fosforilação da proteína G_i, que modula a atividade da adenilato ciclase (AC) (PERSAUD et al., 1993a). De fato, um peptídeo com cerca de 40 KDa (a massa molecular aproximada da subunidade α da proteína G_i) foi descrito como um substrato da PKC em ilhotas intactas (DUNLOP & LARKINS, 1986).

A PKC pode ainda regular a transcrição gênica para subunidades de PKA, mostrando uma relação entre os níveis de PKC e PKA (TASKEN et al., 1990; TASKEN et al., 1992). Mais recentemente, demonstrou-se que a PKC e a PKA podem agir sinérgicamente, a PKA atuando na velocidade de movimentação das vesículas contendo os grânulos de insulina e a PKC agindo na colocalização de enzimas envolvidas no processo secretório e dos grânulos de insulina, auxiliando a movimentação das vesículas para a periferia celular. Células secretoras de insulina (HIT T15), quando em presença de altas concentrações de glicose, estimuladas com Forskolin e TPA, separadamente, secretaram duas vezes mais insulina (em relação a alta glicose). Quando se associou TPA e Forskolin, este aumento foi de quatro

vezes, mostrando mais uma vez a possível interação entre estas duas quinases no processo secretório (WEI et al., 2000).

Levando em consideração a importância das quinases PKA e PKC e possíveis alterações nestas enzimas ocasionadas pela desnutrição (NAIR et al., 1995; BANSAL et al., 1996; KATHAYAT et al., 1997; PAJARI et al., 2000; STEPHEN & NAGY, 1996) é de fundamental importância um estudo mais detalhado da funcionalidade destas duas enzimas.

2. OBJETIVOS

2. OBJETIVO

Geral:

Estudar os efeitos da desnutrição sobre a secreção de insulina e também sobre a expressão de enzimas fundamentais no processo secretório

Específicos:

1. Avaliar a secreção de insulina pelas células B das ilhotas de Langerhans isoladas de ratos submetidos à restrição protéica durante oito semanas.

2. Investigar os níveis de mRNA enzimas PKC e PKA em ilhotas isoladas de ratos submetidos à restrição protéica durante oito semanas .

3. Investigar a expressão das enzimas PKC e PKA em ilhotas isoladas de ratos submetidos à restrição protéica durante oito semanas.

A tese será apresentada no formato de artigo científico, uma vez que os resultados já foram publicados.

3. MATERIAL, MÉTODOS E DISCUSSÃO

ARTIGO 1

Decreased Cholinergic Stimulation of Insulin Secretion by Islets from Rats Fed a Low Protein Diet Is Associated with Reduced Protein Kinase Cα Expression[1]

Fabiano Ferreira, Eliane Filiputti, Vanessa C. Arantes, Luis F. Stoppiglia, Eliana P. Araújo, Viviane Delghingaro-Augusto, Márcia Q. Latorraca,* Marcos H. Toyama,† Antonio C. Boschero and Everardo M. Carneiro[2]

*Departamento de Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil; *Departamento de Nutrição e Dietética, Faculdade de Enfermagem e Nutrição, Universidade Federal de Mato Grosso, Cuiabá, MT, Brasil; and †Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil*

ABSTRACT Undernutrition has been shown to affect the autonomic nervous system, leading to permanent alterations in insulin secretion. To understand these interactions better, we investigated the effects of carbamylcholine (CCh) and phorbol 12-myristate 13-acetate (PMA) on insulin secretion in pancreatic islets from rats fed a normal (17%; NP) or low (6%; LP) protein diet for 8 wk. Isolated islets were incubated for 1 h in Krebs-bicarbonate solution containing 8.3 mmol glucose/L, with or without PMA (400 nmol/L) and CCh. Increasing concentrations of CCh (0.1–1000 μmol/L) dose dependently increased insulin secretion by islets from both groups of rats. However, insulin secretion by islets from rats fed the NP diet was significantly higher than that of rats fed the LP diet, and the dose-response curve to CCh was shifted to the right in islets from rats fed LP with a 50% effective concentration (EC_{50}) of 2.15 ± 0.7 and 4.64 ± 0.1 μmol CCh/L in islets of rats fed NP and LP diets, respectively ($P < 0.05$). PMA-induced insulin secretion was higher in islets of rats fed NP compared with those fed LP. Western blotting revealed that the protein kinase (PK)Cα and phospholipase (PL)Cβ$_1$ contents of islets of rats fed LP were 30% lower than those of islets of rats fed NP ($P < 0.05$). In addition, PKCα mRNA expression was reduced by 50% in islets from rats fed LP. In conclusion, a reduced expression of PKCα and PLCβ$_1$ may be involved in the decreased insulin secretion by islets from LP rats after stimulation with CCh and PMA. J. Nutr. 133: 695–699, 2003.

KEY WORDS: • *low protein diet* • *carbamylcholine* • *insulin secretion* • *protein kinase Cα* • *phospholipase Cβ1.*

A relationship between a high prevalence of malnutrition and diabetes has been observed in developing countries. Malnutrition in general and dietary protein deprivation in particular are associated with low plasma glucose levels in rodents and humans (1–5). Rats fed a diet containing a protein level comparable to that of undernourished humans have reduced insulin secretion as well as increased insulin sensitivity in peripheral tissues (2,4–8). In rats with protein-energy deficiency, the severely blunted insulin secretory response to glucose is related to a reduction in pancreatic B-cell mass and a lower responsiveness to glucose by the remaining B cells (5-10). Thus, the impairment of insulin secretion can be attributed in part to an intrinsic abnormality of the remaining B cells. However, protein-energy restriction may also affect extrapancreatic modulators of B-cell function such as the autonomic nervous system (11).

Cholinergic agents and glucose act synergistically on B cells to increase phosphoinositide (PI)[3] hydrolysis and insulin secretion (12). PI hydrolysis is stimulated by phospholipase (PL)Cβ1 through a mechanism coupled to G-protein. This coupling is initiated with the binding of the cholinergic agonist to muscarinic (M_3) receptors located in the B-cell plasma membrane (13). PI hydrolysis results in the formation of 1,4,5 inositol triphosphate (IP_3), which releases Ca^{2+} from intracellular stores, thereby increasing insulin secretion (14). Cholinergic agonists also activate PLC, which increases diacylglycerol (DAG) formation and stimulates protein kinase (PK)C. Thus, carbamylcholine (CCh)-induced insulin secretion can be reduced by inhibiting PKC (15).

Dietary protein deficiency decreases PKC activity in various rat tissues (16). In this study, we examined the effects of CCh

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[2] To whom correspondence should be addressed. E-mail: emc@unicamp.br.

[3] Abreviations used: Ach, acetylcholine; CCh, carbamylcholine; DAG, diacylglycerol; FFA, free fatty acid; IP_3, 1,4,5 inositol triphosphate; LP, low protein group; M_3, muscarinic; NP, normal protein group; PCR, polymerase chain reaction; PI, phosphoinositide; PKCα, protein kinase Cα; PLCβ$_1$, phospholipase Cβ$_1$; PMA, phorbol 12-myristate 13-acetate; PVX, potato virus X; RT, reverse transcribed; TTBS, Tris-Tween 20 buffered saline.

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on insulin secretion and the expression of PKCα and PLCβ_1 in islets isolated from rats fed a low protein diet.

MATERIALS AND METHODS

Animals and diet. The experiments described here were approved by the institutional (UNICAMP) Committee for Ethics in Animal Experimentation. Groups of 5 male Wistar rats (21 d old) from the breeding colony at UNICAMP were kept at 24°C with a 12-h light:dark cycle. The rats were randomly assigned to groups and fed an isocaloric diet containing 6% (low protein diet, LP) or 17% (normal protein diet, NP) protein for 8 wk. The composition and difference between the two isocaloric diets are described elsewhere (17) and in **Table 1**. During the experimental period, the rats consumed their respective diets and water ad libitum. At the end of the experimental period, the nutritional status of the rats was evaluated by measuring body weight, serum protein (Bio-Rad Laboratories GmbH, Munchen, Germany), albumin (18), glucose (DiaSys Diagnostic Systems, Holzheim, Germany), free fatty acid (FFA) levels (Nonesterified Fatty Acid C kit, Wako Chemicals, Neuss, Germany), and liver glycogen and fat content (19,20).

Insulin secretion. Islets were isolated by collagenase digestion of the pancreas as described (21). For static incubation, groups of five islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer with the following composition (in mmol/L): NaCl, 115; KCl, 5; $CaCl_2$, 2.56; $MgCl_2$, 1; $NaHCO_3$, 24; and glucose, 5.6; the buffer was supplemented with 3 g of bovine serum albumin/L 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 were incubated for 1 h with 8.3 mmol glucose/L and different concentrations of agonists. The insulin content of the medium at the end of the incubation period was measured by RIA (22). The CCh concentration producing a response that was 50% of the maximum (EC_{50}) was expressed as the mean negative logarithm (pD_2).

Western blotting. Groups of islets were pelleted by centrifugation (15,000 × g) and then resuspended in 50–100 μL of homogenization buffer containing protease inhibitors, as described (12,14). After isolation by collagenase digestion of pancreata and subsequent separation on discontinuous lyophilized Ficoll DL-400 gradients, a pool of least 500 clean islets from each experimental group was homogenized by sonication (15 s) 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 70 μg of protein from each experimental group were incubated for 5 min 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 8% polyacrylamide gels at 120 V for 30 min. Electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was done for 1 h at 120 V (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. PKCα and PLCβ_1 were detected in the membranes after a 2-h incubation at room temperature with mouse monoclonal antibodies against PKCα and PLCβ_1 (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:500 in TTBS containing 3% dry skimmed milk). The membranes were then incubated with a rabbit anti-mouse immunoglobulin G (diluted 1:1000 in TTBS containing 3% dry skimmed milk) followed by a further 2 h incubation at room temperature with ^{125}I -labeled protein A (diluted 1:1000 in TTBS containing 1% dry skimmed milk). Radiolabeled protein bound to the antibody was detected by autoradiography. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

mRNA expression. Total RNA from 500 islets was extracted using Trizol reagent (Life Technologies, Paisley, UK). For the polymerase chain reaction (PCR), RNA was reverse-transcribed (RT) using random primers. The resulting cDNA were amplified by PCR using oligonucleotides complementary to sequences in the PKCα gene (5′-CCTGCTCTACGGACTTATC-3′ and 5′-TGTAGTATTCACCCTCCTC-3′) and PVX (potato virus X) gene (5′-CGATCTCAAGCCACTCTCTCCG-3′ and 5′-GTAGTTGAGGTAGTTGACCC-3′), with the latter used as an external control. Actin was not used as an internal control because its expression was altered under our conditions (LP islets). The PCR was done in a 25 μL reaction volume containing 1 μL of cDNA equivalent to 2 μg of RNA, 10 mmol cold dNTP/L (dATP, dCTP, dGTP, dTTP), 50 mmol $MgCl_2$/L, 10X PCR buffer, 10 μmol of appropriate oligonucleotides primers/L, and 2 U of Taq polymerase (Life Technologies). The number of cycles was selected to allow linear amplification of the cDNA under study. The PCR conditions for amplification of PKCα (Gene Bank (X07289), size of fragment obtained by PCR (500 pb), primers position (579–1078) and PVX (Gene Bank (D00344), size of fragment obtained by PCR (106 pb), primers position (5597–5702)) were as follows: 2 min at 94°C followed by 32 cycles (30 s each) at 94°C, 57°C and 72°C (PKCα), and 2 min at 94°C followed by 23 cycles (30 s each) at 94°C, 57°C and 72°C (PVX). PVX RNA was obtained by in vitro transcription with RiboMAX Large Scale RNA Production System-T7 (Promega), following the instructions of the manufacturer. PVX sequence has no homology to any rat sequence as confirmed by BLAST search and RT-PCR (data not shown). Then, an aliquot of the external control was thawed on ice and 0.06 μg was mixed with fresh islets before extraction (23).

The PCR products were separated on 1.5% agarose gels in Tris borate EDTA buffer 1X (TBE 1X) and stained with ethidium bromide. All PCR reactions included a negative control. The absence of genome contamination in the RNA samples was confirmed by the RT-negative RNA samples. Subsequent digitalization and relative band intensities were performed employing an Eagle Eye II documentation system (Stratagene, La Jolla, CA). The results were expressed as a ratio of target to standard signals.

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

TABLE 1

Composition of the normal and low protein diets

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

* Difference between the two isocaloric diets.

RESULTS

Characteristics of the rats. After 8 wk, body weight, serum total protein, albumin and insulin levels of LP rats were lower, whereas serum FFA, liver glycogen and fat concentrations were greater than in NP rats ($P < 0.05$) (**Table 2**). The amount of protein in islets of rats fed NP and LP was similar.

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TABLE 2

Body weight, serum protein, albumin, glucose, insulin, free fatty acid and liver glycogen and fatty acid of normal protein (NP) and low protein (LP) rats after 8 weeks of dietary treatment

Parameters	NP	LP
Body weight, *g*	288.1 ± 7.8	236.5 ± 9.9*
Protein, *g/L*	53.0 ± 2.0	47.0 ± 3.0*
Albumin, *g/L*	35.0 ± 1.0	33.0 ± 1.0*
Glucose, *g/L*	1.27 ± 0.07	1.30 ± 0.15
FFA, *mmol/L*	0.4 ± 0.01	0.6 ± 0.09*
Insulin, *nmol/L*	0.15 ± 0.02	0.12 ± 0.03
Insulin (Fed), *nmol/L*	0.32 ± 0.1	0.11 ± 0.03*
Liver glycogen, *g/100g tissue*	7.0 ± 1.0	11.4 ± 1.0*
Liver fat, *g fatty acid/100 g tissue*	7.0 ± 0.5	12.3 ± 0.9*

Values are the means ± SEM, n = 10–15.
* Different from NP rats, $P < 0.05$.

However, there were differences in the profile of soluble protein in islets of rats fed NP compared with those fed LP (not shown).

Insulin secretion. Increasing concentrations of CCh (0.1–1000 μmol/L) dose dependently increased insulin secretion by islets from rats fed LP and NP, although absolute insulin secretion for each CCh concentration was higher in islets from rats fed NP than in those fed LP (**Fig. 1**). The EC_{50} were 2.15 ± 0.7 μmol/L and 4.64 ± 1.0 μmol/L for islets from rats fed NP and LP, respectively ($P < 0.05$).

PMA (400 nmol/L) also potentiated glucose-induced insulin secretion in both groups of islets. However, the increment in insulin secretion was significantly higher in islets from rats fed NP than in those fed LP ($P < 0.05$) (**Fig. 2**).

PKCα mRNA and protein expression. Western blotting indicated a 30% reduction in the expression of PKCα protein in islets from rats fed LP compared with those fed NP ($P < 0.05$) (**Fig. 3**). Similarly, RT-PCR revealed a 50% reduction in the expression of PKCα mRNA ($P < 0.05$) (**Fig. 4**). The expression of $PLC\beta_1$ protein was also reduced by 25% in islets from rats fed LP compared with those fed NP ($P < 0.05$) (**Fig. 5**).

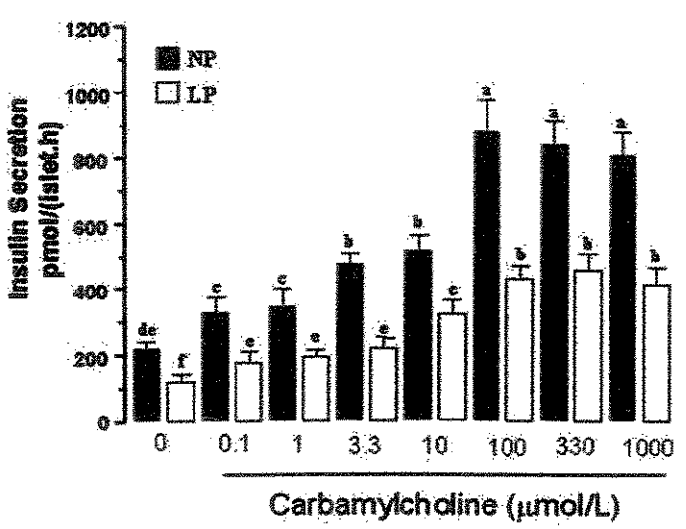

FIGURE 1 Carbamylcholine (CCh) stimulation of insulin secretion in islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. The columns represent the cumulative 1-h insulin secretions and are means ± SEM, n = 4–5 independent experiments. Means without a common letter differ, $P < 0.05$.

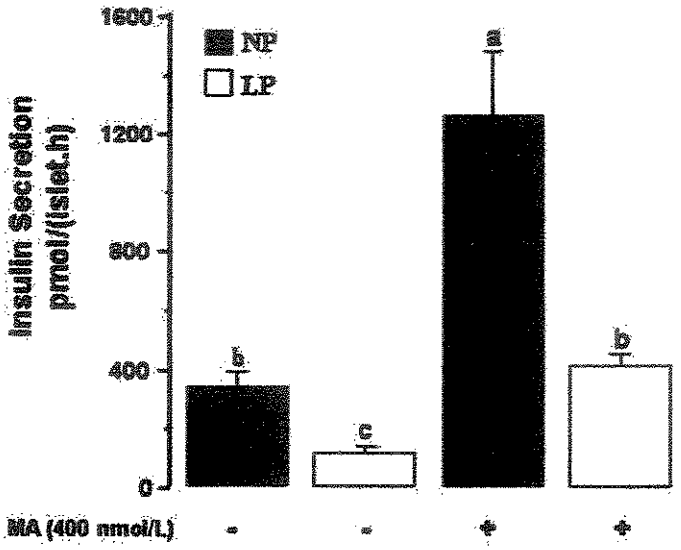

FIGURE 2 Phorbol 12-myristate 13-acetate (PMA) stimulation of insulin secretion in islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. The columns represent the cumulative 1-h insulin secretions and are means ± SEM, n = 4–5 independent experiments. Means without a common letter differ, $P < 0.05$.

DISCUSSION

Young rats fed a low protein (6%) diet for 8 wk exhibited several features similar to those found in malnourished infants

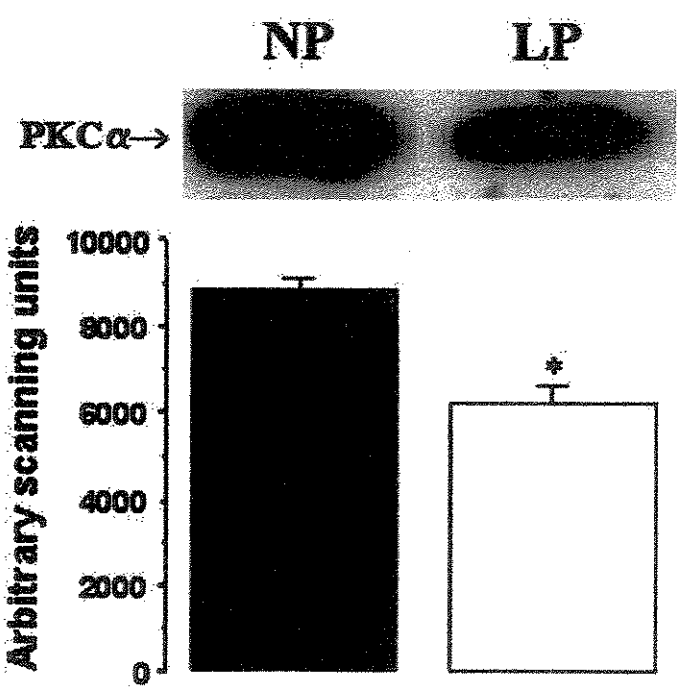

FIGURE 3 Protein kinase Cα (PKCα) content in islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. Values are means ± SEM, n = 4–5 independent experiments. *Different from NP, $P < 0.05$.

and in experimental models. These features included a low body weight, low levels of plasma albumin and insulin (Fed), and high liver glycogen and fat concentrations (3,4,20,24). Thus, these rats were a suitable model for the present study. Although insulinemia was reduced, glycemia did not differ in rats fed LP or NP. These findings may be related to a marked increase in insulin sensitivity explained by an increase in the phosphorylation of the insulin receptor and insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase (17).

The neural modulation of β-cells plays an important role in the control of insulin secretion. A relationship between loss of the first phase of secretion with the onset of type 2 diabetes has been established (25–27). The first phase of insulin secretion is important for glucose tolerance and is partially dependent on the ACh-activation of M_3 receptors present in the B-cell plasma membrane. Acetylcholine increases insulin secretion by activating M_3 receptors (21,28–30). In B cells, the coupling of ACh with this type of receptor stimulates PLC via G proteins (28,31–33) to generate DAG and IP_3, culminating with insulin secretion (29,34).

Because a low protein diet is associated with stress, with possible derangement of the sympathetic/parasympathetic equilibrium, we investigated the modulation of CCh-induced insulin secretion in rats fed a low protein diet. Insulin secretion induced by increasing concentrations of CCh was dose dependently increased in islets from both groups of rats. However, the dose-response curves for insulin secretion, as well as the EC_{50}, indicated that the potency of CCh was significantly reduced in islets from rats fed LP compared with those fed NP (Fig. 1).

Various intracellular messengers regulate insulin secretion in pancreatic islets. DAG and IP_3 are second messengers involved in CCh-induced insulin secretion via PKC. The precise mechanism of action of PKC on insulin stimulation is not yet fully understood, although alterations in K^+ and Ca^{2+} fluxes in β cells are involved (35–38). PKC also stimulates

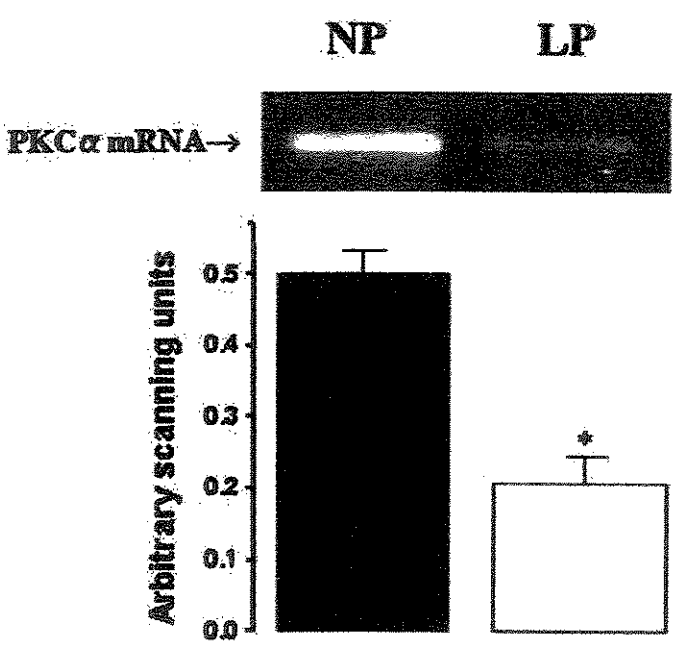

FIGURE 4 Protein kinase alpha (PKCα) mRNA levels in pancreatic islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. The columns are means ± SEM, n = 5–6 independent experiments. *Different from NP, $P < 0.05$.

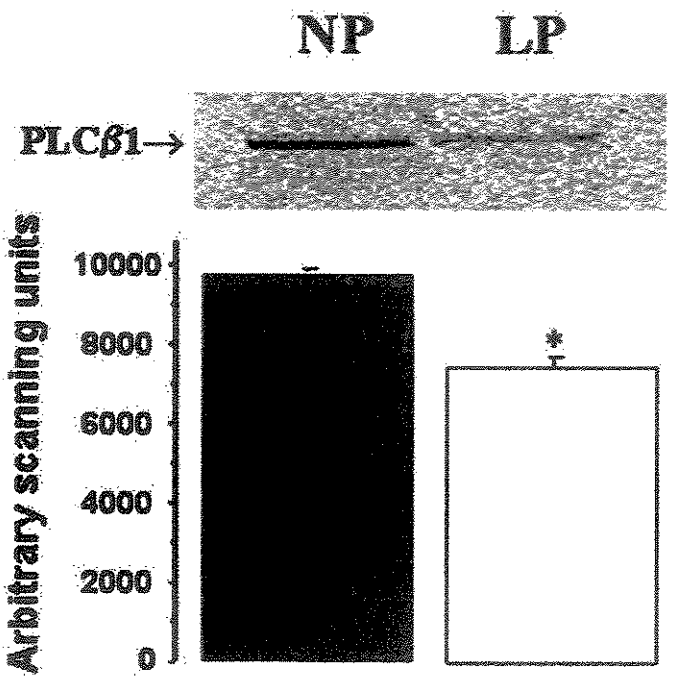

FIGURE 5 Phospholipase $C\beta_1$($PLC\beta_1$) content in islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. The columns are means ± SEM, n = 5–6 independent experiments. *Different from NP, $P < 0.05$.

secretion by facilitating the extrusion of insulin-containing granules (39), and exogenous activators such as phorbol esters (TPA or PMA) or DAG analogs stimulate PKC translocation in rat islet cells (40,41), indicating a relationship between PKC and insulin secretion (35). An attenuated PMA-induced insulin secretion was observed in islets from rats fed LP, suggesting a possible alteration in PKC levels. Several types of PKC are present in β cells, with PKCα as the major component (42–44). Because the content of PKCα was reduced in islets from rats fed LP, this factor could account for a decrease in glucose- and CCh-induced insulin secretion in islets from rats fed LP.

PKC also provokes an apparent paradoxical decrease in the intracellular Ca^{2+} concentration in B cells, probably by reducing Ca^{2+} entry via L-type channels (45). The Ca^{2+} efflux in LP was higher than in NP islets (not shown) confirming, although indirectly, the participation of PKCα in this process.

In conclusion, alterations mainly in PKCα levels and possibly other enzymes involved in this pathway, such as $PLC\beta_1$, may contribute to the poor secretory response induced by glucose, CCh and PMA in islets from rats fed LP. A low protein diet apparently decreases the transcription of genes that encode proteins involved in insulin secretion. When present over a long period of time, these alterations may affect the glucose homeostasis in LP rats.

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ARTIGO 2

Decreased Insulin Secretion in Islets from Rats Fed a Low Protein Diet Is Associated with a Reduced PKAα Expression[1]

Fabiano Ferreira, Helena C. L. Barbosa, Luiz Fabrizio Stoppiglia, Viviane Delghingaro-Augusto, Eliana A. Pereira, Antonio C. Boschero[2] and Everardo M. Carneiro

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

ABSTRACT A low protein diet has been shown to affect the amount and activity of several enzymes and to decrease insulin secretion by islets isolated from rats fed such a diet. To understand the mechanisms involved in this phenomenon, we investigated the effects of forskolin, a stimulator of adenylyl cyclase, on insulin secretion by pancreatic islets from rats fed a normal (17%; NP) or low (6%; LP) protein diet for 8 wk. Isolated islets were incubated for 1 h in Krebs-bicarbonate solution containing 8.3 mmol glucose/L, with or without 10 μmol forskolin/L. The forskolin-induced insulin secretion was higher in islets from NP rats than in those from LP rats ($P < 0.05$). Western blotting revealed that the amount of the α catalytic subunit of protein kinase A (PKAα) was 35% lower in islets from LP rats than in islets from NP rats ($P < 0.05$). Moreover, PKAα mRNA expression was reduced by 30% in islets from LP rats ($P < 0.05$). Our results indicated a possible relationship between a low protein diet and a reduction in PKAα expression. These alterations in PKAα may be responsible in part for the decreased insulin secretion by islets from rats fed a low protein diet. J. Nutr. 134: 63–67, 2004.

KEY WORDS: • *low protein diet* • *forskolin* • *insulin secretion* • *protein kinase α* • *gene expression*

The relationship among overnutrition, obesity and diabetes is well recognized (1), and several studies have also shown an association between undernutrition and alterations in insulin secretion (2–4). Rats fed a diet containing a protein level comparable to that of undernourished humans had decreased insulin secretion but increased insulin sensitivity in peripheral tissues (5–10). In addition, islets isolated from rats fed a low protein diet showed a decrease in the insulin secretory response to glucose, carbamylcholine and phorbol 12-myristate 13-acetate (PMA)[3] (2). This impairment is related at least in part to a reduction in pancreatic B-cell mass (11), lower responsiveness to nutrients by the remaining B cells (2,5–12) and a decrease in protein kinase C (PKC) levels (2).

Insulin secretion by B cells is controlled by various factors, including metabolic fuels, neurotransmitters released from intra-islet nerve endings, paracrine mechanisms and circulating hormones (13,14). Several modulators of insulin secretion act by activating protein kinases and phosphatases (15). One of these kinases is cAMP-dependent protein kinase (PKA), which is the major mediator of the cAMP signal transduction pathway in mammalian cells (16). This enzyme is responsible for the phosphorylation of target cytosolic and nuclear proteins, resulting in pleiotropic effects on cellular metabolism (17). In B cells, this enzyme is important for the phosphorylation reactions required for insulin secretion (18).

PKA is a serine/threonine kinase; structurally, it is a heterotetramer composed of a regulatory subunit (RI or RII) homodimer and two associated catalytic (C) subunits. Activation of the enzyme occurs when two cAMP molecules bind to each R subunit of PKA, resulting in the release of the C subunits (19).

Under normal physiologic conditions, the stimulation of adenylyl cyclase (AC) in B cells occurs through the G protein, mainly via the gut hormone receptors for glucose-dependent insulinotropic factor (GIP) (20) and glucagon-like peptide 1 (GLP-1) (21), whose levels increase after eating (22). Indeed, a strong relationship between gut hormone levels and food intake has been suggested (23).

Although the relationship between dietary protein deficiency and alterations in PKA is unclear and less studied, a relationship between the decrease in the regulatory PKA subunit and low protein diet was established (24). In this study, we examined the effects of forskolin on insulin secretion and the expression of catalytic subunit of PKAα in islets isolated from rats fed a low protein diet.

MATERIALS AND METHODS

Animals and diet. The experiments described here were approved by the institutional Committee for Ethics in Animal Exper-

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[2] To whom correspondence should be addressed.
E-mail: boschero@unicamp.br.

[3] Abbreviations used: AC, adenylyl cyclase; FFA, free fatty acid; GIP, glucose-dependent insulinotropic factor; GLP-1, glucagon-like peptide 1; LP, low protein group; NP, normal protein group; PKAα, protein kinase cAMP-dependent catalytic subunit α; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PVX, potato virus X.

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imentation. Groups of male Wistar rats (n = 5; 21 d old) from the breeding colony at UNICAMP were housed at 24°C on a 12-h light:dark cycle. The rats were separated at random and fed an isocaloric diet containing 6% (low protein diet, LP) or 17% (normal protein diet, NP) protein for 8 wk. The composition of the two isocaloric diets is shown in **Table 1**. During the experimental period, the rats consumed their respective diets and water ad libitum. At the end of 8 wk, the nutritional status of the rats was evaluated (**Table 2**) by measuring their body weight, the serum protein (Bio-Rad Laboratories GmbH, Munich, Germany), albumin (25), glucose (DiaSys Diagnostic Systems GmbH & Co., Holzheim, Germany) and free fatty acid (FFA; Nonesterified Fatty Acid C kit, Wako Chemicals GmbH, Neuss, Germany) levels and the liver glycogen and fat content (26,27).

Insulin secretion. Islets were isolated by collagenase digestion of the pancreas as described (28). For static incubations, groups of five islets were first incubated for 45 min 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 3 g 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 then replaced with fresh buffer and the islets incubated for 1 h with 8.3 mmol glucose/L and 10 μmol forskolin/L. The insulin content of the medium at the end of the incubation period was measured by RIA (29). The amount of protein in groups of 100 islets from LP and NP rats was similar. Thus, the results were expressed as pmol/(islet · h) or fmol/(islet · min).

Immunohistochemistry. To determine the tissue distribution of PKAα, hydrated 5-μm sections of paraformaldehyde-fixed, paraffin-embedded pancreatic tissue were stained with avidin-peroxidase as described (13). The PKAα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blotting. After isolation by collagenase digestion of pancreata and subsequent separation on discontinuous Ficoll DL-400 gradients, groups of islets were pelleted by centrifugation (750 × g for 10 min) and then resuspended in 50–100 μL of homogenization buffer containing protease inhibitors, as described (30–32). The islets were sonicated (15 s) and the protein was determined by the Bradford method (33) using bovine serum albumin as the standard. The volume of the samples was adjusted to provide the same amount of protein added to each lane. Samples containing 70 μg of protein from each experimental group were separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with Ponceau S. No differences in the total amount of protein were observed as judged by densitometric analysis of the stained membranes (not shown). The membranes were subsequently blotted with specific antibodies to PKAα (Santa Cruz). Visualization of specific protein bands was done by incubating the membranes with ^{125}I-protein A followed by exposure to RX-film. The band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

TABLE 1

Composition of the normal and low protein diets[1]

Ingredient	Normal protein (17% protein)	Low protein (6% protein)
	g/kg	
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinized cornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorhydrate	2.5	2.5

[1] See (55) for more details.

TABLE 2

Body weight, serum protein, albumin, glucose, insulin and free fatty acid (FFA) levels and liver glycogen and fatty acid (FA) contents in rats fed a normal protein (NP) or low protein (LP) diet for 8 wk[1]

Variable	NP	LP
Body weight, *g*	274.1 ± 8.5	221.4 ± 7.9*
Protein, *g/L*	55.2 ± 1.8	46.7 ± 3.5*
Albumin, *g/L*	38.0 ± 1.4	31.0 ± 1.2*
Glucose, *mmol/L*	7.3 ± 0.5	7.2 ± 0.9
FFA, *mmol/L*	0.3 ± 0.05	0.7 ± 0.04*
Insulin, *nmol/L*	0.18 ± 0.04	0.14 ± 0.03
Insulin,[2] *nmol/L*	0.35 ± 0.02	0.20 ± 0.03*
Liver glycogen, *g/100 g tissue*	6.8 ± 0.6	12.5 ± 1.3*
Liver fat, *g FA/100 g tissue*	6.7 ± 0.4	13.5 ± 0.7*

[1] Values are the means ± SEM, n = 12. * Different from NP rats, $P < 0.05$.
[2] These values were obtained from fed rats.

mRNA expression. Total RNA from 500 islets was extracted using Trizol reagent (Life Technologies, Paisley, UK). For the PCR, RNA was reverse-transcribed using random primers. The resulting cDNA were amplified by PCR using oligonucleotides complementary to sequences in the PKAα gene (5′-CCAAGAGAGTCAAGGGCAGGAC-3′ and 5′-CAACCTTTCTCGGTAAATCGC-3′) and potato virus X (PVX) gene (5′-CGATCTCAAGCCACTCTCTCCG-3′ and 5′-GTAGTTGAGGTAGTTGACCC-3′), with the latter used as an external control. The reactions were done in a 25-μL reaction volume containing 1 μL of cDNA equivalent to 2 μg of RNA, 10 mmol of cold dNTP/L (dATP, dCTP, dGTP, dTTP), 50 mmol of $MgCl_2$ /L, 10X PCR buffer, 10 μmol of appropriate oligonucleotides primers/L, and 2 U of Taq polymerase (Life Technologies). The number of cycles was selected to allow linear amplification of the cDNA. The PCR conditions for the amplification of PKAα (Gene Bank access no. X53261), size of amplified fragment −369 bp, primer position (−515 to −883) and PVX (Gene Bank access no. D00344), size of amplified fragment 106 bp, primer position (−5597 to −5702) were as follows: 2 min at 94°C followed by 32 cycles (30 s each) at 94°C, 55°C and 72°C (PKAα), and 2 min at 94°C followed by 23 cycles (30 s each) at 94°C, 57°C and 72°C (PVX). PVX RNA was obtained by in vitro transcription using the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI), according to the manufacturer's instructions. The PVX sequence had no homology to any rat sequence, as confirmed by a BLAST search and RT-PCR (data not shown). An aliquot of the external control was thawed on ice and 0.06 μg was mixed with fresh islets before extraction (34).

The PCR products were separated on 1.5% agarose gels in Tris borate 1X EDTA buffer (1X TBE) and stained with ethidium bromide. All PCR reactions included a negative control. The absence of genomic contamination in the RNA samples was confirmed by the RT-negative RNA samples. Subsequent digitalization and measurement of the relative band intensities were done using an Eagle Eye II documentation system (Stratagene, La Jolla, CA). The results were expressed as the ratio of the target to standard signals.

Statistical analysis. Values are means ± SEM. Student's unpaired t test was used to compare the body weight, the serum protein, glucose, albumin, insulin and FFA levels, and the liver glycogen and fat content. For comparing the changes in insulin secretion, the data were log-transformed to correct for heterogeneity in variance and then analyzed by two-way ANOVA, followed by the Tukey-Kramer test to determine significant differences between groups and among glucose and secretagogue concentrations, and to assess the interactions between these factors. The data were analyzed using a statistical software package (Statsoft, Tulsa, OK). The level of significance was set at $P < 0.05$.

RESULTS

After 8 wk, the body weight, total serum protein, serum albumin and insulin levels of LP rats were significantly lower, whereas the serum FFA level and the liver glycogen and fat content were greater than in NP rats ($P < 0.05$) (Table 2).

Under static incubation, insulin secretion in the presence of 2.8 mmol glucose/L did not differ in the two groups (data not shown). In 8.3 mmol glucose/L, insulin secretion in islets from LP rats was lower than in islets from NP rats ($P < 0.05$). Forskolin (10 μmol/L) increased insulin secretion in both groups, although insulin secretion in islets from LP rats was significantly lower than in islets from NP rats ($P < 0.05$) (Fig. 1). Dynamic perifusion showed that in 8.3 mmol glucose/L, the addition of forskolin (10 μmol/L) increased the insulin secretion by both groups of islets, with the levels being lower for islets from LP rats ($P < 0.05$) (Fig. 2). When the areas under the curves were calculated and the basal secretion of each group was subtracted, the lower secretion by islets from LP rats was even more evident ($P < 0.05$) (Inset, Fig. 2).

The localization of PKAα in islets from LP and NP rats was done by immunohistochemistry. Despite the difficulty of quantification by this method, it appears that the amount of PKAα was lower in islets from LP rats than in those from NP rats (Fig. 3).

The apparent reduction in the expression of PKAα in islets from LP rats compared with those from NP rats shown by immunohistochemistry was confirmed by Western blotting which indicated a 35% reduction in the expression of PKAα protein in islets from LP compared with NP rats ($P < 0.05$) (Fig. 4). Similarly, RT-PCR revealed a 30% reduction in the expression of PKAα mRNA in islets from LP rats ($P < 0.05$) (Fig. 5).

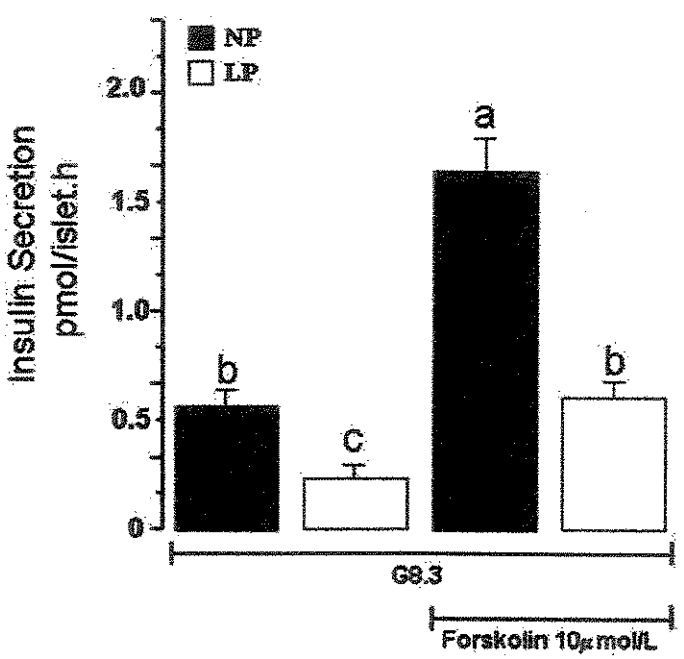

FIGURE 1 Forskolin-induced insulin secretion in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The columns represent the cumulative 1-h insulin secretion and are the means ± SEM; $n = 5$ independent experiments. Means without a common letter differ, $P < 0.05$.

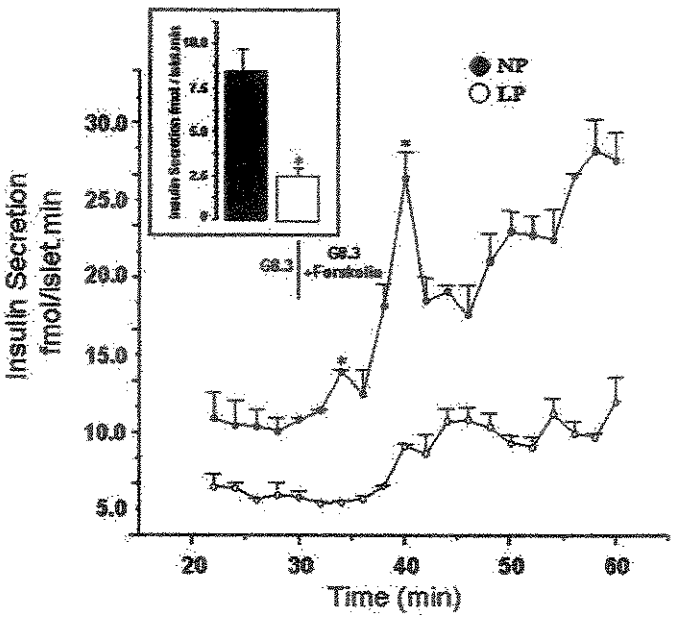

FIGURE 2 Forskolin-induced insulin secretion in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The lines represent the insulin secretion and are the means ± SEM; $n = 5$ independent experiments. The areas under the curves were calculated and the basal secretion of each group was subtracted (*Inset*).

DISCUSSION

Rats fed a low protein diet (6%) for 8 wk displayed several features similar to those found in malnourished infants and in experimental models, including a low body weight, low levels of plasma albumin and insulin, and high liver glycogen and fat contents. Similar results were reported in other studies (2,7,27,35,36). Insulinemia and glycemia in food-deprived rats were unaltered in LP compared with NP rats, whereas the insulinemia in fed rats was higher in NP than in LP rats. These data suggest that glucose homeostasis was maintained in LP rats by increased sensitivity to insulin as a result of alterations in the early steps of the insulin transduction pathway (3,4). The high carbohydrate content of LP diet was not responsible for the alterations in LP rats because recent work showed an increase in insulin secretion and in the activity of PKA and PKC in rats fed high carbohydrate (37).

PKA participates in the potentiation of glucose-induced insulin secretion by gastrointestinal hormones such as GIP and GLP-1 (20,21). This potentiation involves a series of reactions triggered by PKA, including the phosphorylation of vesicular and plasma membrane proteins, voltage-dependent channels and transcription factors (38). Conversely, the inhibition of PKA in isolated islets and insulinoma cells decreased glucose-induced secretion (39).

Because the insulin secretion induced by glucose was lower in islets from LP rats than in islets from NP rats, we used forskolin to investigate whether this reduction involved PKA. In several tissues (40,41), including the endocrine pancreas (42,43), forskolin activates AC to increase cAMP formation, which then stimulates PKA. In the pancreas, this stimulation of PKA leads to increased insulin secretion (44). The addition of forskolin to medium containing 8.3 mmol glucose/L increased the insulin secretion by both groups of islets, although the increase was smaller in islets from LP rats.

Together with previous data (2,3), these results led us to

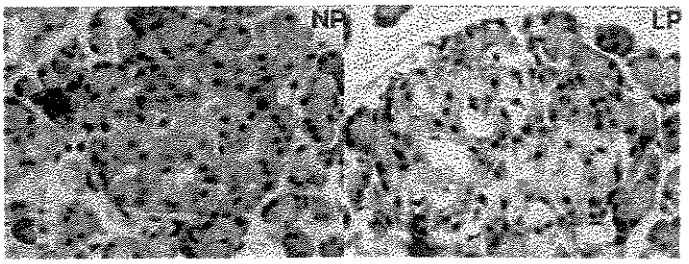

FIGURE 3 Immunohistochemistry of protein kinase cAMP-dependent catalytic subunit α (PKAα). The brown color indicates the localization of PKA in islets of rats fed normal (NP) and low protein (LP) diets for 8 wk.

believe that the lower insulin secretion seen with different secretagogues in islets from LP rats was rather nonspecific. However, this seems not to be the case if one considers that the extent of the reduction in insulin secretion in response to different stimulators was not the same in islets from LP rats. In addition, we observed that oligonucleotide antisense against insulin receptor substrate-1 restored the ability of glucose to stimulate insulin secretion in islets from LP rats (unpublished data).

The changes in forskolin-induced insulin secretion indicated an alteration in one or more steps of the cAMP-PKA pathway. To examine this possibility, we analyzed the expression of the α catalytic subunit of PKA (PKAα), and found that there were lower levels in islets from LP rats. In contrast to these findings, cDNA macroarray experiments showed an increase in the expression of the gene that encodes the regulatory subunit of PKA (unpublished data).

The concomitant reduction in PKAα protein levels and PKAα mRNA expression may be a consequence of the altered protein content in the diet. This conclusion is supported by reports (24,45) that showed a relationship between the levels and activity of PKA and a LP diet. There are two possible, but not exclusive explanations for the decrease in PKA levels and

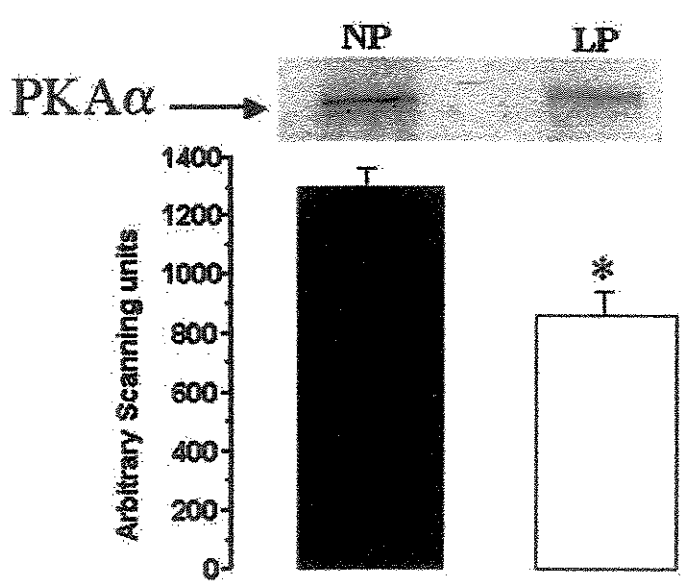

FIGURE 4 Western blot analysis of protein kinase cAMP-dependent catalytic subunit α (PKAα) in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. Values are means ± SEM, $n = 6$ independent experiments. *Different from NP rats, $P < 0.05$.

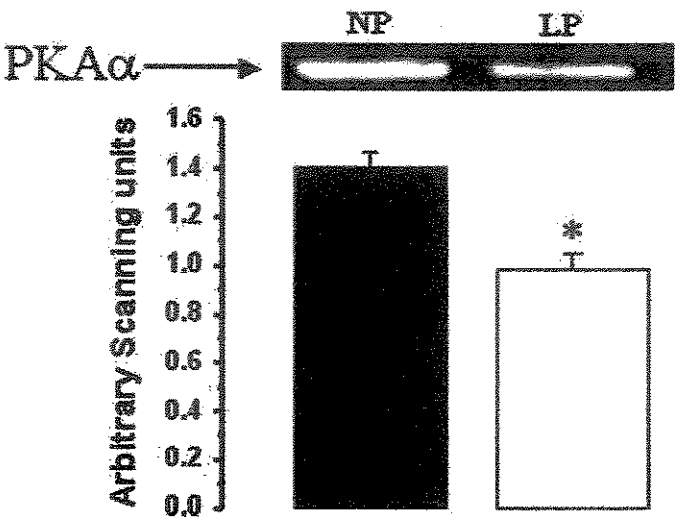

FIGURE 5 Protein kinase cAMP-dependent catalytic subunit α (PKAα) mRNA levels in pancreatic islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The columns are the means ± SEM; $n = 6$ independent experiments. *Different from NP rats, $P < 0.05$.

activity in the pancreatic islets of LP rats. Because the nutritional environment is important for controlling gene expression (46–48), it is conceivable that a low protein diet could directly affect the expression of several genes and their encoded proteins, including key enzymes involved in the secretory process.

Another possibility is that alterations in the protein content in the diet may influence the neuronal-endocrine axis (49), including the regulation of PKA subunit expression by different hormones (50–52). The increase in insulin secretion, in response to a combination of forskolin and PMA, is greater than that observed in the presence of each one of these drugs alone (42), which suggests a synergistic action of PKC and PKA on insulin secretion. Forskolin-stimulated insulin secretion by islets from LP rats was lower than for islets from NP rats (Figs. 1 and 2). Because PKCα expression is also reduced in islets from LP rats (2), it is conceivable that the synergistic effect of these enzymes may be disrupted in islets from LP rats. In addition to its effect on insulin secretion, PKC also modulates the expression of PKA subunit mRNA (53,54).

In conclusion, a low protein diet can decrease PKA levels in pancreatic islets, and this explains the reduced secretion observed in islets from LP rats in response to forskolin.

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4. CONCLUSÃO

- ✓ A desnutrição reduziu a secreção de insulina estimulada por PMA e Forskolin em presença de glicose em ilhotas de Langerhans isoladas de ratos submetidos à restrição protéica.

- ✓ A redução da secreção de insulina estimulada por PMA e Forskolin em presença de glicose está relacionada com uma menor expressão das enzimas PKCα, PLCβ_1 e PKAα em ilhotas isoladas de ratos submetidos à restrição protéica.

5. REFERÊNCIAS

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6. APÊNDICE

ARTIGO 3

A Low Protein Diet Alters Gene Expression in Rat Pancreatic Islets[1]

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

*Departamento de Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil and *Departamento de Fisiologia Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo (USP), São Paulo, SP, Brazil*

ABSTRACT Insulin secretion is regulated mainly by circulating nutrients, particularly glucose, and is also modulated by hormonal and neuronal inputs. Nutritional alterations during fetal and early postnatal periods, induced by either low protein or energy-restricted diets, produce β-cell dysfunction. As a consequence, insulin secretion in response to different secretagogues is reduced, as is the number of β-cells and the size and vascularization of islets. In this study, we used a cDNA macroarray technique and RT-PCR to assess the pattern of gene expression in pancreatic islets from rats fed isocaloric low (6 g/100 g, LP) and normal (17 g/100 g, NP) protein diets, after weaning. Thirty-two genes related to metabolism, neurotransmitter receptors, protein trafficking and targeting, intracellular kinase network members and hormones had altered expression (up- or down-regulated). RT-PCR confirmed the macroarray results for five selected genes, i.e., clusterin, secretogranin II precursor, eukaryotic translation initiation factor 2, phospholipase A_2 and glucose transporter. Thus, cDNA macroarray analysis revealed significant changes in the gene expression pattern in rats fed a low protein diet after weaning. The range of proteins affected indicated that numerous mechanisms are involved in the intracellular alterations in the endocrine pancreas, including impaired glucose-induced insulin secretion. J. Nutr. 134: 321–327, 2004.

KEY WORDS: • *protein restriction* • *gene expression* • *cDNA array* • *pancreatic islets*

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

Several studies have associated the control of gene expression with nutritional signals (2–6). The availability of free amino acids in the diet is important for maintaining protein homeostasis, and a deficiency in any of the essential amino acids can lead to a negative nitrogen balance. Alterations in nitrogen metabolism change the plasma amino acid profile (7–11). As a consequence, gene expression and physiologic functions are altered to cope with the limited availability of amino acids (12–14).

Intrauterine malnutrition induced by feeding dams a low protein diet during pregnancy and lactation also affects the structure and function of several organs in the offspring. A reduction in insulin secretion in response to glucose and different secretagogues, a reduction in the rate of islet-cell proliferation, and a reduction in islet size and vascularization were observed in the endocrine pancreas of malnourished rats (15–18). Alterations in the plasma amino acid profiles of dams and offspring occur when a low protein diet is introduced during gestation.

In this study, we examined the influence of dietary protein restriction on islet gene expression. Pancreatic islet RNA from rats fed low and normal protein diets was reverse-transcribed and hybridized to the Atlas cDNA array (Clontech), a commercial nylon membrane containing 1176 genes. The results of the macroarray analysis were confirmed by RT-PCR for five selected genes.

MATERIALS AND METHODS

Animals and diet. All of the experiments described here were approved by the institutional (UNICAMP) Committee for Ethics in Animal Experimentation. Male Wistar rats (28 d old) from the breeding colony at UNICAMP were maintained at 24°C on a 12-h light:dark cycle and had free access to food and water. The rats were distributed randomly into two groups and were fed a 17% (normal protein, NP)[3] or 6% (low protein, LP) diet for 8 wk, as described

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[2] To whom correspondence should be addressed.
E-mail: boschero@unicamp.br.

[3] Abbreviations used: EIF2-α, eukaryotic translation initiation factor 2 α subunit; ERK, extracellular signal-regulated kinase; FFA, free fatty acid; GLUT2, glucose transporter 2; HC, high carbohydrate; IGF-I, insulin-like growth factor-I; LP, low protein group; MAP2 kinase, microtube-associated protein kinase 2; MAPK2, mitogen-activated protein kinase 2; MMP, metalloproteinases; NP, normal protein group; PFK, phosphofructokinase; PKC, protein kinase C; PLA_2G1B, phospholipase A_2 group IB; PVX, potato virus X; TCA, trichloroacetic acid; TTBS, Tris-Tween 20 buffered saline.

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elsewhere (19). The energy difference between the two diets was balanced with additional carbohydrate instead of protein in the LP diet. At the end of the experimental period, the rats were weighed to measure their nutritional status. After decapitation, blood samples were collected and the sera were stored at −20°C for the subsequent measurement of total serum protein (Bio-Rad Laboratories GmbH, Munchen, Germany), serum albumin (20), serum glucose (DiaSys Diagnostic Systems, Holzheim, Germany), serum free fatty acid (FFA) levels (nonesterified fatty acid C kit, Wako Chemicals, Neuss, Germany), liver glycogen and fat content (21,22), and serum insulin (23).

Amino acid profile. Plasma samples were collected from fed and food-deprived (13 h) rats (NP and LP groups). The samples were deproteinized by adding 1 mL of 25% trichloroacetic acid (TCA) solution to 1 mL of plasma followed by storage at 4°C for 1 h. After centrifugation at 10,000 × *g*, 30 μL of the supernatant was mixed with 60 μL loading sample buffer (Biochrom 20 reagent kit), and 20 μL was analyzed by chromatography on a Biochrom 20 plus (Amersham Pharmacia, Piscataway, NJ) using a specific physiologic amino acid column. FFA standards were analyzed first, followed by the samples. Amino acids were quantified using Biochrom 20 control software version 3.05. Ammonia was also measured as an internal control for estimation of amino acid degradation.

Pancreatic islet isolation and static insulin secretion. Wistar rats were decapitated and the islets were isolated by handpicking under a stereomicroscope after collagenase digestion of the pancreas, as previously described (24). Groups of five islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer with the following composition (mmol/L): NaCl, 115; KCl, 5; $CaCl_2$, 2.56; $MgCl_2$, 1; $NaHCO_3$, 24 and glucose, 5.6, supplemented with 3 g of bovine serum albumin/L 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 were incubated with glucose (5.6; 8.3 or 16.7 mmol/L) for 1 h. The insulin in the medium at the end of the incubation period was measured by RIA (23).

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

The arrays were performed, in parallel, under identical conditions according to the manufacturer's instructions (Clontech). The Atlas rat array 1.2 consisted of 1176 genes spotted on positively charged nylon membranes. Plasmid and bacteriophage DNAs were included as negative controls to confirm hybridization specificity, and housekeeping cDNAs were used as positive controls to normalize the mRNA abundance. All of the cDNAs and controls immobilized on the membrane were grouped into several clusters according to their functions (25).

The membranes were prehybridized in ExpressHyb buffer containing 0.5 mg of heat denatured sheared salmon DNA at 68°C for 30 min. Labeled cDNA probe was added to the prehybridization buffer (30–170 kBq/membrane) and hybridization was continued overnight at 68°C. The membranes were washed and exposed directly to a storage phosphor screen (Molecular Dynamics). The screens were scanned using Storm 840. Signal intensities captured from each spot by Array Vision Evaluation 7.0 software were normalized using the intensities of the housekeeping genes [polyubiquitin, phospholipase A_2 group IB (PLA_2G1B) and ribosomal protein S29] provided in the array. This normalization allowed a quantitative comparison of the signal intensity of each gene on membranes from rats fed LP and NP. Differentially expressed genes were identified using Microsoft Excel XP. The result for each gene was expressed as the fold change in rats fed LP relative to the NP controls (expression arbitrarily designated as 1). The differences in gene expression in rats fed LP were considered relevant when the fold change was ≥2.0 (up-regulated) or ≤0.5 (down-regulated) compared with rats fed NP. The experiment was done twice using cDNA obtained from two different sets of 5 rats and new membranes each time. The genes were classified into different functional clusters on the basis of the putative biological function of the encoded protein, as determined by relevant database searches (26).

RNA isolation and RT-PCR. Semiquantitative RT-PCR was done to validate the findings of gene transcript expression shown by macroarray analysis. Briefly, total RNA was extracted from 300 islets obtained from rats fed LP and NP using the TRIzol reagent/phenol/chloroform procedure (Invitrogen). Before RT, the quality, purity and lack of contamination by genomic DNA were assessed by electrophoresis in denaturing gels, DNase treatment and PCR, respectively. Reverse transcription was done with 2 μg of total RNA using Moloney murine leukemia virus-reverse transcriptase (SuperScript II) and random hexamers, according to the manufacturer's instructions (Invitrogen). The cDNA obtained was stored at −20°C.

The PCR were then done in a 25-μL reaction volume containing 1 μL cDNA, 0.05 mmol/L of each cold dNTP (dATP, dCTP, dTTP, dGTP), 0.37 mmol/L $MgCl_2$, 0.25X PCR buffer, 0.1 μmol/L of appropriate oligonucleotide primers (Table 1) and 1 U of *Taq* DNA polymerase (Invitrogen). The number of cycles was selected to allow linear amplification of the cDNA.

RNA from a modified potato virus X (PVX; GenBank D00344) was used as an external control instead of β-actin because the expression of β-actin was altered in LP islets. PVX RNA was obtained by in vitro transcription with a "RiboMAX Large Scale RNA Production System-T7" (Promega), according to the manufacturer's instruction. The PVX sequence had no homology to any rat sequence, as confirmed by a BLAST search (27) and RT-PCR (data not shown). An aliquot of the external control was thawed on ice and 0.06 μg was mixed with fresh islets (n = 300) before RNA extraction (28).

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

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

RESULTS

Features of the rats. As observed in other studies (29–33), protein deprivation induced many functional and morphological alterations. In agreement with a previous report (33), rats fed the LP diet for 8 wk had a lower body weight, higher serum FFA levels, normoglycemia, decreased total serum protein and albumin levels, increased liver glycogen and fat contents, and lower serum insulin (fed) levels compared with NP-fed rats ($P < 0.05$).

Amino acids profile. Plasma amino acid concentrations in rats fed LP differed from those in the NP rats under both fed and food-deprived conditions (Table 2). Food-deprived LP rats had decreased levels of several amino acids, including taurine, aspartate, glutamate, glutamine, proline, methionine, β-alanine, phenylalanine, homocysteine, ornithine, histidine and increased levels of asparagine, serine, sarcosine, glycine, alanine, leucine, tyrosine, lysine and arginine compared with food-deprived NP rats. In contrast, in the fed state, LP rats had

TABLE 1

Sequences of PCR primers and PCR conditions for the analysis of specific mRNAs[1]

mRNA	GenBank accession no.	Primer sequences (5′–3′)	PCR reaction conditions				Size of PCR product (bp)
			Den	Ann	Ext	Cycles	
PLA_2G1B	D00036	CTGCTGGCTGCTTTGCTCAC ACGGCATAGACAGGAAGTGGC	94	58	72	25	458
EIF2-α	J02646	TCTTGCCCATGTTGCTGAGG TAACCACTTTGGGCTCCATCTG	94	57	72	32	503
SGII	M93669	TGCTGAGGCTTCCTTATGGTCC CCCAGATGCTCCTTGATGGC	94	57	72	28	268
Clusterin	M64723	AAACGACTCGCTCCAGGTGG ATCGCAAGGTGGCTTTATTGG	94	57	72	27	506
GLUT-2	J03145	CATTGCTGGAAGAAGCGTATCAG GAGACCTTCTGCTCAGTCGACG	94	55	72	26	408
PVX	D00344	CGATCTCAAGCCACTCTCCG GTAGTTGAGGTAGTTGACCC	94	57	72	23	106

[1] Abbreviations: Den, denaturation; Ann, annealing; Ext, extension; PLA_2G1B, phospholipase A_2 group IB; EIF2-α, eukaryotic translation initiation factor 2 α subunit; SGII, secretogranin II; GLUT-2, glucose transporter 2; PVX, potato virus X.

decreased levels of taurine, threonine, asparagine, glutamate, proline, valine, methionine, isoleucine, phenylalanine, hydroxylysine and arginine, whereas the levels of serine, glutamine, sarcosine, glycine, alanine, leucine, lysine and β-alanine were increased compared with rats fed NP.

Insulin secretion. Increasing concentrations of glucose (5.6, 8.3 and 16.7 mmol/L) caused a concentration-dependent release of insulin from islets of rats fed NP. Under the same conditions, insulin secretion by islets from rats fed LP was significantly lower than that by islets from rats fed NP (**Fig. 1**).

Gene expression. Of the 1176 genes included in the Atlas rat 1.2 array, 32 genes in islets from rats fed LP showed a significant change in their level of expression compared with islets from rats fed NP, in two different experiments. Of the 32

TABLE 2

Plasma amino acid and other N-containing compound concentrations in fed and food-deprived male Wistar rats fed normal (17 g/100 g, NP) and low (6 g/100 g, LP) protein diets for 8 wk after weaning[1]

	NP		LP	
	Fed	Food-deprived	Fed	Food-deprived
Taurine	1.29 ± 0.082	1.17 ± 0.014	1.01 ± 0.033*	0.53 ± 0.021*
Aspartate	0.21 ± 0.013	0.26 ± 0.050	0.27 ± 0.061	0.19 ± 0.016*
Threonine	1.43 ± 0.056	1.11 ± 0.064	0.95 ± 0.054*	0.99 ± 0.028
Serine	1.25 ± 0.015	2.88 ± 0.024	3.83 ± 0.035*	3.23 ± 0.023*
Asparagine	9.15 ± 0.078	0.31 ± 0.023	1.11 ± 0.057*	0.74 ± 0.042*
Glutamate	6.85 ± 0.023	3.03 ± 0.056	1.75 ± 0.023*	1.28 ± 0.054*
Glutamine	1.86 ± 0.016	7.76 ± 0.920	5.76 ± 0.066*	2.23 ± 0.067*
Sarcosine	4.57 ± 0.082	0.95 ± 0.029	7.02 ± 0.240*	8.45 ± 0.780*
Proline	4.84 ± 0.560	6.23 ± 0.052	3.26 ± 0.065*	4.20 ± 0.260*
Glycine	2.96 ± 0.063	2.45 ± 0.058	3.94 ± 0.057*	3.41 ± 0.076*
Alanine	2.68 ± 0.035	2.49 ± 0.036	6.05 ± 0.780*	5.52 ± 0.066*
Citrulline	0.86 ± 0.072	0.57 ± 0.036	0.86 ± 0.013	0.83 ± 0.032
Valine	1.99 ± 0.032	1.57 ± 0.042	1.62 ± 0.052*	1.33 ± 0.042
Cysteine	0.06 ± 0.009	0.10 ± 0.013	0.09 ± 0.014	0.21 ± 0.056*
Methionine	0.61 ± 0.014	0.61 ± 0.048	0.36 ± 0.080*	0.36 ± 0.018*
Isoleucine	0.97 ± 0.028	0.66 ± 0.028	0.76 ± 0.057*	0.62 ± 0.026
Leucine	1.17 ± 0.027	1.07 ± 0.032	1.42 ± 0.054*	1.33 ± 0.078*
Tyrosine	1.22 ± 0.019	1.06 ± 0.049	1.25 ± 0.069	1.40 ± 0.056*
β-Alanine	0.23 ± 0.012	0.88 ± 0.075	0.41 ± 0.018*	0.18 ± 0.064*
D,L-β-Aminoisobutyrate	2.46 ± 0.046	2.76 ± 0.083	2.36 ± 0.052	0.06 ± 0.036*
Phenylalanine	0.92 ± 0.027	0.58 ± 0.063	0.58 ± 0.082*	0.08 ± 0.036*
Homocysteine	0.03 ± 0.001	0.48 ± 0.094	0.02 ± 0.018	0.09 ± 0.072*
Ethanolamine	1.68 ± 0.063	1.13 ± 0.073	1.52 ± 0.026	0.79 ± 0.049*
Hydroxylysine	0.24 ± 0.050	0.32 ± 0.082	0.03 ± 0.005*	0.47 ± 0.046
Ornithine	0.72 ± 0.024	1.25 ± 0.057	0.68 ± 0.035	0.54 ± 0.056*
Lysine	2.71 ± 0.035	2.49 ± 0.078	3.63 ± 0.084*	2.92 ± 0.078*
Histidine	0.56 ± 0.091	0.63 ± 0.096	0.56 ± 0.053	0.47 ± 0.036*
Arginine	1.29 ± 0.027	1.07 ± 0.032	1.11 ± 0.078*	1.22 ± 0.036*

[1] Values are means ± SEM, $n = 4$. * Different from the corresponding NP group, $P < 0.05$.

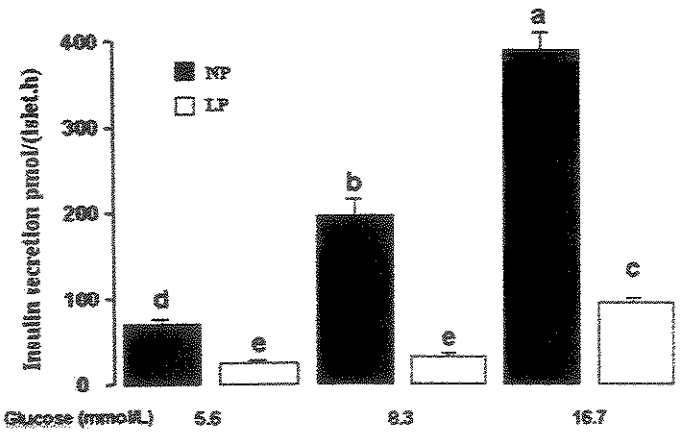

FIGURE 1 Insulin secretion by islets from rats fed normal (NP) and low protein (LP) diets at different concentrations of glucose. Five islets were preincubated in Krebs-Ringer bicarbonate buffer containing 5.6 mmol/L glucose for 45 min. Islet insulin-secretory responsiveness was determined after incubation in medium containing 5.6, 8.3 and 16.7 mmol/L glucose. Values are means ± SEM, $n = 10$. Means without a common letter differ, $P < 0.05$.

genes, 17 had a greater than twofold increase, whereas in 15 genes, the expression decreased by more than twofold. The genes involved were related to metabolism, hormones, trafficking and targeting proteins, extracellular kinase networks and other categories (**Tables 3** and **4**).

RT-PCR. Semiquantitative RT-PCR was done to validate the findings of the atlas cDNA array. The genes selected for analysis were eukaryotic translation initiation factor 2 α subunit (EIF2-α), secretogranin precursor, glucose transporter-2 (GLUT2), clusterin and PLA_2G1B. RT-PCR confirmed the macroarray results for all of these genes. The EIF2-α and PLA_2G1B mRNA concentrations did not differ in islets from NP and LP rats (**Fig. 2***A*, *E*). In contrast, the mRNA concentrations for secretogranin (32%), GLUT-2 (53%) and clusterin (31%) were significantly lower in islets from LP rats ($P < 0.05$) (Fig. 2*B*, 2*D*). The expression of PVX did not differ in islets from LP and NP rats when analyzed by RT-PCR (Fig. 2*F*). This result validates the use of this external standard/control for normalizing the gene expression in RT-PCR.

DISCUSSION

Recent studies have reported that nutritionally adverse conditions during the early and postnatal periods of life lead to alterations in pancreatic endocrine functions (31–36). Nutritional disturbances caused by a low protein diet during pregnancy can alter the endogenous amino acid profiles and lead to physiologic adaptations, including changes in gene expression, to cope with the limited amino acid availability (14).

As expected, LP rats showed features typical of malnutrition, including low body weight, low levels of serum albumin and insulin, and higher liver glycogen and fat contents. Despite a significant reduction in insulinemia, glycemia was unaltered in rats fed LP compared with rats fed NP. These findings may be related to a marked increase in insulin sensitivity through an increase in the phosphorylation of the insulin receptor and insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase (18,19,35).

Levels of valine, isoleucine, methionine, phenylalanine and threonine were lower in LP-fed rats. These amino acids are considered essential and a deficiency in any of them could lead to a negative nitrogen balance with clinical consequences (13). In addition, the amino acid taurine was reduced in both fed and food-deprived LP rats. Taurine influences glucose metabolism and consequently increases insulin secretion. A deficiency in taurine slows growth, decreases insulin secretion and induces cardiac dysfunction and immunological insufficiency (37–39).

Leucine, a stimulator of insulin secretion was also diminished in rats fed LP. The metabolization of leucine via the Krebs' cycle or by mitochondrial oxidation in β-cells can elevate the ATP/ADP ratio; this in turn may stimulate the release of insulin (40,41). Previous work showed that leucine-induced insulin secretion is affected by a low protein diet (18).

To assess the possible global transcriptional modifications caused by a low protein diet, a cDNA macroarray was used to monitor and compare gene expressions. The Atlas cDNA array 1.2, which contains 1176 genes, was used in this study. In islets from rats fed LP, 32 genes showed an altered expression when compared with islets of NP-fed rats. Tables 3 and 4 show that there were metabolic and structural adaptations in pancreatic islets from rats fed LP.

The expression of the GLUT-2 gene and its respective protein (measured by Western blotting, not shown) was re-

TABLE 3

Identification of up-regulated rat islets genes induced by a low protein (6 g/100 g) diet

Protein/gene	Accession number	Fold induction
Membrane channels and transporters		
Multidrug resistance protein	X96394	7.5
Simple carbohydrate metabolism		
Cytosolic hydroxymethylglutaryl-CoA synthase	X52625	4.0
Energy metabolism/other intracellular transducers, effectors and modulators		
Mitochondrial H^+ transporting ATP synthase F1 complex α subunit isoform 1	X56133	2.7
Muscle phosphofructokinase	U25651	3.2
Metalloproteinases		
Matrix metalloproteinase 14; membrane-type matrix metalloproteinase 1	X83537	2.0
Oncogenes and tumor suppressors		
Wilms' tumor protein homolog 1 (WT1)	X69716	21.1
Nucleotide metabolism		
cAMP-dependent protein kinase type-I α regulatory subunit	M17086	2.5
Growth factors, cytokines and chemokines		
Insulin-like growth factor II (IGF2)	M13969	3.6
Hormone receptors/G protein-coupled receptors		
Thyrotropin-releasing hormone	D17469	15.0
Estrogen receptor β (ESR2); nuclear receptor subfamily 3 group A member 2	U57439	7.3
Calcium-binding proteins		
NVP-3; neural visinin-like Ca^{2+} binding protein	D13126	2.0
Neurotransmitter receptors/ligand-gated ion channels		
γ-Aminobutyric acid receptor $\alpha 2$ subunit	L08492	4.3
Gastric inhibitory polypeptide receptor; glucose-dependent	L19660	3.1
Amino- and carboxypeptidases/other trafficking and targeting proteins		
Carboxypeptidase D	U62897	3.3
G proteins/other trafficking and targeting proteins		
Ras-related protein RAB26	U18771	4.0

TABLE 4

Identification of down-regulated genes in rat islets induced by a low protein (6 g/100 g) diet

Protein/gene	Accession number	Fold inhibition
Other trafficking and targeting proteins		
Sterol carrier protein 2 (SCPX); nonspecific lipid transfer protein	M34728	0.28
Simple carbohydrate metabolism		
Fructose-biphosphate aldolase A	M12919	0.23
Symporters and antiporters		
Solute carrier family 13 member 1	L19102	0.17
Intracellular transducers, effectors and modulators, transcription activators and repressors		
Signal transducer and activator of transcription 3 (STAT3)	X91810	0.23
Voltage-gated ion channel		
Voltage-gated ion channel		
Voltage-gated K+ channel protein	M59980	0.24
Intracellular kinase network proteins		
Mitogen-activated protein kinase 3, extracellular signal-regulated kinase 1 (ERK1), insulin-stimulated microtube-associated protein kinase 2 (MAP2 kinase)	M61177	0.46
Mitogen-activated protein kinase 2, extracellular signal-regulated kinase 2 (ERK2)	M64300	0.22
Calcium/calmodulin-dependent protein kinase type II beta subunit (CAM kinase II beta)	M16112	0.11
Protein phosphatase receptors		
Pheochromocytoma-derived protein tyrosine phosphatase-like protein	D38222	0.20
Phospholipases and phosphoinositol kinases/ kinase activators and inhibitors/amino acid metabolism		
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ/δ, protein kinase C inhibitor protein 1, mitochondrial import stimulation factor S1 subunit	D17615	0.16
Metalloproteinases		
Carboxypeptidase E	M31602	0.27
Cysteine proteases		
Cathepsin L	Y00697	0.25
Other extracellular communication proteins/trafficking and targeting proteins		
Secretogranin III	U02983	0.25
Facilitated diffusion proteins		
Glucose transporter	J03145	0.18
Amino and carboxypeptidases		
Metabolism of cofactors, vitamins and related substances		
Dipeptidase (DPEP1)	M94056	0.26
Stress response proteins		
Clusterin	M64723	0.24
Adenylate/guanylate cyclase and diesterases		
Adenylyl cyclase type VI, ATP pyrophosphate-lyase, Ca^{2+}-inhibitable adenylyl cyclase	L01115	0.27

duced in islets from rats fed LP. Using knockout mice for GLUT-2, Thorens et al. (42) showed a total loss of the first phase of glucose-stimulated insulin secretion and a reduced second phase during perfusion experiments. A similar pattern of insulin secretion was observed for islets from pups of dams fed a low protein diet during pregnancy and lactation (35). These results are consistent with GLUT-2 acting as a glucose sensor and regulator of glucose metabolism.

Islets from rats fed LP showed increased expression of muscle phosphofructokinase (PFK), a tetrameric protein with three isoforms, designated M (muscle), P (platelet) (or C) and L (liver), involved in the glycolytic pathway (43,44). All three isoforms were detected in pancreatic islets and clonal pancreatic β-cells (INS-1) (44,45). The autocatalytic activation of PFK by its product, fructose 1,6-bisphosphate, generates glycolytic oscillations. The overexpression of PFK in transgenic mice results in defective glucose metabolism followed by impaired glucose-induced insulin secretion (46). Thus, alterations in the levels of PFK isoforms may lead to changes in the enzymes properties and activity, and this could decrease glucose-induced insulin secretion.

Voltage-gated ion channels play an important role in the insulin secretion stimulated by glucose and other insulinotropic agents. An increase in the ATP/ADP ratio through glucose metabolism closes K_{ATP} channels and depolarizes β-cells. This depolarization leads to the opening of voltage-dependent Ca^{2+} channels and an influx of calcium that triggers insulin granule exocytosis (47,48). In islets from rats fed LP, there is a decreased movement of intracellular calcium and a reduced expression of protein kinase C (PKC)α (18,33,47). As shown here, there is also a reduced gene expression of voltage-gated K^+ channel proteins. This finding could explain in part the poor secretory response to glucose and other secretagogues in rats fed LP.

Alterations in the expression of the G-protein Rab 26 were observed. Although the functions of this protein are not completely understood, some Ras-related proteins have been implicated in intracellular vesicular trafficking along the biosynthetic and endocytic pathways, with functional links to SNARE complexes (49–51). Sequence comparisons between Rab 26 and other Ras proteins described in the literature revealed closest homology to Rab 8, Rab 3A, Rab 15 and

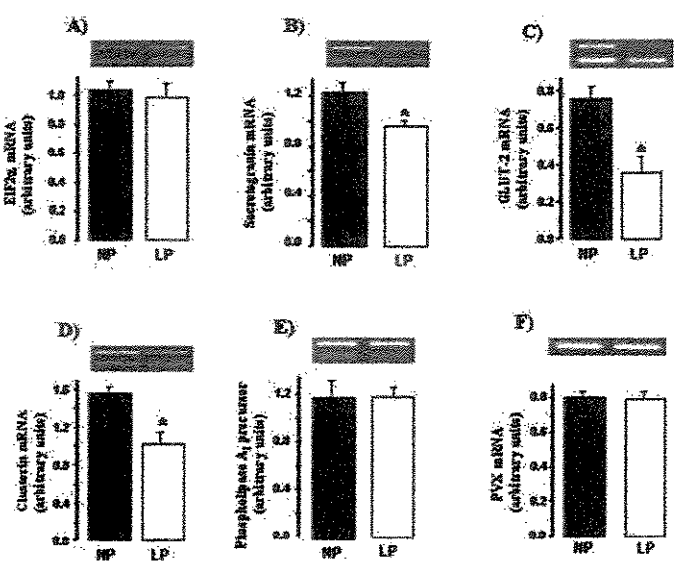

FIGURE 2 Relative expression of mRNA levels for eukaryotic translation initiation factor 2 α subunit (EIF2α) (*A*), secretogranin (*B*), glucose transporter (GLUT)-2 (*C*), clusterin (*D*), phospholipase A_2 (*E*) and potato virus X (PVX) (*F*) in islets from rats fed low (LP) and normal (NP) protein diets. mRNA levels were semiquantified by RT-PCR. In each panel, a representative ethidium bromide-stained agarose gel shows the mRNA levels in islets from rats fed LP and NP diets. In each gel, the upper band corresponds to the cDNA for the specific mRNA indicated and the lower band corresponds to the external control (PVX). The level of mRNA expression for each gene in NP and LP was expressed relative to PVX. Values are means ± SEM, $n = 8$. *Different from NP, $P < 0.05$.

SEC4 (52). To our knowledge, there is no information on the effects of Rab 26 on insulin secretion. However, other members of the Rab family, such as Rab 3A, Rab 3B and Rab 3D, negatively modulate Ca^{2+}-triggered exocytosis in β-cells (53,54). Thus, it is conceivable that the increase in Rab 26 expression may be related to the reduced insulin secretion by islets from rats fed LP. In contrast, a decrease in calcium/calmodulin-dependent protein kinase expression was observed. This protein kinase is related to Ca^{2+} modulation and induction of insulin secretion and is the major Rab 3A-associating protein in pancreatic β-cells (55,56).

Rats fed LP also had a 3.3-fold increase in carboxypeptidase D, an important enzyme present in the *trans*-Golgi network. This enzyme is responsible for the production of the peptides and proteins that transit the secretory and endocytic pathways, including the cleavage of C-terminal residues from prohormones (57). Interestingly, there was a reduction in the expression of secretogranin III. This protein is involved in the production or release of peptide hormones from the storage vesicles of neuroendocrine cells involved in the biogenesis of secretory granules (58,59).

A reduction in insulin-like growth factor-I (IGF-I) and IGF-II mRNA was observed in β-cells from neonatal rats of dams fed a low protein diet during pregnancy (31). These growth hormones are regulated by the nutritional supply of dietary energy and protein and are able to increase islet cell DNA synthesis and proliferation, thus providing an important link between nutrition and growth (31). In the present study, we observed an increase in IGF-II mRNA, suggesting an increase in proliferation rates.

Elevated levels of matrix metalloproteinase (MMP) 14 were also observed in our analysis. Matrix metalloproteinases are extracellular proteinases important for cell migration, invasion, proliferation and apoptosis; their main function is presumed to be remodeling of the extracellular matrix (60). In contrast to MMP-14, other genes related to cell growth, differentiation and cellular responses to infection/injury, such as STAT3 (61), were concomitantly diminished.

The decreased expression in LP of genes belonging to the intracellular kinase network, such as mitogen-activated protein kinase 2 (MAPK2) and insulin-stimulated microtubule-associated protein kinase 2 (MAP2 kinase), suggested alterations in certain signal transduction pathways. Previous studies in INS-1 cells and rat islets indicated that MAP were not involved in insulin secretion (62), but may be related to other β-cell functions, such as metabolism, transcription, cell cycle progression, cytoskeletal rearrangements, cell movement, apoptosis and differentiation (63). In addition, there was a decrease in the mRNA of extracellular signal-regulated kinase 1 and 2 (ERK1, ERK2), which are involved in the regulation of meiosis and mitosis in differentiated cells.

In rats fed a high-carbohydrate (HC) diet milk, there was an immediate onset of hyperinsulinemia and up-regulation of preproinsulin gene transcription. cDNA array analysis of pancreatic islets from these rats showed that the HC diet up-regulated genes involved in metabolic pathways, ion channels, signal transduction, the cell cycle, protein synthesis and apoptosis (64,65). Increased expression of genes related to insulin biosynthesis/secretion, such as insulin, PDX-1, ACC, Reg III, Isl-1, GLUT-2, IRS-1 and IRS-2, was also observed. In contrast, we observed a decreased expression of PDX-1 (34), PKCα (33), and PKAα, insulin, GLUT-2 and IRS-1, as well as glucose metabolism (data not shown). Together, these findings indicate that it is unlikely that the carbohydrate added to the LP diet to compensate for the lack of protein was responsible for the alterations observed in the cDNA array. However, we

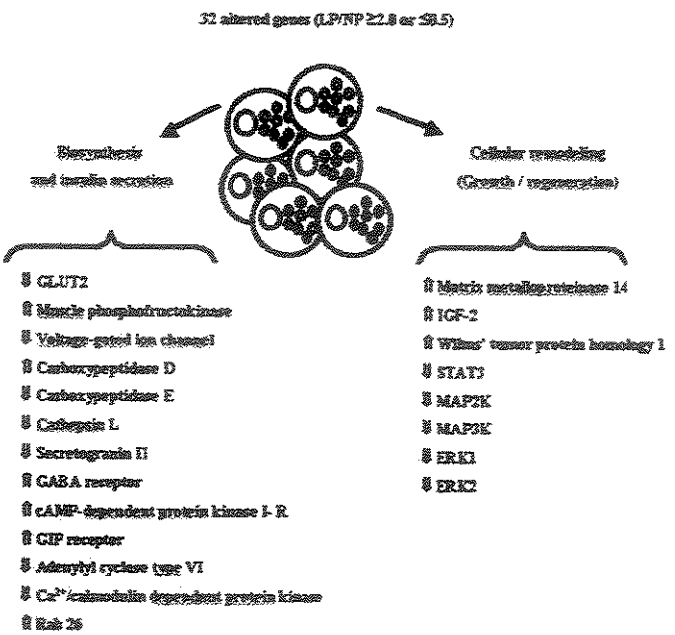

FIGURE 3 Summary of the major findings of changes in gene expression observed in this study based on the macroarray analysis of gene transcript levels in pancreatic islets from rats fed a normal (NP) or low (LP) protein diet. Abbreviations: GLUT, glucose transporter; GABA, γ-amino butyric acid; IGF, insulin-like growth factor; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

cannot dismiss the possibility that the expression of some genes was affected by the higher glucose intake of rats fed LP compared with those fed NP.

In conclusion, the results of this study showed that a low protein diet altered the expression of several genes that encode for proteins related to insulin biosynthesis, secretion and cellular remodeling in rat pancreatic islets (Fig. 3). This altered expression could explain the reduced insulin secretion by islets from rats fed LP after stimulation.

Although the results obtained by the macroarray technique do not provide information about protein levels or enzyme activity, our results suggest that a low protein diet after weaning can lead to metabolic, structural and physiologic adaptations in pancreatic islets. In addition, modifications in the expression of several genes in the endocrine pancreas of rats fed LP could explain in part the decreased insulin secretion observed during islet stimulation by different insulinotropic agents.

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