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O EFEITO DA FARINHA DE SOJA NA RECUPERAÇÃO DO ESTADO NUTRICIONAL E NA SECREÇÃO DE INSULINA DE RATOS SUBMETIDOS À RESTRIÇÃO PROTÉICA DURANTE A VIDA INTRA-UTERINA E NA LACTAÇÃO.

Tese de Doutorado apresentado ao Instituto de Biologia para a obtenção do Título de Doutor em Biologia Funcional e Molecular na área de Fisiologia.

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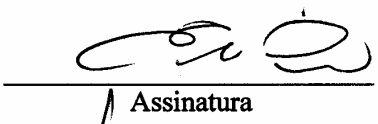

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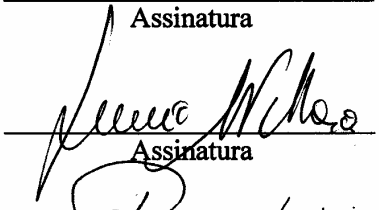

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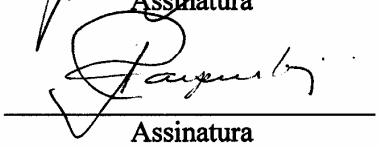

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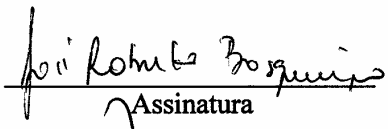

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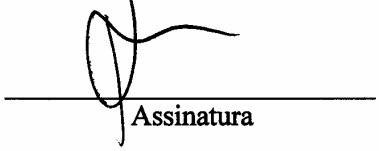

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A minha esposa Tereza Christina e aos meus filhos Paulo Victor, Ludmila e Isadora, dedico esse trabalho.

Ilusões da Vida

Quem passou pela vida em branca nuvem

E em plácido repouso adormeceu;

Quem não sentiu o frio da desgraça,

Quem passou pela vida e não sofreu;

Foi espectro de homem, não foi homem,

Só passou pela vida, não viveu.

Francisco Otaviano

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RESUMO

A restrição protéica materna reduz o crescimento e promove alterações permanentes na estrutura e função de órgãos da prole, contribuindo para o desenvolvimento de doenças cardiovasculares, obesidade e diabetes. O consumo de alimentos à base de soja está associado a um menor risco de desenvolver diabetes pelo seu conteúdo de isoflavonas e pela composição de aminoácidos de sua proteína que contribuem para uma melhora na secreção de insulina. Tem sido sugerido que a genisteína, uma isoflavona da soja, modula a secreção de insulina através das vias AMPc/PKA e PLC/PKC. Deste modo, nós avaliamos o valor biológico da dieta à base de farinha de soja e seus efeitos sobre o crescimento de órgãos, o perfil de aminoácidos, insulina e metabólitos séricos em ratos submetidos à restrição protéica na infância e recuperados com essa dieta após o desmame, bem como o efeito da dieta à base de soja sobre a secreção de insulina em resposta a glicose e a ativadores do adenilato ciclase e proteína quinase C, além da expressão da PKAα e PKCα em ilhotas pancreáticas de ratos adultos. Ratos de mães alimentadas com 17% ou 6% de proteína (caseína) durante a gestação e lactação foram mantidos com dieta contendo 17% de proteína à base de caseína (grupos CC e RC) ou dieta com 17% de proteína à base de farinha de soja (grupos CS e RS) e dieta com 6% de proteína (grupo HP). Após 90 dias de idade, proles de mães alimentadas com dieta hipoprotéica exibiram déficit permanente de peso corpóreo e de concentrações séricas de insulina, taurina, glutamina, fenilserina e lisina, porém apresentaram um aumento no peso relativo dos órgãos, exceto do fígado. A dieta à base de farinha de soja reduziu o peso relativo do fígado e aumentou as concentrações séricas de insulina, taurina, ornitina e fenilserina. Embora ratos recuperados

com soja (RS) tenham ingerido mais dieta proporcionalmente ao peso corpóreo do que os ratos recuperados com caseína (RC) eles mostraram menor coeficiente de eficácia alimentar, e resultou em peso corpóreo final similar entre esses grupos. As concentrações séricas de albumina e proteínas totais não diferiram entre os grupos RS e RC. A dieta à base de soja melhorou a resposta de células beta de ratos controles em concentrações fisiológicas de glicose, enquanto em ilhotas de ratos recuperados isso ocorreu na presença de concentrações suprafisiológicas de glicose. A presença de PMA induziu uma resposta secretória com potência similar em ilhotas dos grupos RS e CS e a expressão de PKCα foi similar em todos os grupos, exceto no grupo HP, que expressou menores concentrações dessa proteína. A adição de forskolin ao meio de incubação aumentou a secreção de insulina em ratos recuperados e naqueles mantidos com caseína e a expressão de PKAα foi maior no grupo RS em relação ao grupo CS. Esses resultados sugerem que dieta à base de farinha de soja é capaz de promover a recuperação nutricional em animais submetidos à restrição protéica em fases críticas de desenvolvimento, melhorando o perfil de aminoácidos séricos que estimulam a secreção de insulina. Além disso, a melhora na secreção de insulina parece não ser devido a ativação das vias AMPc/PKA e inositol fosfato/PKC.

ABSTRACT

Maternal protein restriction leads to reduction in the growth of organs, permanent changes in their structure and functions contributing to development of cardiovascular disease, obesity and diabetes. Consumption of soy-based foods is associated to lower risk of diabetes by its isoflavone content and amino acid composition of its protein that contribute to improve of insulin secretion. It has been suggested that genistein, soy isoflavone, modulates the insulin secretion through of cAMP/PKA and PLC/PKC pathways. Thus, we evaluated the biological value of soybean flour diet and its effects on organ growth, serum amino acids, insulin and metabolites profile in rats submitted to protein restriction in early life and recovered with those diet after weaning, as well as, the effect of soybean diet on insulin secretion in response to glucose and activators of adenylate cyclase and PKC, besides the expression of PKAα and PKCα in pancreatic islets from adult rats. Rats from mothers fed with 17% or 6% protein (casein) during pregnancy and lactation were maintained with 17% casein (CC and CR groups) or soybean (SC and SR groups) diet and with 6% casein (LP groups) diet. At 90d of age offspring of protein-restricted-mothers exhibited permanent deficit of body weight, serum insulin, taurine, glutamine, phenylserine and lysine concentrations, but increased relative organs' weight, except liver. Soybean flour diet reduced the relative liver weight and increased serum insulin, taurine, ornithine and phenylserine concentrations. Although SR rats had eaten proportionally to body weight more diet than CR rats they showed lower feed conversion efficiency which resulted in the final body weight similar between these groups. The SR and CR also exhibited similar serum albumin and protein concentrations. Soybean diet improved the response of ß-cells from control rats to a physiological concentration of glucose, whereas in islets from

recovered rats this occurred in presence of a supra-physiological glucose concentration. PMA induced similar potent secretory response in islets from SR and SC groups and PKCα expression was similar in all groups, except LP that expressed lower levels this protein. Forskolin increased the insulin secretion in recovered rats and in those maintained with casein diet and PKA expression was higher in SR than in SC rats. These results suggest that soybean flour diet is able to promote the nutritional recovery in animals submitted to protein restriction in critical phase of development improving serum amino acid levels that have the stimulatory effect on insulin release. Moreover the improve of insulin secretion seemed does not to be due the activation of the cAMP/PKA and inositol phosphate/PKC pathways.

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INTRODUÇÃO

1. INTRODUÇÃO

A desnutrição protéico-calórica (DPC) em crianças menores de cinco anos de idade continua sendo um dos mais importantes problemas de saúde pública nos países em desenvolvimento. No início do ano 2000, estimava-se que cerca de 30% da população mundial sofria de alguma forma de desnutrição (ONIS *et al*., 2000), e em 2005, estima-se uma prevalência de desnutrição infantil de 29%; o continente africano apresentando a maior taxa, com 33,8%, seguido da Ásia com 29,9% e da América Latina com 9,3% (MACKEY & MONTGOMERY, 2004). É uma doença de origem multifatorial, resultando da inter-relação de diversos fatores, como fome, pobreza, processos infecciosos e baixa ingestão calórica e protéica (MONTEIRO, 2003).

No Brasil, como em muitos países em desenvolvimento, o estado nutricional de crianças abaixo de cinco anos tem melhorado nos últimos anos, devido ao extraordinário desenvolvimento econômico e pela expansão dos programas e serviços de saúde (MONTEIRO, 1995; BATISTA FILHO & RISSIN, 2003).

Nos últimos anos os efeitos adversos da desnutrição materna sobre o crescimento fetal e neonatal e suas repercussões na vida adulta têm despertado grande interesse da comunidade científica. A hipótese do "fenótipo econômico", postulada na década de 1990 na tentativa de explicar a elevada prevalência de doenças crônicas na vida adulta propõe que o baixo peso ao nascer e no primeiro ano de vida, resultante de deficiência nutricional materna, está associado a um maior risco de desenvolvimento do diabetes tipo 2 e da síndrome metabólica na vida adulta (HALES & BARKER, 1992; HALES *et al*., 1991; BARKER *et al*., 1993). Alguns pesquisadores têm sugerido que a relação entre alterações do crescimento em fases críticas do desenvolvimento e diabetes tipo 2 se deve às desordens

estruturais e funcionais da célula β pancreática (HALES *et al*., 1991; WILLS *et al*., 1996; PHILLIPS *et al*., 1994).

O tipo de deficiência nutricional que torna o feto ou infante suscetível ao diabetes mellitus tipo 2 não é conhecido. Grande atenção tem sido dada à deficiência protéica, devido à importância dos aminoácidos para o crescimento fetal, desenvolvimento das células β. pancreáticas e secreção de insulina (HALES & BARKER, 1992). Além disso, alimentos fontes de proteína são geralmente escassos e dispendiosos em comunidades que apresentam alta prevalência de diabetes e da síndrome metabólica (HALES *et al*., 1997).

A maioria dos estudos que testou a hipótese do "fenótipo econômico" avaliou roedores submetidos à restrição protéica durante a vida intra-uterina e/ou lactação ou uma combinação de vida intra-uterina, lactação e após o desmame.

A restrição protéica imposta durante a vida intra-uterina e a lactação é capaz de produzir danos morfológicos e funcionais irreversíveis no pâncreas endócrino. Estudos morfológicos indicam que proles de ratas mantidas com dieta hipoprotéica durante a prenhez e a lactação, possuem número reduzido de células β e aumentado de células α pancreáticas (BERNEY *et al*., 1997). Ocorre também redução do tamanho e vascularização das ilhotas pancreáticas, (SNOECK *et al*., 1990) bem como redução da densidade, massa absoluta e conteúdo de insulina das células β (GAROFANO *et al*., 1997). Essas alterações têm sido atribuídas à redução da capacidade proliferativa (SNOECK *et al*., 1990) e a diferenciação celular anormal. Corroborando com a hipótese de que a restrição protéica na vida intra-uterina promove diferenciação celular anormal, ARANTES *et al*. (2002) verificaram em neonatos de mães submetidas à restrição protéica na vida intra-uterina redução da expressão do fator de transcrição PDX-1 (*pancreatic duodenal homeobox*),

responsável pela diferenciação das células das ilhotas pancreáticas e pela transcrição do gene da insulina.

Ilhotas fetais e neonatais de ratos submetidos à restrição protéica apresentam redução significativa da quantidade de insulina liberada em resposta à glicose, aminoácidos (leucina, arginina, taurina, glutamina) e a estímulos não nutrientes (TPA: éster de forbol, forskolin, teofilina, acetilcolina e KCl) (LATORRACA *et al*., 1998; DAHRI *et al*., 1991; CHERIF *et al*., 1996; CHERIF *et al*., 1997). Em ratos submetidos à restrição protéica na vida intra-uterina e na lactação foram observadas que algumas etapas do processo de acoplamento estímulo-secreção parecem estar alteradas: 1) alterações do metabolismo da glicose na via glicolítica (WILSON & HUGHES, 1997); 2) a redução da atividade da glicerolfosfato desidrogenase na mitocôndria (RASSCHAERT *et al*., 1995); 3) diminuição da captação de Ca^{2+} pelas ilhotas pancreáticas (LATORRACA *et al*., 1999); 4) alteração da produção e/ou ativação de mensageiros intracelulares (AMP cíclico e diacilglicerol) (CHERIF *et al*., 1996; WILSON & HUGHES, 1997). As alterações morfológicas e funcionais produzidas pela restrição protéica intra-uterina e/ou lactação não são revertidas pela recuperação nutricional com dieta normoprotéica, utilizando como fonte protéica a caseína (LATORRACA *et al*., 1998; GAROFANO *et al*., 1997; DAHRI *et al*., 1994a; BARBOSA *et al*., 1993; HALES *et al*., 1996).

Intervenções dietéticas têm se mostrado úteis no tratamento e na prevenção de doenças crônicas e a soja (*Glycine max (L.) Merr*.) tem sido associada a inúmeros benefícios à saúde, incluindo menor risco de doenças crônicas, dentre elas o diabetes mellitus tipo 2 (BHATHENA & VELASQUEZ, 2002; SETCHELL, 1998; MESSINA, 1999; ANDERSON *et al*., 1990; ADLERCREUTZ & MAZUR, 1997; ALI *et al*., 2004).

A soja tem sido amplamente utilizada como fonte de óleo comestível e proteína para alimentação humana e animal. A existência da soja é descrita desde 1000 anos antes de Cristo no Japão e na China e somente a partir do século XIX esta leguminosa passou a ter importância econômica.

A farinha de soja contém cerca de 40% de proteína e a partir do desengorduramento é possível produzir vários produtos para consumo humano e animal, tais como, concentrados, isolados e texturizados protéicos (SNYDER & KWON, 1987).

A soja tem sido descrita por conter antinutrientes que limitam a sua utilização. O mais importante e extensivamente investigado dos antinutrientes protéicos foram os inibidores de proteases (PUSZTAI *et al*., 1991; SANT'ANA *et al*., 2000). Estes antinutrientes apresentam especificidade de inibir as enzimas proteolíticas e, conseqüentemente, reduzem a digestão protéica de alimentos, proporcionando diminuição no ganho de peso e crescimento dos animais. Também estão relacionados ao aumento no peso do pâncreas de ratos e aves. Desta forma, para aumentar o valor nutricional da soja e seus produtos, há necessidade de processamentos térmicos para inativá-los (LIENER, 1994; MIURA *et al*., 2001).

A soja é um alimento rico em proteínas, ácidos graxos insaturados, ferro, potássio, cálcio e vitaminas do complexo B. Essa leguminosa tem maior destaque nutricional em relação aos outros alimentos do mesmo gênero por possuir um composto chamado isoflavona em sua composição. Esse composto caracteriza a soja como um alimento funcional, ou seja, além dos grãos fornecerem nutrientes essenciais ao organismo também trazem benefícios à saúde prevenindo algumas doenças crônicas.

As isoflavonas são compostos químicos fenólicos, pertencentes à classe dos flavonóides e estão amplamente distribuídos no reino vegetal. As concentrações desses

compostos são relativamente maiores nas leguminosas e, em particular na soja, sendo que as principais isoflavonas encontradas na soja e seus derivados são a daidzeína, a genisteína e a gliciteína. Nas últimas décadas, tem havido um grande interesse nos fitoestrógenos e em particular nos potenciais benefícios que uma dieta rica nesses compostos pode conferir no controle de muitas doenças crônicas. O maior interesse dos pesquisadores é na farmacologia e fisiologia das isoflavonas, pois apresentam estrutura não esteroidal, mas comportam-se como estrógenos na maioria dos sistemas biológicos, além de serem as mais abundantes dentre os fitoestrógenos. Em adição à sua atividade anti-estrogênica, estes compostos possuem diversas propriedades biológicas (atividade antioxidante, inibição da atividade enzimática) que podem influenciar em muitos processos bioquímicos e fisiológicos (SETCHELL, 1998).

A evidência de que as isoflavonas protegem contra várias doenças crônicas é baseada em estudos experimentais e epidemiológicos (BRANDI, 1997). Os efeitos da genisteína na regulação da secreção de insulina também têm sido demonstrados (SORENSON *et al*., 1994; LIU *et al*., 2006). Os mecanismos pelos quais as isoflavonas podem exercer estes efeitos parecem depender, em parte, de suas propriedades agonistas-antogonistas dos estrógenos. Outros mecanismos hipotéticos poderiam derivar de outras propriedades bioquímicas, tais como inibição da atividade enzimática e efeito antioxidante (BRANDI, 1997). Os efeitos das isoflavonas variam de tecido para tecido e em cada tipo, estas apresentam afinidade por receptores específicos. Embora os efeitos moleculares ainda não estejam suficientemente elucidados, estudos têm demonstrado que as isoflavonas possuem mecanismos gerais de ação que podem interferir no metabolismo de muitos nutrientes (ANDERSON & GARNER, 1998). Um possível mecanismo de ação geral das isoflavonas inclui efeitos estrogênicos e anti-estrogênicos, regulação da atividade de

proteínas (especialmente das tirosina quinases), regulação do ciclo celular e efeitos antioxidantes (KURZER & XU, 1997). Estudos em humanos, animais e sistemas de culturas de células sugerem que as isoflavonas, especificamente a genisteína e a daidzeína desempenham um papel importante na prevenção de doenças crônicas tais como, osteoporose, doenças do coração e diabetes.

No pâncreas endócrino, a genisteína parece exercer seus efeitos por modular a atividade das proteínas tirosina-quinase. A fosforilação/desfosforilação de várias proteínas intracelulares por proteínas tirosina-quinase/fosfatases é um importante mecanismo regulatório para diversas funções celulares, tais como crescimento, diferenciação, proliferação e ativação celular (PARK *et al*., 1992). Existe prova convincente de que a ativação de proteínas tirosina-quinase é importante para que células endócrinas precursoras se diferenciem em células β pancreáticas, e que sua ativação promova o crescimento de células β fetais (ÖBERG *et al*., 1994; KANAKA-GANTENBEIN *et al*., 1995; OTONKOSKI *et al*., 1996).

Evidências experimentais sugerem que a genisteína tem papel permissivo na regulação da secreção de insulina, por competir com os sítios de ligação do ATP das proteínas tirosina-quinase, inibindo-as. Entretanto, OHNO *et al*. (1993) e JONAS *et al*. (1995), têm sugerido que o efeito estimulatório da genisteína sobre a secreção de insulina pode ocorrer independentemente da inibição da atividade de proteínas tirosina-quinase e resultaria do aumento da concentração de AMPc, pelo menos na presença de baixa concentração de genisteína (OHNO *et al*., 1993). Ilhotas de Langerhans cultivadas com genisteína e linhagens de células secretoras de insulina (INS-1, MIN6) quando estimuladas com essa isoflavona, apresentam aumento da secreção de insulina nos estados basal e

estimulado, bem como da sensibilidade à glicose, (SORENSON *et al*., 1994; OHNO *et al*., 1993; VERSPOHL *et al*., 1995; JONES & PERSAUD, 1994) apesar da redução da proliferação das células da ilhota, em concentração de 100μmol/L de genisteína (SORENSON *et al*., 1994). Em oposição a esses estudos, PERSAUD *et al*. (1999) mostraram a genisteína inibindo a atividade da tirosina-quinase e a liberação de insulina estimulada por glicose e por sulfoniluréias, sem afetar o metabolismo da glicose, a atividade da proteína quinase dependente de AMPc (PKA) e da proteína quinase dependente de Ca^{2+}-calmodulina (CaMK), mas diminuindo a atividade da proteína quinase dependente de Ca^{2+} (PKC).

Portanto, ainda existem dúvidas a respeito do real papel da genisteína sobre o processo de acoplamento estímulo-secreção de insulina.

A proteína da soja pode também afetar a homeostase glicêmica, por aumentar a secreção de insulina e alterar a sensibilidade dos tecidos periféricos a esse hormônio. O aumento da secreção de insulina tem sido atribuído à composição de aminoácidos da proteína da soja, especialmente às altas concentrações de arginina, um estimulador da secreção de insulina (FAJANS *et al*., 1997; SANCHEZ *et al*., 1991). A arginina exerce ação insulinotrópica por seu acúmulo no interior das células β, que provoca a despolarização da membrana e a entrada de cálcio via canais de Ca^{2+} voltagem-dependente (BLACHIER *et al*., 1989). O [Ca^{2+}] tem um papel crucial na regulação da secreção da insulina pelas células β pancreáticas em resposta a vários estímulos, incluindo nutrientes (PRENTKI & MATSCHINSKY, 1987).

Assim, por seu elevado valor nutritivo, baixo custo e pelos seus efeitos sobre a secreção de insulina, a utilização da soja em modelo animal propenso a desenvolver

diabetes na vida adulta devido a perda da capacidade de secretar insulina, poderia ser benéfico na prevenção e tratamento dessa enfermidade.

OBJETIVOS

2. OBJETIVOS

2.1 GERAL

- Avaliar o efeito da recuperação nutricional de ratos desnutridos durante a vida intra-uterina e a lactação com dieta à base de farinha de soja sobre os mecanismos de secreção de insulina.

2.2 ESPECÍFICOS

- Determinar o consumo alimentar absoluto e relativo, a proteína ingerida, o coeficiente de eficácia alimentar e o crescimento somático na fase de recuperação nutricional nos grupos controle, recuperado e desnutrido.
- Verificar os parâmetros biológicos de peso e nitrogênio fecais e digestibilidade durante a recuperação nutricional nos cinco grupos estudados.
- Identificar o efeito da recuperação nutricional sobre as concentrações séricas de proteínas totais, albumina, ácidos graxos livres, aspartato aminotransferase (AST) e alanina aminotransferase (ALT) em ratos controles, recuperados e desnutridos.
- Determinar o peso absoluto e relativo dos órgãos pulmão, coração, baço, fígado e rins dos animais controles, recuperados e desnutridos no final da recuperação nutricional.
- Estabelecer o perfil aminoacídico sérico dos animais controles, recuperados e desnutridos após a fase de recuperação nutricional.
- Verificar a glicemia e insulinemia nos estados pós absortivo e pós prandial em ratos adultos nos cinco grupos de tratamento.

- Verificar a resposta insulínica à glicose *in vivo* em ratos adultos recuperados com dietas controle, à base de farinha integral de soja e hipoprotéica.
- Determinar a tolerância à glicose e a sensibilidade à insulina exógena *in vivo* em ratos adultos recuperados com dietas controle, à base de farinha integral de soja e hipoprotéica.
- Verificar os efeitos da dieta à base de farinha de soja durante a recuperação nutricional sobre as vias AMPc/PKA e PLC/PKC, bem como a expressão dessas proteínas em ilhotas pancreáticas.

ARTIGO 1

Role of soybean flour in the nutritional recovery of rats submitted to protein restriction[1]

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Running title: Nutritional recovery with soybean diet.

ABSTRACT

Backgroud Maternal protein restriction leads to reduction in the growth of organs, permanent changes in their structure and functions contributing to the development of cardiovascular disease, obesity and diabetes. The consumption of soy-based foods is associated to lower risk of these diseases by its isoflavone content and amino acid composition of its protein. *Aim* We evaluated the biological value of the soybean flour diet and its effects on organ growth, serum amino acids, insulin and metabolites profile in rats submitted to protein restriction in early life and recovered with this diet after weaning. *Design* Rats from mothers fed with 17% or 6% protein (casein) during pregnancy and lactation were maintained with 17% casein (CC and CR groups) or a soybean (SC and SR groups) diet and with 6% casein (LP groups) diet. *Results* At 90d of age offspring of protein-restricted-mothers exhibited permanent deficit of body weight, serum insulin, taurine, glutamine, phenylserine and lysine concentrations, but presented increased relative organs' weight, except liver. The soybean diet reduced the relative liver weight and increased serum insulin, taurine, ornithine and phenylserine. Although SR rats had eaten proportionally to body weight more diet than CR rats they showed lower feed conversion efficiency which resulted in the final body weight similar between these groups. The SR and CR rats also exhibited similar serum albumin and protein concentrations. *Conclusion* These results suggest that a soybean flour diet is able to promote the nutritional recovery in animals submitted to protein restriction in critical phase of development improving serum amino acid levels that have the stimulatory effect on insulin release.

KEY WORDS: intrauterine malnutrition, soya flour, nutritional recovery, organ growth, amino acid profile, rat

INTRODUCTION

Malnutrition is the second most frequent cause of death in children under the age of 5 in developing countries [1] and the World Health Organization estimates that, every year, more than 20 million children present low weight at birth and that approximately 150 million children younger than 5 years have low weight patterns for their age [2]. Low-weight babies who have intrauterine growth retardation are born with malnutrition, which can have a number of different causes, but world-wide maternal malnutrition is probably the most common. Current evidences indicate that low weight at birth is associated with a greater risk for chronic diseases in adulthood, such as type 2 diabetes, hypertension and cardiovascular diseases [3].

Experimental studies have reinforced this association, and attention has been drawn to the potential role of protein deficiency, due to the importance of amino acids in fetal growth, pancreatic β-cell development and insulin secretion [4]. It has been shown that maternal protein restriction during pregnancy and/or lactation produce alterations in the structure and function of several organs in the offspring, which appear to be permanent, organ-selective and sex-dependent [5]. Alterations in the plasma amino acid profiles of dams and offspring occur when a low protein diet is introduced during pregnancy [6]. An impaired insulin secretion in response to glucose and different secretagogues, a reduction in the islets-cell proliferation rate, in the islets size and vascularization were observed in the endocrine pancreas of malnourished rats [7-10]. Moreover, alterations in the function of liver, skeletal muscle and adipose tissue as well as in the intermediary metabolism were noted in rats with early growth restriction, which provide support for the “thrifthy phenotype” hypothesis and clues to the mechanism [11].

Dietary interventions can be a useful and efficient tactic to prevent and to treat chronic disease and the consumption of soy-based foods is associated with a number of health benefits, including lower risk of cardiovascular disease, diabetes mellitus, breast cancer and prevention of bone stress [12-15].

Soy is the most important oleaginous cultivated in the world. It contains lipids with high concentration of polyunsaturated fatty acid, vitamins (A, B_1, B_2, B_6 and folic acid), minerals (Fe, Zn, Mg, K, Ca, Mn and Se), phytoestrogens, fibers and antinutritional factors. Among those factors are the protease inhibitors and lectins which interfering with the absorption of nutrients and inhibition of growth. Although soy contains a large amount of proteins, it is deficient in sulfur-containing amino acids such as methionine and cystine [16]. When soy protein is consumed in a normal concentration (18%) it does not meet the protein requirement and support adequate growth rates in rats [17]. The benefic effects of soybean have been attributed to its isoflavone and lignans contents and the protein composition that modulates pancreatic insulin secretion improves glucose tolerance; reduces body weight, and plasma cholesterol and triacylglicerol concentrations [18-20].

By its reduced cost, elevated nutritional value and beneficial role in obesity and diabetes, the soybean could be an excellent alternative feed in the recovery of the nutritional status. Few studies have been carried out to explore the late repercussion of its use in the recovery of animal models of protein restriction during critical phases of development. Therefore, we investigate the effect of the soybean flour diet on the nutritional recovery of rats submitted to the protein restriction during the intrauterine life and the lactation with emphasis on organ growth, serum amino acid profile and blood variable such as insulin, glucose and free fat acids (FFA).

MATERIALS AND METHODS

Animals and diets

All of the animal experiments were approved by the State University of Campinas Ethics Committee (São-Paulo, Brazil). Virgin female Wistar rats (90 d old) were obtained from the University's own breeding colony. Mating was performed by housing males with females overnight, and pregnancy was confirmed by the examination of vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained from the first day of pregnancy until the end of lactation on isocaloric diets containing 6% protein (LP diet, n=8) or 17% protein (C diet, n=6) as described previously [21]. Spontaneous delivery took place on day 22 of pregnancy after which, at 3 d of age, large litters were reduced to six male pups to ensure a standard litter size per mother. Five groups of adult male rats (90 d old) were used in this study: *i*) CC (n=8), consisting of rats born from and suckled by mothers fed a control diet, and subsequently fed a control diet after weaning, *ii*) SC (n=8), consisting of offspring born from and suckled by mothers fed a C diet and subsequently fed a soybean flour isocaloric diet with 17% protein after weaning; iii) LP (n=6), consisting of the offspring of mothers fed a LP diet and subsequently fed the same diet after weaning; iv) CR (n=8), consisting of the offspring of mothers fed a LP diet, but fed a C diet after weaning; v) SR (n=8), consisting of offspring of mothers fed a LP diet, but fed an integral and inactive micronizada soybean flour diet with 17% protein after weaning. The diet components are described in (Table 1). The protein in the LP diet was replaced by the same amouth of carbohydrate. In the soybean flour diet, adjustments were made to equalize the carbohydrate, lipid, fiber contents and energy value to casein diet, suppressing the soybean oil and reducing fiber amount.

Experimental procedures

All of the offspring were weaned at the 4th wk after birth. Throughout the experimental period, the rats were given free access to food and water. They were kept under standard lighting conditions (12-h light/dark cycle) at a temperature of 24°C. All the animals were weighed at the beginning of the biological assay and each 4 days, during all the experiment. The food intake was recorded four times per week. The feces were discarded in the first three days of the experimental protocol and collected each 2 days during 10 days of the experiment. Feces were dried in a ventilated stove at 50°C, for 12 hours, cleaned of coats and food residues, weighed and macerated until a fine and homogeneous dust was attained.

Biological methods for evaluation of diets

Feed Conversion Efficiency (FCE) was determined by: FCE = Feed offered (g)/Weight gained (g), and; Digestibility (D) = [(Nitrogen intake- Fecal nitrogen)/Nitrogen intake]x100 [22]. The fecal nitrogen was measured by Micro-Kjeldahl method [23].

Amino acid profile

At 90 days of life, blood samples were collected from fed and food-deprived (13h) rats from all groups. The samples were deproteinized by adding 1 mL of 25% trichloroacetic acid (TCA) solution to 1 mL of serum 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 were analyzed by chromatography on a Biochrom 20 plus (Amersham Pharmacia, Piscataway, NJ) using a specific physiologic amino acid column. FFA standards were analyzed in paralell. 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.

Biochemical and hormonal profile

After 90 days of life, weaning rats were killed by decapitation and blood samples were collected, allowed to clot and the sera stored at -20°C for the subsequent measurement of insulin by radioimmunoassay [24]. The following determinations were done immediately after decapitation: serum glucose [25], serum albumin [26], total serum protein [27] and serum-free fatty acids using commercial Kit (FFA; Nonesterified Fatty Acid C kit, Wako Chemicals GmbH, Neuss, Germany).

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels

Measurements of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were done by spectrophotometry using commercial kits (Kinetic without Pyridoxal Phosphate, Roche). The AST to ALT ratio was determined from the absolute aminotransferase levels.

Organ weights

After medial laparotomy, the lung, heart, liver, spleen and kidney were quickly removed for determination of fresh weight.

Statistical analysis

The results are presented as the means ± SD for the number of rats (*n*) indicated. When comparing the data from CC, SC, CR and SR groups a two-way analysis of variance (nutritional status effect and diet effect) was used. The same data were analyzed by one-way analysis of variance when comparing CR, SR and LP groups to verify if the diet was efficient in improving the nutritional status. When necessary, these analyses were complemented by the Tukey test to determine the significance of individual differences. Bartlett's test for the homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric analysis of variance. When necessary the data were log-transformed to correct for variance heterogeneity or non-normality [28]. The data were analyzed using the Statistic Software package (Statsoft, Tulsa, OK).

RESULTS

Rats from dams maintained with protein restriction during pregnancy and lactation (CR, SR and LP) started the recovery phase with body weight significantly lower than those whose mothers were fed with normal protein diet (CC and SC). At the end of the recovery time CR and SR groups had lower final body weight ($F_{1,18}$=83.47, p<0.001), weight gain ($F_{1,18}$=27.02, p<0.001) and total food and protein intake ($F_{1,18}$= 42.42, p<0.001) than CC and SC groups. The soybean diet also reduced final body weight ($F_{1,18}$=17.76, p<0.001), weight gain ($F_{1,18}$=18.38, p<0.001) and total food and protein intake ($F_{1,18}$=18.99, p<0.001) when compared with casein diet. The food intake relative to body weight of SR group was higher than CR group (p=0.046) but similar to CC and SC groups. The FCE was lower in the SR rats than in CR rats, but did not differ from that of CC and SC groups. Although LP rats had initial body weight higher than SR and CR rats (p<0.02 and p<0.01, respectively), the weight gain from LP group was 1.8 and 2.3 times lower than those of SR and CR groups (p<0.02 and p<0.001, respectively) and, consequently, at the end of the experimental period, the body weight of LP rats was lower than in the two recovered groups (p<0.02 and p<0.001). No difference was verified in final body weight of SR and CR groups (p=0.139). The total food intake by SR group did not differ from CR and LP groups, whereas total protein intake was similar in SR and CR groups and these groups had eaten more protein than LP group (p<0.001). During the nutritional recovery period, the food intake relative to body weight was similar between SR and CR groups and lower than LP groups (p<0.001). The FCE was also similar between SR and CR groups and significantly higher than LP group (Table 2).

The nitrogen ingested by SR and CR groups was similar and lower than by SC and CC groups ($F_{1,18}$= 4.87, $p<0.05$). The fecal nitrogen and feces weight were lower in SR and CR groups than in CC and SC groups ($F_{1,18}$=10.39, $p<0.01$ and $F_{1,18}$=10.46, $p<0.01$, respectively). The amount of fecal nitrogen and feces weight of SR and SC groups were higher than of CR and CC groups ($F_{1,18}$=129.70, $p<0.001$ and $F_{1,18}$= 6.99, $p<0.05$, respectively). The digestibility was affected by the previous nutritional status ($F_{1,18}$=8.73, $p<0.01$), by diet used during experimental phase ($F_{1,18}$=628.29, $p<0.001$) as well as by the interaction between these two factors ($F_{1,18}$=7.59, $p<0.02$). SR and SC groups had digestibility similar and significantly lower than CR and CC groups. No difference was observed in the nitrogen ingested by SR and CR, and these groups had this variable higher than LP group. The fecal nitrogen was higher in SR group than in CR and LP groups ($p<0.001$), whereas the feces weight of SR group were higher than those of LP group ($p<0.01$) but similar to CR group. The digestibility of SR group was lower than of CR and LP groups (Table 3).

The absolute weights of all organs were lower in SR and CR groups than in SC and CC groups. Also, the organ weights were lower in groups maintained with soybean diet than those fed with casein diet after weaning. When expressed per 100g body weight lung, heart, spleen and kidney were higher in SR and CR groups than in SC and CC groups. The liver weight relative to body weight was lower in the groups maintained with soybean diet than in those groups fed on casein diet after weaning. When evaluating the effect of diets on nutritional recovery, it was observed that the absolute weights of lung, spleen and liver of SR group were lower than CR group but higher than LP group ($p<0.05$). Absolute weight of heart did not differ between SR and CR groups, but this variable was around two times higher in those groups in relation to LP group ($p<0.05$). Kidney weights of SR and

LP were similar and significantly lower than in CR group ($p<0.05$). Rats of SR and CR groups showed similar weight of lung per 100g body weight and were significantly lower than LP groups ($p<0.05$). The relative weight of heart in SR group was not different from CR and LP groups. The weight of spleen, liver and kidney per 100g body weight were similar among SR, CR and LP groups (Table 4).

The serum total protein concentrations in fed state were significantly lower in SR and CR groups than in SC and CC groups ($F_{1,15}=11.44$, $p<0.01$), whereas in fast state they did not differ among groups. In both states, the serum albumin and serum glucose concentrations were equal in the four groups. The FFA levels in fasted state were similar in all groups and in fed state there were no differences between SR and CR groups but in SC group the FFA levels were lower than in the CC group ($p<0.01$). Serum insulin concentration in fed state was lower in SR and CR groups than in SC and CC groups ($F_{1,19}=5.77$, $p<0.03$). Furthermore, rats maintained after weaning with the soybean diet (SR and SC groups) showed higher serum insulin levels than those fed with the casein diet (CR and CC groups) ($F_{1,19}=14.82$, $p<0.01$). The serum insulin concentration in fast state, AST and ALT concentrations, and AST to ALT ratio were similar in all groups. Serum albumin and protein concentrations in the fed and fast states, basal glycemia and fed insulin concentrations were similar in SR and CR groups and significantly higher than in LP group. ALT concentration was higher in LP groups when compared with SR and CR groups ($p<0.05$ and $p<0.01$), which were similar. No difference was observed among these groups for fed serum glucose concentration, fasting FFA and insulin concentration, and AST to ALT ratio (Table 5).

The fast serum taurine ($F_{1,11}=5.40$, $p<0.05$), glutamine ($F_{1,11}=7.38$, $p<0.02$), lysine ($F_{1,11}=8.39$, $p<0.02$) and phenylserine ($F_{1,11}=5.41$, $p<0.05$) concentrations were lower and

the urea concentration was higher in SR and CR groups than in SC and CC groups. In this same state, serum phenylserine ($F_{1,11}$=32.36, $p<0.001$) and urea ($F_{1,11}$=7.23, $p<0.02$) concentrations were lower in rats maintained with a soybean diet than those maintained with casein. The fast serum glutamate concentrations were higher in the SR rats than in CR rats ($p<0.05$) and similar to CC and SC rats. Evaluating the effect of nutritional recovery, it was verified that in fast serum urea and phenylserine concentrations were similar in SR and LP groups and lower in the CR groups ($p<0.05$ and $p< 0.01$, respectively) (Table 6).

In the fed state, the serum taurine, ornithine and phenylserine concentrations were higher in rats maintained with a soybean diet than with a casein diet. ($F_{1,12}$=5.48, $p<0.05$, $F_{1,12}$=11.69, $p<0.01$ and $F_{1,12}$=7.09, $p<0.02$ respectively). After nutritional recovery, the serum phenylalanine concentrations from LP and CR groups were similar but lower than in SR group ($p<0.05$), whereas serum ornithine concentrations did not differ between LP and SR groups and they were higher than in the CR group ($p<0.05$) (Table 6). Finally, serum glycine, cysteine, isoleucyne, alanine, tyrosine, ammonia, arginine, serine, ethanolamine hydroxylysine and phenylethylamine concentrations in fed and fast states did not differ among groups (data not shown).

TABLE 1. Composition of the normal, low-protein and soy flour inactive diets

Ingredient	Normal (AIN-93G)[1] (170 g protein/Kg)	Low protein (60 g protein/Kg)	Soya flour inactive + (AIN-93G) (170 g protein/Kg)
Soya flour inactive[2]	-	-	415.0
Casein (850 g protein/Kg)	202.0	71.5	-
Cornstarch	397.0	480.0	312.2
Dextrinized cornstarch	130.5	159.0	103.7
Sucrose	100.0	121.0	78.60
Soybean oil	70.0	70.0	-
Fiber	50.0	50.0	40.0
Mineral mix (AIN93G-MX)	35.0	35.0	35.0
Vitamin mix (AIN93-VX)	10.0	10.0	10.0
L-Cystine	3.0	1.0	3.0
Choline chlorydrate	2.5	2.5	2.5

[1] For detailed composition see [53].
[2] Perdigão Indústria e Comércio Ltda, SC, Brasil.

TABLE 2. Initial and final weight, weight gain, absolute and relative food intake, protein intake, feed efficiency ratio (FER) in the five treatment groups

Parameters	GROUPS				
	CC	**SC**	**CR**	**SR**	**LP**
Initial weight, g	81±3	81±2	27±2 ^{b}	29±2 ^{b}	32±2 ^{a}
Final weight, g	389±12	327±22	261±40 ^{a}	212±44 ^{a}	132±29 ^{b}
Weight gain, g	307±13	246±21	234±38 ^{a}	183±45 ^{a}	99±28 ^{b}
Total food intake(g)	1045±36	827±66	746±116^{a}	635±120 ^{ab}	549±56 ^{b}
(g/100g BW)	340±13^{AB}	337±20 ^{AB}	319±7^{Bb}	352±27^{Ab}	576±90^{a}
Protein intake, g	178±6	141±11	127±20 ^{a}	108±20 ^{a}	33±3 ^{b}
FCE	0.29±0.01^{AB}	0.30±0.02 ^{AB}	0.31±0.01^{Aa}	0.29±0.02 ^{Ba}	0.18±0.03 ^{b}

Values are expressed as means ± SD for the 5-6 rats per group. Means with different superscript capital letters are significantly different by two-way ANOVA followed by Tukey test ($p<0.05$) and with superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey ($p<0.05$).

TABLE 3. Nitrogen ingested, fecal nitrogen, feces weight, apparent digestibility ratio (DRa) during 10 days of the experiment in the five treatment groups

Parameters	GROUPS				
	CC	SC	CR	SR	LP
Nitrogen ingested, g	5.8±0.4	5.7±0.5	5.1±0.6 ^{a}	5.1±1.0 ^{a}	1.5±0.1 ^{b}
Fecal nitrogen, g	0.30±0.03	0.57±0.06	0.20±0.04 ^{b}	0.50±0.09 ^{a}	0.11±0.03 ^{b}
Feces, g	12.0±1.2	13.4±1.5	9.2±1.7 ^{ab}	11.6±2.2 ^{a}	6.9±2.0 ^{b}
Digestibility	94.8±0.3 ^{B}	90.1±0.5 ^{C}	96.0±0.6 ^{Aa}	90.2±0.5 ^{Cc}	92.8±1.9 ^{b}

Values are expressed as means ± SD for the 5-6 rats per group. Means with different superscript capital letters are significantly different by two-way ANOVA followed by Tukey test ($p<0.05$) and with superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey ($p<0.05$).

TABLE 4. Absolute and relative weight of the lung, heart, spleen, liver and kidney in the five treatment groups

Parameters	GROUPS				
	CC	**SC**	**CR**	**SR**	**LP**
Lung,					
(g)	1.52±0.20	1.42±0.21	1.26±0.07^{a}	1.02±0.13^{b}	0.83±0.07^{c}
(g/100g)	0.39±0.05	0.44±0.07	0.49±0.10^{b}	0.49±0.08^{b}	0.65±0.11^{a}
Heart,					
(g)	1.30±0.10	1.08±0.08	0.96±0.10^{a}	0.82±0.1^{a}	0.58±0.04^{b}
(g/100g)	0.33±0.03	0.33±0.01	0.37±0.02^{b}	0.39±0.04^{ab}	0.45±0.07^{a}
Spleen,					
(g)	0.78±0.07	0.62±0.05	0.62±0.05^{a}	0.48±0.07^{b}	0.26±0.04^{c}
(g/100g)	0.20±0.02	0.19±0.02	0.24±0.03	0.23±0.03	0.20±0.02
Liver,					
(g)	12.8±1.9	8.9±1.0	8.0±1.1^{a}	6.2±1.1^{b}	3.8±0.5^{c}
(g/100g)	3.3±0.5	2.7±0.2	3.1±0.1	2.9±0.1	2.9±0.3
Kidney,					
(g)	2.8±0.2	2.5±0.3	2.1±0.2^{a}	1.7±0.3^{b}	1.2±0.1^{b}
(g/100g)	0.72±0.04	0.76±0.07	0.82± 0.08	0.79±0.06	0.96±0.16

Values are expressed as means ± SD for the 5-6 rats per group. Means with different superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey ($p<0.05$).

TABLE 5. Serum albumin, serum total protein, serum glucose, FFA and serum insulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and AST/ALT ration in the five treatment groups

	GROUPS				
Parameters	**CC**	**SC**	**CR**	**SR**	**LP**
Serum albumin, g/dl					
fed	2.6±0.1	2.6±0.1	2.4±0.2^{a}	2.4±0.1^{a}	1.4±0.4^{b}
fast	2.3±0.2	2.1±0.1	2.4±0.2^{a}	2.4±0.1^{a}	1.8±0.2^{b}
Serum total protein, g/dl					
fed	47±1	47±2	43±3^{a}	43±4^{a}	28±4^{b}
fast	43±1	39±4	44±3^{a}	41±2^{a}	27±0.01^{b}
Serum glucose, mg/dl					
fed	157±34	160±16	200±61	181±16	159±9
fast	86±27	132±27	139±42^{a}	124±18^{a}	32±2^{b}
FFA, nmol/l					
fed	0.42±0.06^{A}	0.31±0.07^{B}	0.32±0.05^{B}	0.33±0.04^{AB}	0.24±0.09
fast	1.2±0.2	1.0±0.4	0.8±0.2	1.0±0.3	1.0±0.1
Serum insulin, ng/ml					
fed	0.91±0.49	2.80±1.15	0.68±0.24^{a}	1.42±1.09^{a}	0.25±0.09^{b}
fast	0.34±0.14	0.33±0.07	0.43±0.05	0.41±0.15	0.54±0.03
AST, U/l	158±43	179±47	119±21	149±31	130±21
ALT, U/l	40±12	51±18	30±4^{b}	36±3^{b}	55±10^{a}
AST/ALT	4.0±0.2	3.5±0.3	4.0±0.6	4.2±1.2	2.4±0.6

Values are expressed as means ± SD for the 3-8 rats per group. Means with different superscript capital letters are significantly different by two-way ANOVA followed by Tukey test ($p<0.05$) and with superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey ($p<0.05$).

TABLE 6. Serum amino acid and other N-containing compound concentration in fed and fast Wistar rats in the five treatment groups

Amino Acids	GROUPS				
	CC	**SC**	**CR**	**SR**	**LP**
Fast state					
Glutamate	1.36±0.21^{AB}	1.14±0.02^{AB}	1.04±0.21^{B}	1.65±0.59^{A}	0.83±0.16
Glutamine	4.72±0.22	4.45±0.48	3.99±0.52	3.96±0.47	3.25±0.74
Leucine	1.04±0.08	0.74±0.62	1.02±0.16	1.18±0.48	0.63±0.46
Lysine	2.35±0.23	2.52±0.53	2.14±0.18	1.70±0.40	2.29±0.73
Ornithine	0.45±0.29	0.34±0.10	0.35±0.08	0.33±0.06	0.41±0.11
Phenylalanine	0.60±0.53	0.84±0.70	0.68±0.46	1.20±0.60	0.29±0.07
Phenylserine	0.42±0.01	0.35±0.05	0.41±0.04^{a}	0.27±0.04^{b}	0.30±0.04^{b}
Taurine	2.27±0.35	2.07±0.54	1.81±0.70	1.37±0.20	0.86±0.29
Urea	1.24±0.21	1.08±0.27	1.87±0.26^{a}	1.39±0.18^{b}	1.22±0.33^{b}
Fed state					
Glutamate	1.45±0.57	0.95±0.22	1.02±0.42	1.48±0.62	0.92±0.15
Glutamine	3.32±0.52	3.52±0.72	3.37±0.13	2.50±1.66	3.95±0.50
Leucine	0.64±0.49	0.65±0.35	0.89±0.27^{ab}	0.97±0.35^{a}	0.23±0.30^{b}
Lysine	2.02±0.78	1.81±0.14	2.17±0.46	1.85±0.45	2.49±0.25
Ornithine	0.22±0.07	0.67±0.35	0.28±0.11^{b}	0.49±0.10^{a}	0.43±0.08^{a}
Phenylalanine	0.83±0.60	0.71±0.45	0.43±0.33^{b}	1.07±0.49^{a}	0.26±0.08^{b}
Phenylserine	0.23±0.06	0.36±0.05	0.30±0.04	0.35±0.11	0.45±0.07
Taurine	0.76±0.21	1.10±0.22	0.91±0.20	1.08±0.24	1.10±0.24
Urea	1.96±0.57	2.11±0.16	1.99±0.39	2.11±0.44	1.38±0.16

Values are expressed as means ± SD for 3-4 rats per group. Means with different superscript capital letters are significantly different by two-way ANOVA followed by Tukey test ($p<0.05$) and with superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey ($p<0.05$).

DISCUSSION

The present study confirmed previous observations [5, 29-30] that protein restriction during intrauterine and lactational periods affects overall growth and produces selective changes in specific organs. In rats recovered with soybean or casein diet, organs such as lung, heart, spleen and kidney were proportionally higher than body weight, but this result does not necessarily reflect normality of their structure and function.

The typical metabolic alterations commonly seen in malnourished animals (hypoalbuminemia, hypoglicaemia and elevated FFA) were not verified in recovered animal independently of protein quality. However, in the fed state, serum total protein concentrations were lower in the recovered groups compared to control groups, possibly at the expenses of reduced globulin concentrations, since serum albumin concentrations were similar in those groups. Although serum albumin concentrations from recovered groups had returned to similar values shown by the control groups, some amino acids remained altered in the fasting state. Lysine, which is commonly better preserved than other essential amino during protein deprivation [31], possibly due to its capacity for storage and slower catabolism [32], remained reduced after nutritional recovery. Its deficiency is associated with alteration in the circadian release of the neurotransmitter serotonin in the central amygdale, a brain area implicated in emotion regulation, food preferences, fear and response to stress [33]. Reduced serum levels of glutamine in recovered animals may indicate catabolic stress [34], whereas low serum taurine concentration may be a consequence of its elevated urinary excretion due to decreased renal reabsorption [35-36], since very low birth-weight shows impaired renal function [37]. Taurine deficiency is associated with various pathological lesions of diabetes (rethinopathy, neuropathy,

nephropathy, cardiomyophatya, etc.) and their supplementation improves insulin secretion and reduces glucose levels in diabetic patients [38]. Finally, the high concentrations of urea in serum of recovered rats could reflect the increase of its production [39]. Hence, these results reinforce the “thrifty phenotype” hypothesis regarding the possible relationship between intrauterine growth retardation (IUGR) and the development of metabolic syndrome later in life [4].

Although rats recovered with a soybean flour diet had eaten, proportionally to body weight, more than those recovered with a casein diet the feed conversion efficiency (FCE) from the first diet was lower than the last one, resulting in the similar final body weight, which could be an indication of reduced quality and/or digestibility of soybean protein. There is a good correlation between plasma urea concentration and protein quality [40] and it has been shown that soy protein is proportionally more degraded to urea than casein protein [41]. In this study the serum urea concentration in animals fed with a soybean diet was lower than those maintained with casein. Thus, our results suggest that the biological value of soybean protein is not inferior to that of casein protein. Several factors are involved in the soybean digestibility, and the most important is the presence of antinutritional factors and fiber amount [42]. Considering that the soybean flour utilized by us in this study was submitted to a thermal treatment, it is improbable that the anti-nutritional factors such as proteolytic enzymes and lectins have interfered in the bioavailability of their amino acids and protein. As judged by the high feces weight, it is possible that the lower digestibility and consequently FCE in rats maintained with soybean diet could be due to a higher amount of fiber in this diet.

Although the rats maintained with soybean diet had less growth than those maintained with casein diet, their organs weight/body weight ratios were similar, except for

the liver that was reduced. However, the unchanged serum AST and ALT concentrations discard the possibility of liver cell damage for both acute and chronic hepato-cellular injuries [43]. The reduced liver weight may be explained by the low glycogen concentration observed in rats maintained with soybean diet (Oliveira EA, Veloso RV, Arantes VC, Carneiro EM, Boschero AC, Latorraca MQ., unpublished results) and/or by reduced triglyceride accumulation in the liver or may be due to the reduction of lipogeneses [44].

Protein restriction during intrauterine and lactation periods induced a reduction in insulin secretion but did not change glucose homeostasis, since it was paralleled by an increase in insulin sensitivity [30]. Nutritional intervention studies performed in animals and humans suggest that the ingestion of soy protein associated with isoflavones improves glucose control, insulin resistance and increases insulin secretion [45]. In the present study, soybean diet enhanced serum insulin levels in the fed state. The rise of insulinaemia in rats maintained with soybean diet may reflect the increase in the insulin secretion due to the inhibitory effect of isoflavone genistein on a protein tyrosine kinase that exerts multiple actions enhancing the insulin release from pancreatic islet cells [20, 46] or the amino acid composition of its protein [47]. The latter hypothesis is reinforced by the serum amino acid composition of rats fed with soybean diet that showed higher serum ornithine, taurine, phenylserine and glutamate levels. The stimulatory effect of taurine and ornithine amino acids on insulin release is attributable mainly to their depolarizing effect on plasma membrane of insulin secreting cells [48]. Glutamate may play a direct or indirect (via generation of putative messengers of mitochondrial origin) role by amplifying pathways involving Ca^{2+} signal in nutrient-stimulated insulin secretion [49] and by inhibiting some protein phosphatases what, in turn, favors insulin exocytosis in pancreatic beta-cells [50].

Despite higher serum insulin levels in the fed state, animals maintained with soybean diet exhibited glycemia similar to rats fed with casein diet, an indication of lower insulin sensitivity of tissues. It has been demonstrated that genistein and/or soybean protein decrease glucose uptake in skeletal muscle cell [51], reduces insulin-induced lipogenesis and enhances epinephrine-induced lipolysis in adipose tissue [52].

In conclusion these results suggest that soybean flour promotes nutritional recovery in rats submitted to protein restriction in a critical phase of development. The nutritional recovery is represented by the augmentation of some serum amino acid levels that stimulate insulin release. The augmented serum insulin concentration appears to be compensated by the increase of insulin resistance, which contributes to the maintenance of glucose homeostasis. These findings underscore the importance of investigating the effects of the use of this source of protein with high quality and low cost in the long run on the glucose homeostasis in this animal model.

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ARTIGO 2

Nutritional Recovery With Soybean Diet Improves Insulin Secretion Through Activation of cAMP/PKA Pathway[1]

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Running title: Soybean diet improves insulin secretion

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ABSTRACT

Maternal malnutrition leads to permanent alterations in insulin secretion of offspring and the soybean diet contributes to improve insulin release. At least a soy component, genistein, seems to increase the insulin secretion by activating the cAMP/PKA and PLC/PKC pathways. Here, we investigated the effect of the soybean diet during nutritional recovery on the expression of PKAα and PKCα, and insulin secretion in response to glucose and activators of adenylate cyclase and PKC in adult pancreatic rat islets. Rats from mothers fed with 17% or 6% protein (casein) during pregnancy and lactation were maintained with 17% casein (CC and CR groups) or soybean (SC and SR groups) diet and with 6% casein (LP groups) diet until 90-d of life. The soybean diet improved the insulin response to a physiological concentration of glucose in control islets, but only in presence of supra-physiological concentrations of glucose in islets from recovered rats. PMA also improved the insulin response in islets of SC and SR groups. The expression of PKCα was similar in all groups, except for LP that expressed lower levels of this protein. Forskolin increased the insulin secretion; however the magnitude of the increment was lower in islets from recovered than in control animals and in those from rats maintained with soybean diet than in rats fed with casein diet. The PKAα expression was similar between SR and CR groups and lower in SC than in CC islets. Thus, nutritional recovery with the soybean diet improved the secretory pattern of β–cell, at least in part, by activating the cAMP/PKA-signaling cascade.

KEY WORDS: insulin secretion, malnutrition, nutritional recovery, PKAα, PKCα, soybean diet

INTRODUCTION

Nutritional deprivation before and after birth impairs the structure and function of ß cells [1], [2], [3] and [4]. The structural and functional damage during these phases represents a potential hazard for the development of diabetes mellitus in adult life. Because the growth of ß cells and insulin secretion during fetal life are predominantly regulated by amino acids, protein restriction in early life may play a major role in the appearance of type 2 diabetes [5].

Recent studies performed in animals with insulin resistance and in type 2 diabetic patients have shown that ingestion of soy protein and isoflavones improves the hyperglycemia and insulin sensitivity [6] and [7], as well as the insulin secretion [8]. In addition, rats recovered from protein restriction with the soybean diet in critical phase of development exhibited increased serum insulin levels in fed state and total area under the insulin curves (ΔI) in response to a glucose load [9].

The effect of soy on insulin secretion is attributed to its content of isoflavones and the amino acidic composition of its protein. Isoflavones, particularly genistein, may favor glucose homeostasis by enhancing insulin secretion [10]. The potentiating effect of genistein on insulin secretion has been attributed to its ability to inhibit islet tyrosine kinase activity [11]. It was suggested that the stimulatory effect of genistein on insulin secretion can occur independently of inhibition of protein tyrosine kinase, but it could result from an enhancement of cAMP concentration [8], [12] and [13]. The elevation of cAMP level and the consequent activation of PKA in ß-cells play an important role in incretin-stimulated insulin secretion [14] and [15]. In opposition to these studies, it was shown that genistein inhibited protein tyrosine kinase activity and increased the insulin secretion in response to

glucose and sulphonylurea without affecting the glucose metabolism, cAMP-dependent protein kinase (PKA) activity but decreased the protein kinase C (PKC) activity [16].

Since in protein-restricted rats the alterations in the cAMP/PKA and PLC/PKC systems appear to be involved in the reduction of insulin secretion [17] and [18], and since soybean diet modulates these pathways, we investigated the effect of soybean diet during nutritional recovery on insulin secretion and on the expression of PKA and PKC in pancreatic islets.

MATERIALS AND METHODS

Animals and diets

All of the animal experiments were approved by the State University of Campinas Ethics Committee (São Paulo, Brazil). Male and virgin female Wistar rats (85-90 d old) obtained from the University's breeding colony were housed in individual cages on a 12 h light/dark cycle at 24°C with free access to food and water throughout the experimental period. Mating was done by housing males with females overnight, and pregnancy was confirmed by the examination of vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained from the first day of pregnancy until the end of lactation on an isocaloric diet containing 6% (low protein or LP diet) or 17% (control or C diet) protein. Spontaneous delivery took place on day 22 of pregnancy after which, at 3 d of age, large litters were reduced to eight male pups to ensure a standard litter size per mother. At weaning males were divided into five groups: CC, consisting of offspring born to and suckled by mothers fed a C diet and subsequently fed the same diet after weaning; SC, consisting of offspring born to and suckled by mothers fed a C diet and subsequently fed a soybean flour diet with 17% protein after weaning; LP, consisting of the offspring of mothers fed an LP diet and subsequently fed the same diet after weaning; CR, consisting of the offspring of mothers fed an LP diet, but fed a C diet after weaning; SR, consisting of the offspring of mothers fed an LP diet, but fed a diet of whole soybean flour containing 17% protein after weaning. Adjustments in the soybean diet to equalize the carbohydrate, lipids and fiber contents and energy value to casein diet, suppressing the soybean oil and fiber were made. The diets are described in Table 1. The whole soybean flour was obtained by industrial processing (thermal treatment, peeling, grinding and

micronization) that reduced the enzymatic and anti-trypsin factor content, and contained 80% of the nutritional value of animal casein.

Glucose-tolerance test

A GTT was done in 90-d-old rats of the five groups. After 15 h fast, glucose (200 g/L) was administered intra peritoneal at a dose of 2 g/kg of body weight. Blood samples were obtained from the cut tip of the tail 0, 30, 60 and 120 min later for the determination of serum glucose [19] and insulin [20] concentrations. The glucose and insulin responses during the glucose-tolerance test were calculated by estimating the total area under the glucose (ΔG) and insulin (ΔI) curves, using the trapezoidal method [21].

Insulin secretion

Islets were isolated by collagenase digestion of the pancreas as described [22]. 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 replaced with fresh buffer and then islets were further incubated for 1 h with the following secretagogues: *1*) glucose (2.8 mmol/L, 8.3 mmol/L and 22.2 mmol/L); 2) glucose (8.3 mmol/L) in the absence and presence of phorbol 12-myristate 13-acetate (PMA; 100 nmol/L; Sigma); 3) glucose (8.3 mmol/L) in the absence and presence of forskolin (1 µmol/L). The insulin content of the medium, at the end of the incubation period, was measured by RIA [20].

Western blotting

Groups of islets were pelleted by centrifugation (15,000 x *g*) and then resuspended in 50–100 μL of homogenization buffer containing protease inhibitors, as described [23] and [24]. After isolation by collagenase digestion of pancreata and subsequent separation on discontinuous lyophilized Ficoll DL-400 gradients, a pool of at 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. PKA and PKC were detected in the membranes after 2-h incubation at room temperature with mouse monoclonal antibodies against PKAα and PKCα (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).

Statistical analysis

The results are presented as the mean ± SD. Two-way analysis of variance (ANOVA; effects of nutritional status and diet) was used to compare the glucose-insulin secretion data from the CC, SC, CR and SR groups. When analyzing the forskolin or PMA effects three-way analysis of variance (ANOVA; effects of nutritional status, diet and potentiators) was used to compare the data from the CC, SC, CR and SR groups. The same data were analyzed by one-way ANOVA when assessing whether diet was effective in improving the nutritional status in the CR, SR and LP groups. When necessary, these analyses were complemented by the Tukey test to determine the significance of individual differences. Bartlett's test for the homogeneity of variances was initially used to check the fit of the data to the assumptions for parametric analysis of variance. To correct for variance heterogeneity or non-normality data were log-transformed [25]. P values <0.05 were considered to indicate a significant difference.

RESULTS

The body weights of recovered rats were similar to control rats and higher than LP rats independently of the diet used during the recovery period (Table 2).

The fasting serum glucose and insulin concentrations, as well as the mean total areas under the ΔG curves in response to a glucose load, were not significantly different among the groups. For the mean total areas under the ΔI curves, ANOVA revealed no effect of previous nutritional status, but a main effect of diet used during recovery ($F_{1,21}$=28.61, P<0.001) and interaction between these two effects ($F_{1,21}$=8.44, P<0.01). Thus, SR rats had similar mean total areas under the ΔI curves to CC and SC rats and higher than CR rats. The ΔG:ΔI ratios were influenced by the nutritional status ($F_{1,21}$=9.23, P<0.01), the diet ($F_{1,21}$=23.59, P<0.001) and the interaction of these effects ($F_{1,21}$=9.91, P=0.05), showing that SR rats had a lower ΔG:ΔI ratio than CR, CC and SC rats. Following nutritional recovery, the SR group had higher mean total area under the ΔI curves and lower ΔG:ΔI ratios than CR and LP groups (P<0.01 and P<0.05, respectively). The fasting serum glucose and insulin concentrations, and the mean total area under the ΔG curves did not differ among groups (Table 2).

Insulin secretion in the presence of 2.8 mmol/L glucose did not differ among groups, since that ANOVA using nutritional status before the recovery phase and diet used during recovery as factors yielded no reliable main effects or interactions (Figure 1A). In 8.3 mmol/L glucose two-way ANOVA revealed a significant effect of nutritional status before the recovery phase ($F_{1,40}$=24.02, P<0.001) and diet used during recovery ($F_{1,40}$=11.06, P<0.01) as well as an interaction between nutritional status by diet ($F_{1,40}$=4.89, P<0.05). The insulin secretion by islets from SR and CR rats was similar (1.74 ± 0.55

ng/islet.h, n=12 and 1.49 ± 0.45 ng/islet.h, n=10, respectively), whereas in SC islets the insulin release was higher than in CC islets (3.32 ± 1.00 ng/islet.h, n=12 and 2.09 ± 0.78 ng/islet.h, n=10, respectively, $P<0.01$). Islets from LP group showed lower insulin secretion than those from SR and CR groups (0.59 ± 0.15 ng/islets.h, n= 9, $P<0.001$) (Figure 1B). After incubation in 22.2 mmol/L glucose two-way ANOVA showed no reliable effect of diet used during recovery ($F_{1,54}=2.22$, $P<0.05$) but revealed a main effect of nutritional status before the recovery phase ($F_{1,54}=7.19$, $P<0.01$) as well as an interaction between nutritional status by diet ($F_{1,54}=29.78$, $P<0.001$). The insulin secretion by islets from SR group was higher than that from CR group (14.94 ± 2.94 ng/islet.h, n=14 and 11.16 ± 2.96 ng/islet.h, n=15, respectively, $P<0.05$), whereas the insulin released by islets from SC group was lower when compared to islets from CC group (12.29 ± 3.33 ng/islet.h, n=14 and 18.92 ± 4.88 ng/islet.h, n=15, $P<0.001$). Islets from LP group released less insulin than islets from SR and CR groups (6.80 ± 1.25 ng/islet.h, n=9, $P<0.001$) (Figure 1C).

When PMA (100 nmol/L) was combined with glucose (8.3 mmol/L) the insulin response was increased in all groups ($F_{1,45}=575.45$, $P<0.001$). Again the recovered rats exhibited lower insulin secretion than control rats ($F_{1,45}=15.47$, $P<0.001$) and the soybean diet produced an increase in the insulin secretion to a higher extent than the casein diet ($F_{1,45}=14.62$, $P< 0.001$). Although PMA induced a 6.3-fold and 3.9-fold enhance in the insulin secretion in the SR and SC groups, respectively, the difference of magnitude of potentiation did not approach statistical significance ($F_{1,45}=3.24$, $P=0.078$). In the LP group PMA produced a 9.5 fold increase of insulin secretion reaching an amount of secretion similar to that observed in CR group (Figure 2).

Western blotting showed that PKCα expression was similar among CC, SC, CR and SR groups, and significantly lower in islets from LP rats compared with SR and CR groups (Figure 3).

In the absence of forskolin, insulin secretion by islets from recovered rats was lower than control islets ($F_{1,46}$=10.36, P<0.01). Addition of 1 μmol/L forskolin increased insulin secretion ($F_{1,46}$=63.18, P<0.001), however the magnitude of the increment was lower in recovered than in control islets ($F_{1,46}$=11.32, P<0.01) as well as in islets from rats maintained with soybean diet than in rats fed with casein diet ($F_{1,46}$=6.45, P<0.05). In the presence of forskolin, insulin secretion in LP islets was similar to SR group and lower than in CR group (p<0.05) (Figure 4).

Concerning PKAα expression, ANOVA revealed no significant effect of previous nutritional status and diet used during the recovery period, however there was an interaction between these effects ($F_{1,23}$=6.94, P<0.02). Thus, the expression of PKAα was similar between SR and CR groups and lower in SC than in CC groups. The PKAα content in LP islets was lower than those from SR and CR groups (Figure 5).

TABLE 1. Composition of the normal, low-protein and soy flour diets

Ingredient	**Normal (AIN-93G)[1] (170 g protein/Kg)**	**Low protein (60 g protein/Kg)**	**Soya flour inactive + (AIN-93G) (170 g protein/Kg)**
Soya flour inactive[2]	-	-	415.0
Casein (850g protein/Kg)	202.0	71.5	-
Cornstarch	397.0	480.0	312.2
Dextrinized cornstarch	130.5	159.0	103.7
Sucrose	100.0	121.0	78.60
Soybean oil	70.0	70.0	-
Fiber	50.0	50.0	40.0
Mineral mix (AIN93G-MX)	35.0	35.0	35.0
Vitamin mix (AIN93-VX)	10.0	10.0	10.0
L-Cystine	3.0	1.0	3.0
Choline chlorydrate	2.5	2.5	2.5

[1] For detailed composition see [38].
[2] Perdigão Indústria e Comércio Ltda, SC, Brasil.

TABLE 2. Body weight, fasting glucose and insulin concentrations, total areas under the glucose (ΔG) and insulin (ΔI) curves obtained from the oral glucose-tolerance test and ΔG:ΔI ratio in the five treatment groups

Parameters	GROUPS				
	CC (6)	**SC** (6)	**CR** (6)	**SR** (7)	**LP** (5)
Body weight, g	230±10	220±20	241±37[a]	217±16[a]	167±34[b]
Fasting glucose, mmol/L	3.8 ± 0.5	4.3 ± 0.6	4.1 ± 0.3	4.3 ± 0.8	7.0 ± 2.8
Fasting insulin, pmol/L	54±21	59± 13	53±26	62±22	59±22
ΔG, mmol/L.120 min	753±148	775±64	674±130	824±155	833±270
ΔI, pmol/L.120 min	13442±2073[A]	16246±2499[A]	8013±1562[Bb]	17480±4222[Aa]	9617±2396[b]
ΔG:ΔI, mmol/pmol	0.06 ± 0.01[B]	0.05±0.01[B]	0.09±0.02[Aa]	0.05±0.01[Bb]	0.09±0.04[a]

Values are means ± SD for the number of rats in parentheses. Means with different superscript capital letters are significantly different by two-way ANOVA and with superscript minuscule letters are significantly different by one-way ANOVA followed by Tukey test ($p<0.05$).

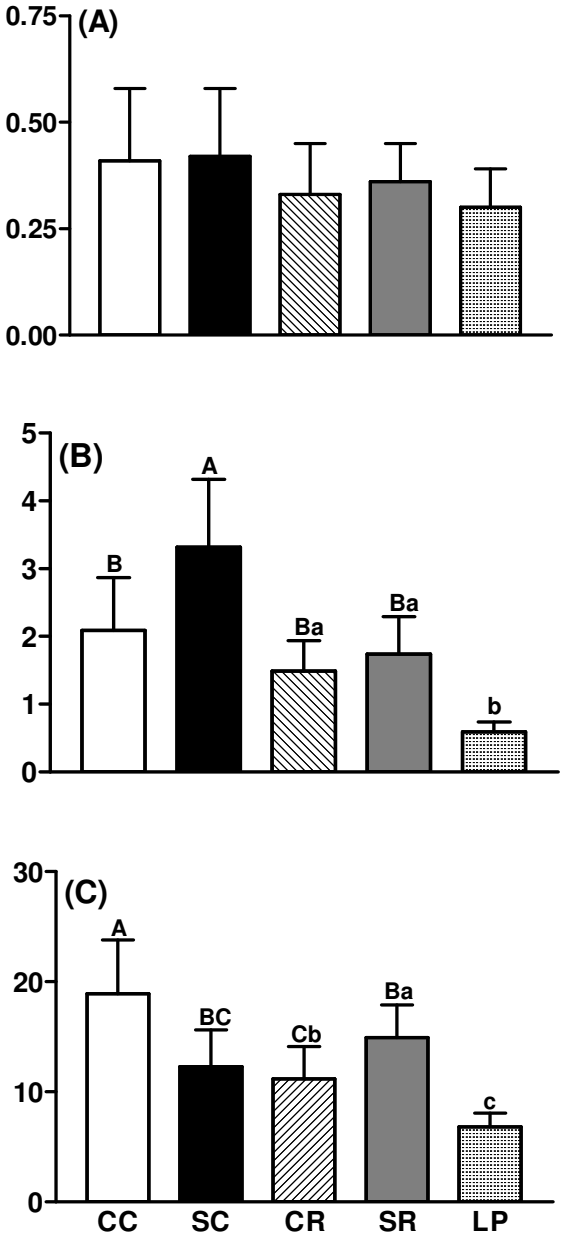

FIGURE 1. Insulin secretion in response to 2.8 mmol/L **(A)**, 8.3 mmol/L **(B)** and 22.2 mmol/L **(C)** glucose by in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) and low protein (LP) diets after weaning. The columns represent the cumulative 1 h insulin secretion and are expressed in means ± SD, n=6 independent experiments. Means with different superscript capital letters are significantly different by two-way ANOVA and with superscript minuscule letters are significantly different by one-way ANOVA followed by followed by Tukey test (p<0.05).

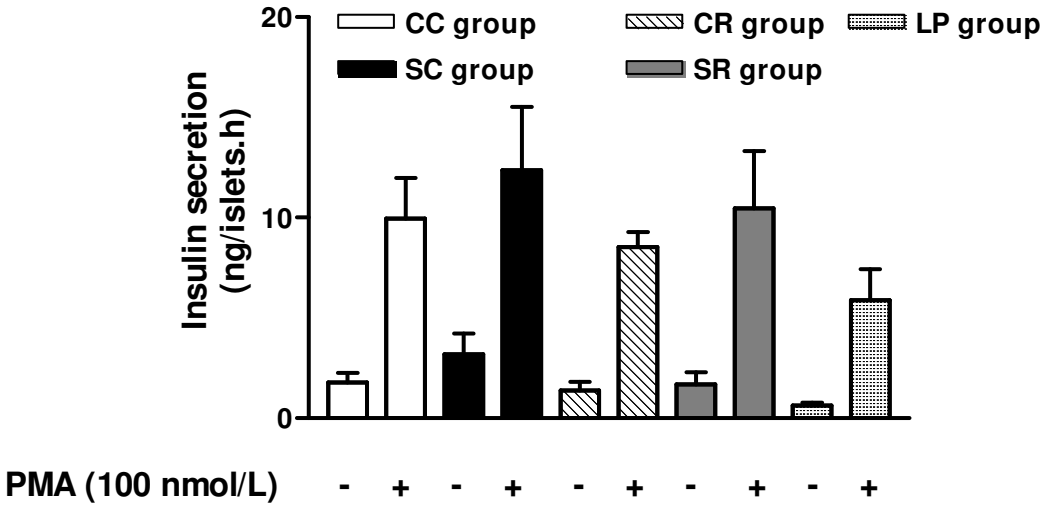

FIGURE 2. Phorbol 12-myristate 13-acetate (PMA) in 8.3 mmol/L glucose stimulation of insulin secretion in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) and low protein (LP) diets after weaning. The columns represent the cumulative 1-h insulin secretions and are expressed in means ± SD, *n*=6 independent experiments.

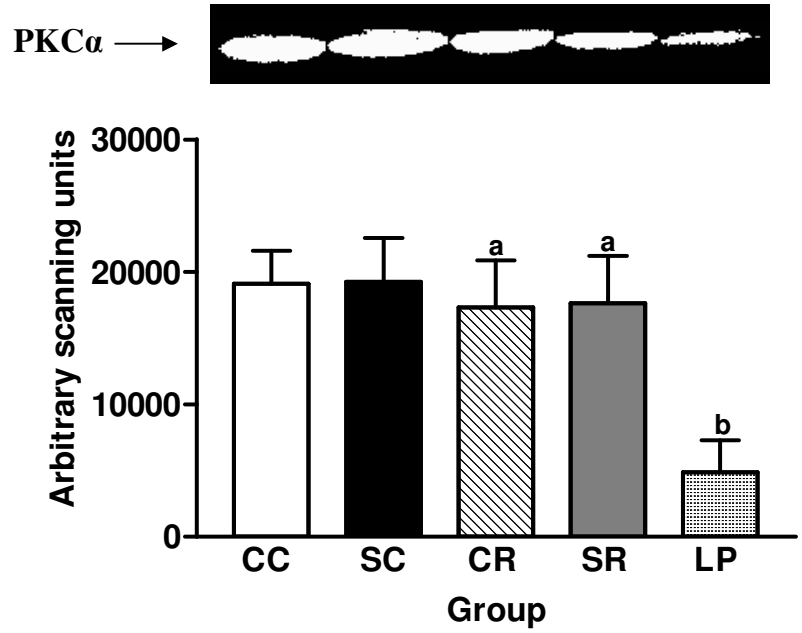

FIGURE 3. Protein Kinase Cα (PKCα) expression in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. Values are expressed in means ± SD, *n*=6 independent experiments. Means with different superscript minuscule letters are significantly different by one-way ANOVA followed by followed by Tukey test ($p<0.05$).

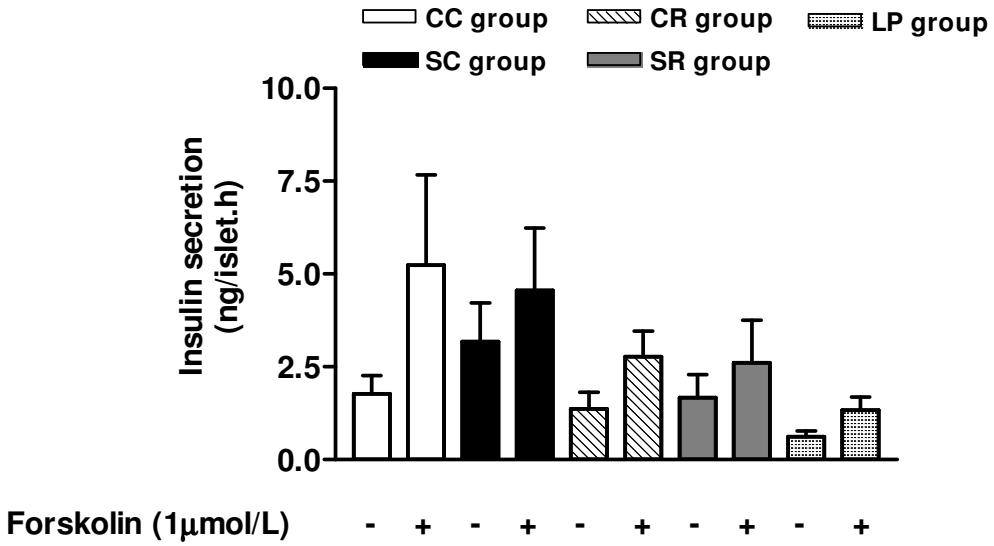

FIGURE 4. Forskolin (1μmol/L) in 8.3 mmol/L glucose induced insulin secretion in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) and low protein (LP) diets after weaning. The columns represent the cumulative 1-h insulin secretion and are expressed in means ± SD, *n*=6 independent experiments.

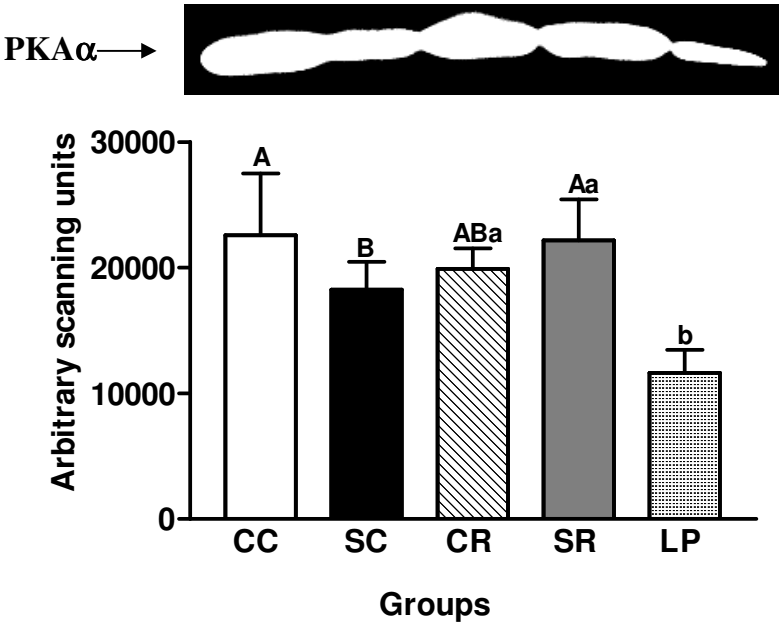

FIGURE 5. Western blot analysis of protein kinase cAMP-dependent catalytic subunit α(PKAα) in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) and low protein (LP) diets after weaning. Values are expressed in means ± SD, *n*=6 independent experiments. Means with different superscript capital letters are significantly different by two-way ANOVA and with superscript minuscule letters are significantly different by one-way ANOVA followed by followed by Tukey test ($p<0.05$).

DISCUSSION

In this study we have shown that the soybean diet induced a body weight gain similar to that observed in rats recovered with casein, in disagreement to the observation that soy does not meet the protein requirements to support the adequate growth rates in rat [26]. Also, the soybean diet during nutritional recovery increased the total areas under the ΔI curves in response to glucose load and reduced the ΔG:ΔI ratio, indicating an improvement of the ß-cells function. However, the insulin secretion in islets from recovered rats was lower than in control rats in physiological and supra-physiological glucose concentrations. These results were not surprising since peripheral insulin levels may not reflect true insulin secretion [27] and [28]. We should emphasize that soybean diet improved the response of ß-cells from control rats to a physiological concentration of glucose, whereas in islets from recovered rats this occurred only in the presence of a supra-physiological glucose concentration. The increase in insulin secretion obtained in islets from SC group could be due to genistein, a component of soy that produces a shift to the left in the glucose-dose response curve in rat pancreatic islets, enhancing the insulin secretion even with 50 mg/dl glucose, indicating that this compound is a potent regulator of the glucose-stimulated insulin secretion [11]. However, this effect did not occur in islets from SR rats, which showed increase in the insulin secretion only in supra-physiological glucose concentration.

Activators of the cAMP/PKA and inositol phosphate/PKC pathways potentiate glucose-induced insulin secretion and their importance in this process has been investigated [29]. Genistein acutely stimulates insulin secretion in pancreatic beta cells through a cAMP-dependent protein kinase pathway [8]. Because protein restriction modifies

cAMP/PKA and inositol phosphate/PKC pathways [17] and [18], we examined the insulin secretory response to PMA and forskolin, activators of PKC and adenylate cyclase as well as PKC and PKA expressions, respectively. PMA induced similar potent secretory response in islets from SR and SC groups, suggesting no alterations in the PKC levels. Several types of PKC are present in ß cells, with PKCα as the major component [30] and [31]. Independently of the source of protein or the previous nutritional status, the PKCα expression was similar among groups, and islets from SR and CR groups had higher PKCα content than LP islets. Because the content of PKCα and the secretory response to PMA was not modified among groups, this pathway should not be accounted for the increased sensitivity verified in islets from SC group or for the unaltered effect observed in the SR group at physiological glucose concentration.

In several tissues [32] and [33], including the endocrine pancreas [34] and [35], forskolin activates adenylate cyclase increasing cAMP formation, which stimulates PKA. In the pancreas, the stimulation of PKA leads to increased insulin secretion [36]. Addition of 1 µmol/L forskolin to medium containing 8.3 mmol glucose/L produced a lower effect on insulin secretion in islets from rats recovered than control rats as well as in those maintained with the soybean diet compared with rats fed with casein diet. At least in the SC group the low sensitivity to forskolin could be attributed to a decrease in PKA expression. However, this argument is not consistent if one considers the PKA levels and the magnitude of the rise in insulin release induced by forskolin in the CR and SR islets. Because genistein, at physiological concentrations, exhibits the same effect showed by forskolin, we speculate that the low responsiveness to this agent by islets from rats fed with soybean diet could be a reflex of desensitization of cAMP/PKA pathway due to chronically

higher levels of cAMP. Another possibility is that the additive effect of two potent stimulators of adenylate cyclase turned 1 μmol/L forskolin an overdose to β-cells from rats maintained with the soybean diet. Especially in islets deriving from SC group, it is possible that the overproduction of cAMP has been counter-regulated by diminished PKA expression in an attempt to reestablish normality of cAMP/PKA pathway. The unchanged PKA expression in islets from SR animals could be caused by a lower cAMP synthesis than in SC rats due to previous protein deprivation that permanently affects the production of this intracellular messenger [37].

In conclusion, this study shows that the soybean diet during nutritional recovery improved the insulin secretion, at least in supra-physiological glucose concentrations. This effect seems to be mediated, at least in part, by the cAMP/PKA pathway.

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CONCLUSÕES

4. CONCLUSÕES

Com base nos objetivos traçados e de acordo com os resultados obtidos concluímos que:

- A farinha de soja é capaz de promover a recuperação do estado nutricional em animais submetidos à restrição protéica em fases críticas do desenvolvimento.
- A recuperação do estado nutricional é representada pelo aumento das concentrações séricas de aminoácidos e compostos nitrogenados, tais como glutamato, ornitina, fenilserina e taurina que estimulam a liberação de insulina.
- O aumento da concentração de insulina sérica parece ser compensado pelo aumento da resistência à insulina, que contribui para manutenção da homeostase da glicose.
- Dieta à base de farinha de soja durante a recuperação nutricional melhora a secreção de insulina, pelo menos em concentrações suprafisiológicas de glicose. Em animais controles esta melhora ocorreu em concentrações fisiológicas de glicose. Entretanto, em ambas as situações este efeito parece ser devido à ativação da via AMPc/PKA.
- Outros estudos devam ser realizados com o intuito de investigar a longo tempo, repercussões da utilização desta fonte de proteína com alta qualidade e baixo custo sobre a homeostase da glicose neste modelo animal.

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CERTIFICADO

Certificamos que o Protocolo nº **1150-1**, sobre **"O papel da farinha de soja na recuperação nutricional e na secreção de insulina de ratos submetidos à restrição proteica durante a vida intra-uterina e na lactação"**, sob a responsabilidade de **Prof. Dr. Everardo Magalhães Carneiro / Roberto Vilela Veloso**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 29 de novembro de 2006.

CERTIFICATE

We certify that the protocol nº **1150-1**, entitled **"Role of soybean flour in the nutritional recovery and in the insulin secretion of rats submitted to protein restriction during pregnancy and lactation period"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on November 29, 2006.

Campinas, 29 de novembro de 2006.

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