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Soybean diet improves insulin secretion through activation of cAMP/PKA pathway in rats☆

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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 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 until 90 days of life. The soybean diet improved the insulin response to a physiological concentration of glucose in control islets, but only in the presence of supra-physiological concentrations of glucose in islets from CR and SR groups. PMA also improved the insulin response in islets of SC and SR groups. The expression of PKCα was similar in all groups. Forskolin increased the insulin secretion; however, the magnitude of the increment was lower in islets from CR and SR groups 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, soybean diet improved the secretory pattern of β cells, at least in part, by activating the cAMP/PKA-signaling cascade.

Keywords: Insulin secretion; Malnutrition; PKAα; PKCα; Soybean diet

1. Introduction

Nutritional deprivation before and after birth impairs the structure and function of β cells [1–4]. 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 β 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 [6,7].

Isoflavones, particularly genistein, may favor glucose homeostasis by enhancing insulin secretion [8]. The potentiating effect of genistein on insulin secretion has been attributed to its ability to inhibit islet tyrosine kinase activity [9]. It was suggested that the stimulatory effect of genistein on insulin secretion can occur independently of inhibition of protein tyrosine kinase [10], and it could result from an enhancement of cAMP concentration [8,11].

The elevation of cAMP level and the consequent activation of PKA in β cells play an important role in incretin-stimulated insulin secretion [12,13]. In opposition to these studies, it was shown that genistein inhibited protein tyrosine kinase activity and increased the insulin secretion in response to glucose without affecting the glucose metabolism and cAMP-dependent protein kinase (PKA) activity but decreased the protein kinase C (PKC) activity [14].

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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 [15,16], and since soybean diet modulates these pathways, we investigated the effect of soybean diet on insulin secretion and on the expression of PKA and PKC in pancreatic islets.

2. Materials and methods

2.1. 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 days 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. The control diet followed the AIN-93G recommendations. Spontaneous delivery took place on Day 22 of pregnancy after which, at 3 days of age, large litters were reduced to eight male pups to ensure a standard litter size per mother. At weaning, males were divided into four groups: CC, consisting of offspring born to and suckled by mothers fed a C diet and subsequently fed the same diet after weaning (n=6); 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 (n=6); CR, consisting of the offspring of mothers fed an LP diet, but fed a C diet after weaning (n=6); SR, consisting of the offspring of mothers fed an LP diet, but fed a diet of whole soybean flour containing 17% protein after weaning (n=7). Adjustments in the soybean diet were made to equalize the carbohydrate, lipids and fiber contents and energy value to casein diet. The diets are described in Table 1. The whole soybean flour was obtained by industrial processing (thermal treatment, peeling, grinding and micronization), which reduced the enzymatic and anti-trypsin factor content, and contained 80% of the nutritional value of animal casein. The soy flour used in this study is called inactive because the thermal treatment inhibits the antinutritional factors, such as hemaglutinine and anti-trypsin. Fat content was similar to that of AIN-93G diet. First, we analyzed the composition of soy flour (protein, carbohydrate, lipids and fibers), then added to the flour the other AIN-93 diet components to keep the same proportion of the macronutrients above and the same amount of calories (Table 1).

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

Ingredient	Normal (AIN-93G)[a] (170 g protein/kg)	Soybean flour[b] (170 g protein/kg)	Low protein (60 g protein/kg)
Soybean flour[c]	–	415.0	–
Casein (850 g protein/kg)	202.0	–	71.5
Cornstarch	397.0	312.2	480.0
Dextrinized cornstarch	130.5	103.7	159.0
Sucrose	100.0	78.6	121.0
Soybean oil	70.0	70.0	70.0
Fiber	50.0	50.0	50.0
Mineral mix (AIN93G-MX)	35.0	35.0	35.0
Vitamin mix (AIN93G-VX)	10.0	10.0	10.0
L-Cystine	3.0	3.0	1.0
Choline chlorydrate	2.5	2.5	2.5

[a] For detailed composition see Ref. [17].
[b] Perdigão Indústria e Comércio Ltda, SC, Brazil.
[c] Isoflavonóides: 100.0 mg/100 g (genistein: 1.9 mg/100 g; daidzein: 2.6 mg/100 g).

2.2. Glucose-tolerance test

A glucose-tolerance test (GTT) was done in 90-day-old rats of the four groups. After a 15-h fast, glucose (200 g/L) was administered intraperitoneally 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 [18] and insulin [19] concentrations. The glucose and insulin responses during the GTT were calculated by estimating the total area under the glucose (ΔG) and insulin (ΔI) curves, using the trapezoidal method [20]. The rats were sacrificed for islets studies 2 days after the GTT.

2.3. Insulin secretion

Islets were isolated by collagenase digestion of the pancreas as described [21]. 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 the islets were further incubated for 1 h with the following secretagogues: (1) glucose (2.8, 8.3 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 [19].

2.4. Western blotting

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 were 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, USA). Samples containing 70 μg of protein from each experimental group were incubated for 5 min at 80°C with 4× 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 a 2-h incubation at room temperature with mouse monoclonal antibodies against PKAα and PKCα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (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, USA) [22].

2.5. Statistical analysis

The results are presented as mean±S.D. 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 (effects of nutritional status, diet and potentiators) was used to compare the data from the CC, SC, CR and SR 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 [23]. P values <.05 were considered to indicate a significant difference.

3. Results

The body weights of SR and CR rats were similar to control rats independently of the diet used after weaning (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 after weaning ($F_{1,21}$=28.61, P<.001) and interaction between these two effects ($F_{1,21}$=8.44, P<.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<.01), the diet ($F_{1,21}$=23.59, P<.001) and the interaction of these effects ($F_{1,21}$=9.91, P=.05), showing that SR rats had a lower ΔG/ΔI ratio than CR, CC and SC rats (Table 2).

Insulin secretion in the presence of 2.8 mmol/L glucose did not differ among groups, since the ANOVA using nutritional status before the weaning (LP and CC groups) and diet used after weaning (SC, CC, CR and SR) as factors yielded no reliable main effects or interactions (Fig. 1A). In 8.3 mmol/L glucose, two-way ANOVA revealed a significant effect of nutritional status before the weaning ($F_{1,40}$=24.02, P<.001) and diet used after weaning ($F_{1,40}$=11.06, P<.01) as well as an interaction between nutritional status by diet ($F_{1,40}$=4.89, P<.05). The insulin secretion by islets from SR and CR rats was similar (1.74±0.55 ng/islet per hour, n=12; 1.49±0.45 ng/islet per hour, n=10, respectively), whereas in

Table 2
Body weight, fasting glucose and insulin concentrations, total areas under the glucose (ΔG) and insulin (ΔI) curves obtained from the oral GTT and ΔG/ΔI ratio in the four treatment groups

Parameters	GROUPS			
	CC (6)	SC (6)	CR (6)	SR (7)
Body weight, g	230±10	220±20	241±37	217±16
Fasting glucose, mmol/L	3.8±0.5	4.3±0.6	4.1±0.3	4.3±0.8
Fasting insulin, pmol/L	54±21	59±13	53±26	62±22
ΔG, mmol/L per 120 min	753±148	775±64	674±130	824±155
ΔI, pmol/L per 120 min	13442±2073^{A}	16246±2499^{A}	8013±1562^{B}	17480±4222^{A}
ΔG/ΔI, mmol/pmol	0.06±0.01^{B}	0.05±0.01^{B}	0.09±0.02^{A}	0.05±0.01^{B}

Values are mean±S.D. for the number of rats in parentheses. Means with different superscript letters are significantly different by two-way ANOVA followed by Tukey test (P<.05).

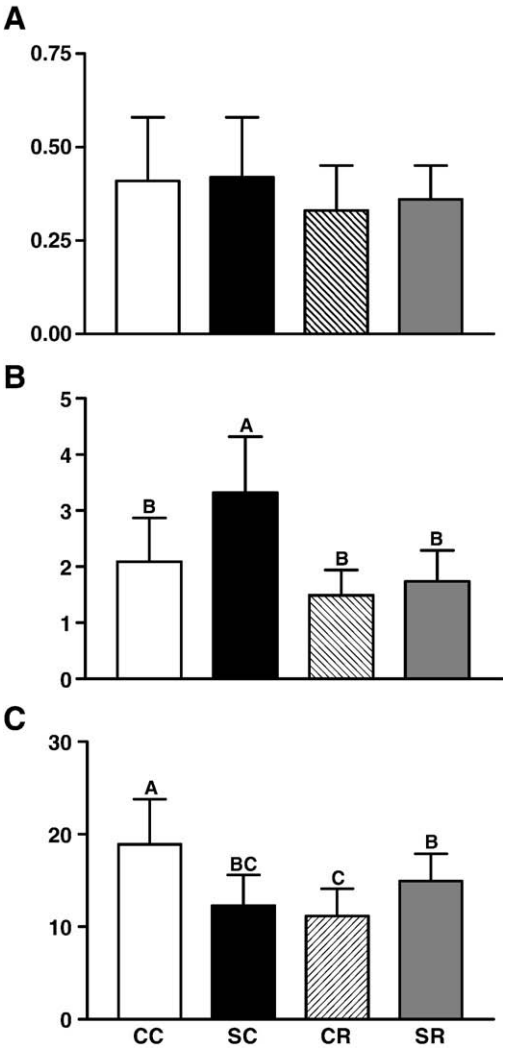

Fig. 1. Insulin secretion in response to 2.8 mmol/L (A), 8.3 mmol/L (B) and 22.2 mmol/L (C) glucose by islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. The columns represent the cumulative 1-h insulin secretion and are expressed in mean±S.D.; n=6 samples of pooled islets in two separate experiments. Means with different superscript letters are significantly different by two-way ANOVA followed by Tukey test (P<.05).

SC islets the insulin release was higher than in CC islets (3.32±1.00 ng/islet per hour, n=12; 2.09±0.78 ng/islet per hour, n=10, respectively, P<.01) (Fig. 1B). After incubation in 22.2 mmol/L glucose, two-way ANOVA showed no reliable effect of diet used after weaning ($F_{1,54}$=2.22, P<.05) but revealed a main effect of nutritional status before the weaning ($F_{1,54}$=7.19, P<.01) as well as an interaction between nutritional status by diet ($F_{1,54}$=29.78, P<.001). The insulin secretion by islets from the SR group was higher than that from the CR group (14.94±2.94 ng/islet per hour, n=14; 11.16±2.96 ng/islet per hour, n=15, respectively, P<.05), whereas the insulin released by islets from the SC group was lower when compared to that from the CC group (12.29±3.33 ng/islet per hour, n=14; 18.92±4.88 ng/islet per hour, n=15, P<.001) (Fig. 1C).

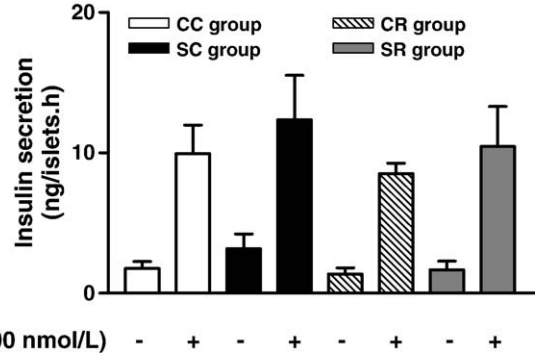

Fig. 2. Phorbol 12-myristate 13-acetate (PMA) in 8.3 mmol/L glucose stimulation of insulin secretion in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. The columns represent the cumulative 1-h insulin secretions and are expressed in mean±S.D.; n=6 samples of pooled islets in two separate experiments.

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<.001). Again the CR and SR groups exhibited lower insulin secretion than control rats ($F_{1,45}$=15.47, P<.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<.001). Although PMA induced a 6.3-fold and 3.9-fold enhancement in the insulin secretion in the SR and SC groups, respectively, the difference in magnitude of potentiation did not approach statistical significance ($F_{1,45}$=3.24, P=0.078) (Fig. 2).

Western blotting showed that PKCα expression was similar among CC, SC, CR and SR groups (Fig. 3).

In the absence of forskolin, insulin secretion by islets from CR and SR groups was lower than that by control islets ($F_{1,46}$=10.36, P<.01). Addition of 1 μmol/L forskolin increased insulin secretion ($F_{1,46}$=63.18, P<.001); however, the magnitude of the increment was lower in the CR and SR

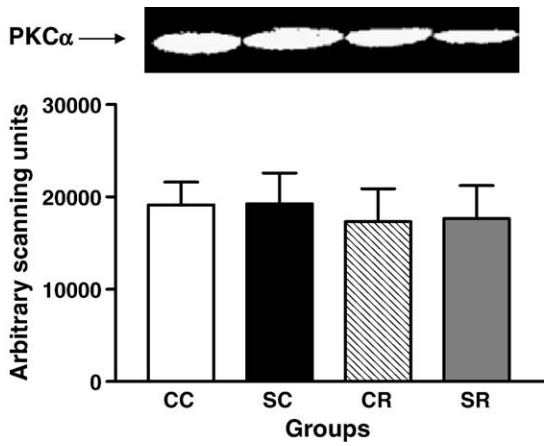

Fig. 3. Protein kinase Cα (PKCα) content in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. Values are expressed in mean±S.D.; n=6 samples of pooled islets in two separate experiments.

groups than in control islets ($F_{1,46}$=11.32, P<.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<.05) (Fig. 4).

Concerning PKAα expression, ANOVA revealed no significant effect of previous nutritional status and diet used after weaning; however, there was an interaction between these effects ($F_{1,23}$=6.94, P<.02). Thus, the expression of PKAα was similar between SR and CR groups and lower in the SC than in the CC groups (Fig. 5).

4. Discussion

In this study we have shown that the soybean diet induced a body weight gain similar to that observed in rats fed casein diet after weaning, in agreement to the observation that soy does meet the protein requirements to support the adequate growth rates in rat [24]. Also, the soybean diet after weaning increased the total areas under the ΔI curves in response to glucose load and reduced the ΔG/ΔI ratio, indicating an improvement of the pancreatic function. However, in the presence of physiological and supra-physiological glucose concentrations the insulin secretion in islets from rats previously submitted a low protein diet (CR and SR) was lower than that from control rats. These results were not surprising since peripheral insulin levels may not reflect true insulin secretion [25,26]. We should emphasize that soybean diet improved the response of β cells from CC and SC rats to a physiological concentration of glucose, whereas in islets from SR and CR rats this occurred only in the presence of a supra-physiological glucose concentration. The increase in insulin secretion obtained in islets from the 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 [9]. However, this effect did not occur in islets from SR rats, which showed an increase in the insulin secretion only in supra-physiological glucose concentration.

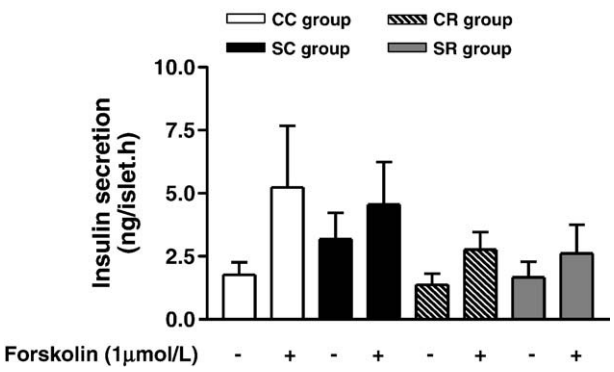

Fig. 4. Forskolin (1 μmol/L) in 8.3 mmol/L glucose-induced insulin secretion in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. The columns represent the cumulative 1-h insulin secretion and are expressed in mean±S.D.; n=6 samples of pooled islets in two separate experiments.

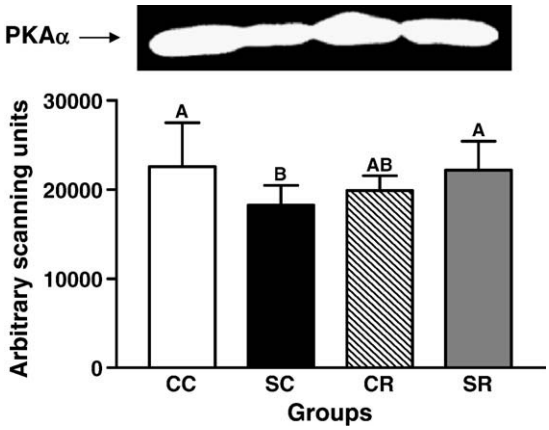

Fig. 5. Western blot analysis of protein kinase cAMP-dependent catalytic subunit α(PKAα) in islets from adult rats maintained on control (CC and CR) or soybean flour (SC and SR) diets after weaning. Values are expressed in mean±S.D.; n=6 samples of pooled islets in two separate experiments. Means with different superscript letters are significantly different by two-way ANOVA followed by Tukey test (P<.05).

Activators of the cAMP/PKA and inositol phosphate/PKC pathways potentiate glucose-induced insulin secretion, and their importance in this process has been investigated [27]. Genistein acutely stimulates insulin secretion in pancreatic β cells through a cAMP-dependent protein kinase pathway [8]. Because protein restriction modifies cAMP/PKA and inositol phosphate/PKC pathways [15,16], 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 a similar potent secretory response in islets from the SR and SC groups, suggesting no alterations in the PKC levels. Several types of PKC are present in β cells, with PKCα as the major component [28,29]. Independently of the source of protein or the previous nutritional status, the PKCα expression was similar among groups. 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 the SC group or for the unaltered effect observed in the SR group at physiological glucose concentration.

In several tissues [30,31], including the endocrine pancreas [32,33], forskolin activates adenylate cyclase increasing cAMP formation, which stimulates PKA. In the pancreas, the stimulation of PKA leads to increased insulin secretion [34]. Addition of 1 μmol/L forskolin to medium containing 8.3 mmol glucose/L produced a lower effect on insulin secretion in islets from the CR and SR groups than that from the CC and SC groups 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 made 1 μmol/L forskolin an overdose to β cells from rats maintained with the soybean diet. Especially in islets from the 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 [35].

In conclusion, this study shows that the soybean diet 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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