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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

Insulin secretion. Islets were isolated by collagenase digestion of the pancreas as described (28). For static incubations, groups of five islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer with the following composition (mmol/L): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES and 5.6 glucose, supplemented with 3 g of bovine serum albumin/L and equilibrated with a mixture of 95% O_2:5% CO_2 to give a pH of 7.4. This medium was then replaced with fresh buffer and the islets incubated for 1 h with 8.3 mmol glucose/L and 10 μmol forskolin/L. The insulin content of the medium at the end of the incubation period was measured by RIA (29). The amount of protein in groups of 100 islets from LP and NP rats was similar. Thus, the results were expressed as pmol/(islet · h) or fmol/(islet · min).

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

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

TABLE 1

Composition of the normal and low protein diets[1]

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

[1] See (55) for more details.

TABLE 2

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

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

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

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

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

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

RESULTS

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

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

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

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

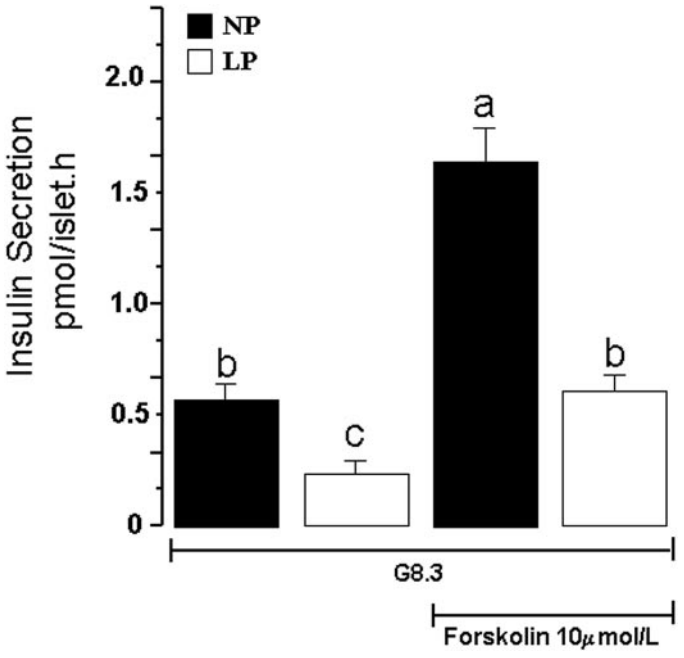

FIGURE 1 Forskolin-induced insulin secretion in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The columns represent the cumulative 1-h insulin secretion and are the means ± SEM; $n = 5$ independent experiments. Means without a common letter differ, $P < 0.05$.

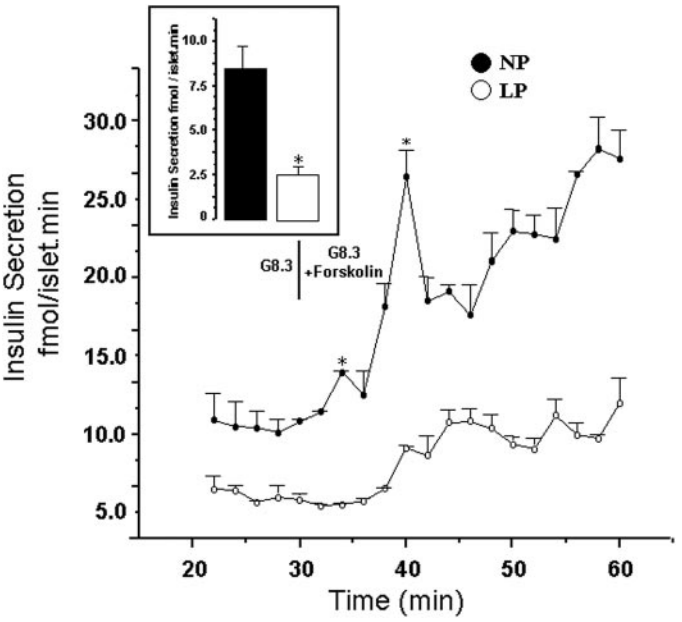

FIGURE 2 Forskolin-induced insulin secretion in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The lines represent the insulin secretion and are the means ± SEM; $n = 5$ independent experiments. The areas under the curves were calculated and the basal secretion of each group was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

DISCUSSION

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

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

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

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

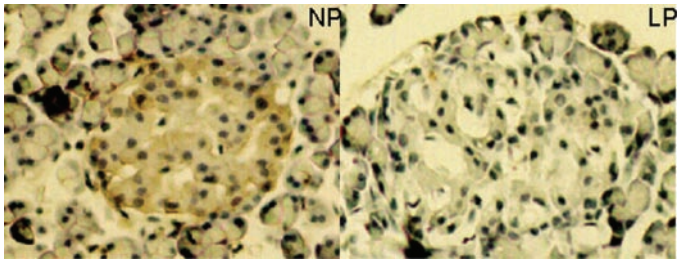

FIGURE 3 Immunohistochemistry of protein kinase cAMP-dependent catalytic subunit α (PKAα). The brown color indicates the localization of PKA in islets of rats fed normal (NP) and low protein (LP) diets for 8 wk.

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

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

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

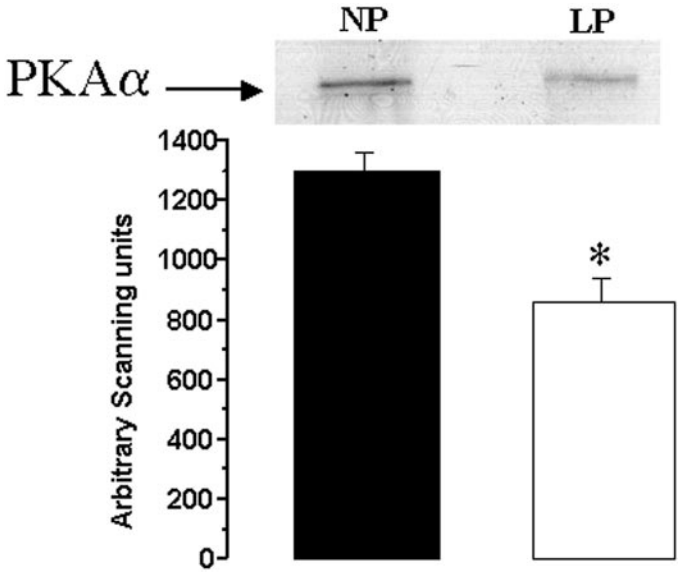

FIGURE 4 Western blot analysis of protein kinase cAMP-dependent catalytic subunit α (PKAα) in islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. Values are means ± SEM, $n = 6$ independent experiments. *Different from NP rats, $P < 0.05$.

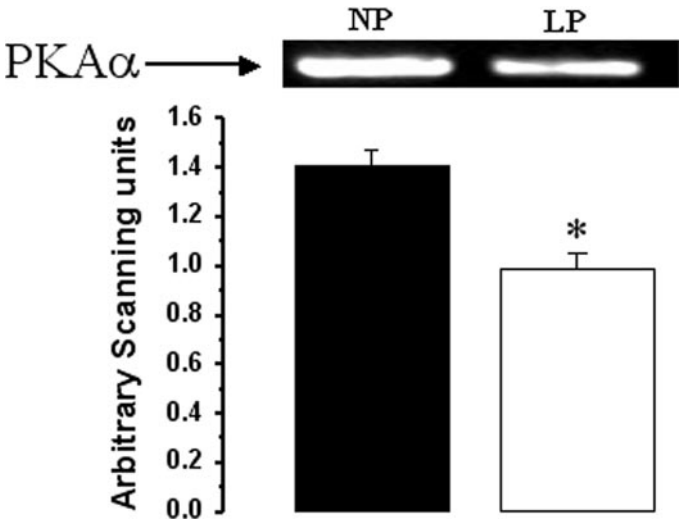

FIGURE 5 Protein kinase cAMP-dependent catalytic subunit α (PKAα) mRNA levels in pancreatic islets from rats fed normal (NP) and low protein (LP) diets for 8 wk. The columns are the means ± SEM; $n = 6$ independent experiments. *Different from NP rats, $P < 0.05$.

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

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

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

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