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


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ABSTRACT Undernutrition has been shown to affect the autonomic nervous system, leading to permanent alterations in insulin secretion. To understand these interactions better, we investigated the effects of carbamylcholine (CCh) and phorbol 12-myristate 13-acetate (PMA) on insulin secretion in pancreatic islets from rats fed a normal (17%; NP) or low (6%; LP) protein diet for 8 wk. Isolated islets were incubated for 1 h in Krebs-bicarbonate solution containing 8.3 mmol glucose/L, with or without PMA (400 nmol/L) and CCh. Increasing concentrations of CCh (0.1–1000 μmol/L) dosedependently increased insulin secretion by islets from both groups of rats. However, insulin secretion by islets from rats fed the NP diet was significantly higher than that of rats fed the LP diet, and the dose-response curve to CCh was shifted to the right in islets from rats fed LP with a 50% effective concentration (EC50) of 2.15 ± 0.7 and 4.64 ± 0.1 μmol CCh/L in islets of rats fed NP and LP diets, respectively (P < 0.05). PMA-induced insulin secretion was higher in islets of rats fed NP compared with those fed LP. Western blotting revealed that the protein kinase (PK)Cα and phospholipase (PL)Cβ1 contents of islets of rats fed LP were 30% lower than those of islets of rats fed NP (P < 0.05). In addition, PKCα mRNA expression was reduced by 50% in islets from rats fed LP. In conclusion, a reduced expression of PKCα and PLβ1 may be involved in the decreased insulin secretion by islets from LP rats after stimulation with CCh and PMA. J. Nutr. 133: 695–699, 2003.

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

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

Cholinergic agents and glucose act synergistically on B cells to increase phosphoinositide (PI)3 hydrolysis and insulin secretion (12). PI hydrolysis is stimulated by phospholipase (PL)Cβ1 through a mechanism coupled to G-protein. This coupling is initiated with the binding of the cholinergic agonist to muscarinic (M3) receptors located in the B-cell plasma membrane (13). PI hydrolysis results in the formation of 1,4,5 inositol triphosphate (IP3), which releases Ca2+ from intracellular stores, thereby increasing insulin secretion (14). Cholinergic agonists also activate PLC, which increases diacylglycerol (DAG) formation and stimulates protein kinase (PK)C. Thus, carbamylcholine (CCh)-induced insulin secretion can be reduced by inhibiting PKC (15).

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

3 Abbreviations used: Ach, acetylcholine; CCh, carbamylcholine; DAG, diacylglycerol; FFA, free fatty acid; IP3, 1,4,5 inositol triphosphate; LP, low protein group; M3, muscarinic; NP, normal protein group; PCR, polymerase chain reaction; PI, phosphoinositide; PKCα, protein kinase Cα; PLCβ1, phospholipase Cβ1; PMA, phorbol 12-myristate 13-acetate; PVX, potato virus X; RT, reverse transcribed; TTBS, Tris-Tween 20 buffered saline.
on insulin secretion and the expression of PKCα and PLCβ1 in islets isolated from rats fed a low protein diet.

MATERIALS AND METHODS

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

Insulin secretion. Islets were isolated by collagenase digestion of the pancreas as described (21). For static incubation, groups of five islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer with the following composition (in mmol/L): NaCl, 115; KCl, 5; CaCl2, 2.56; MgCl2, 1; NaHCO3, 24; and glucose, 5.6; the buffer was supplemented with 3 g of bovine serum albumin/L and equilibrated with a mixture of 95% O2:5% CO2, pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with 8.3 mmol glucose/L and different concentrations of agonists. The insulin content of the medium at the end of the incubation period was measured by RIA (22). The CCH concentration producing a response that was 50% of the maximum (EC50) was expressed as the mean negative logarithm (pD2).

Western blotting. Groups of islets were pelleted by centrifugation (15,000 × g) and then resuspended in 50–100 μL of homogenization buffer containing protease inhibitors, as described (12,14). For static incubation, groups of five islets were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer with the following composition (in mmol/L): NaCl, 115; KCl, 5; CaCl2, 2.56; MgCl2, 1; NaHCO3, 24; and glucose, 5.6; the buffer was supplemented with 3 g of bovine serum albumin/L and equilibrated with a mixture of 95% O2:5% CO2, pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with 8.3 mmol glucose/L and different concentrations of agonists. The insulin content of the medium at the end of the incubation period was measured by RIA (22). The CCH concentration producing a response that was 50% of the maximum (EC50) was expressed as the mean negative logarithm (pD2).

Composition of the normal and low protein diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Normal protein (17% protein)</th>
<th>Low protein (6% protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein (84% protein)</td>
<td>202.0</td>
<td>71.5*</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.0</td>
<td>480.0*</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>130.5</td>
<td>150.0*</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>121.0*</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix (AIN-93)*</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93)*</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Difference between the two isocaloric diets.

RESULTS

Characteristics of the rats. After 8 wk, body weight, serum total protein, albumin and insulin levels of LP rats were lower, whereas serum FFA, liver glycogen and fat concentrations were greater than in NP rats (P < 0.05) (Table 2). The amount of protein in islets of rats fed NP and LP was similar.
However, there were differences in the profile of soluble protein in islets of rats fed NP compared with those fed LP (not shown).

**Insulin secretion.** Increasing concentrations of CCh (0.1–1000 μmol/L) dose dependently increased insulin secretion by islets from rats fed LP and NP, although absolute insulin secretion for each CCh concentration was higher in islets from rats fed NP than in those fed LP (Fig. 1). The EC₅₀ were 2.15 ± 0.7 μmol/L and 4.64 ± 1.0 μmol/L for islets from rats fed NP and LP, respectively (P < 0.05).

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

**PKCα mRNA and protein expression.** Western blotting indicated a 30% reduction in the expression of PKCα protein in islets from rats fed LP compared with those fed NP (P < 0.05) (Fig. 3). Similarly, RT-PCR revealed a 50% reduction in the expression of PKCα mRNA (P < 0.05) (Fig. 4). The expression of PLCβ₁ protein was also reduced by 25% in islets from rats fed LP compared with those fed NP (P < 0.05) (Fig. 5).

**DISCUSSION**

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

**TABLE 2**

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

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

![Figure 1](https://example.com/figure1.png)

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

![Figure 2](https://example.com/figure2.png)

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

![Figure 3](https://example.com/figure3.png)

**FIGURE 3** Protein kinase Cα (PKCα) content in islets from rats fed normal (NP) and low (LP) protein diets for 8 wk. Values are means ± SEM, n = 4–5 independent experiments. *Different from NP, P < 0.05.
and in experimental models. These features included a low body weight, low levels of plasma albumin and insulin (Fed), and high liver glycogen and fat concentrations (3,4,20,24). Thus, these rats were a suitable model for the present study. Although insulinemia was reduced, glycemia did not differ in rats fed LP or NP. These findings may be related to a marked increase in insulin sensitivity explained by an increase in the phosphorylation of the insulin receptor and insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase (17).

The neural modulation of β-cells plays an important role in the control of insulin secretion. A relationship between loss of the first phase of secretion with the onset of type 2 diabetes has been established (25–27). The first phase of insulin secretion is important for glucose tolerance and is partially dependent on the ACh-activation of M3 receptors present in the B-cell plasma membrane. Acetylcholine increases insulin secretion by activating M3 receptors (21,28–30). In B cells, the coupling of ACh with this type of receptor stimulates PLC via G proteins (28,31–33) to generate DAG and IP₃, culminating with insulin secretion (29,34).

Because a low protein diet is associated with stress, with possible derangement of the sympathetic/parasympathetic equilibrium, we investigated the modulation of CCh-induced insulin secretion in rats fed a low protein diet. Insulin secretion induced by increasing concentrations of CCh was dose dependently increased in islets from both groups of rats. However, the dose-response curves for insulin secretion, as well as the EC₅₀, indicated that the potency of CCh was significantly reduced in islets from rats fed LP compared with those fed NP (Fig. 1).

Various intracellular messengers regulate insulin secretion in pancreatic islets. DAG and IP₃ are second messengers involved in CCh-induced insulin secretion via PKC. The precise mechanism of action of PKC on insulin stimulation is not yet fully understood, although alterations in K⁺ and Ca²⁺ fluxes in β cells are involved (35–38). PKC also stimulates secretion by facilitating the extrusion of insulin-containing granules (39), and exogenous activators such as phorbol esters (TPA or PMA) or DAG analogs stimulate PKC translocation in rat islet cells (40,41), indicating a relationship between PKC and insulin secretion (35). An attenuated PMA-induced insulin secretion was observed in islets from rats fed LP, suggesting a possible alteration in PKC levels. Several types of PKC are present in B cells, with PKCα as the major component (42–44). Because the content of PKCα was reduced in islets from rats fed LP, this factor could account for a decrease in glucose- and CCh-induced insulin secretion in islets from rats fed LP.

PKC also provokes an apparent paradoxical decrease in the intracellular Ca²⁺ concentration in B cells, probably by reducing Ca²⁺ entry via L-type channels (45). The Ca²⁺ efflux in LP was higher than in NP islets (not shown) confirming, although indirectly, the participation of PKCα in this process.

In conclusion, alterations mainly in PKCα levels and possibly other enzymes involved in this pathway, such as PLCβ₁, may contribute to the poor secretory response induced by glucose, CCh and PMA in islets from rats fed LP. A low protein diet apparently decreases the transcription of genes that encode proteins involved in insulin secretion. When present over a long period of time, these alterations may affect the glucose homeostasis in LP rats.

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**LITERATURE CITED**

2. Escriva, F., Rodriguez, C., Cacho, J., Alvarez, C., Portha, B. & Pascual-


