Programming into adulthood of islet adaptations induced by early nutritional intervention in the rat

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Aalinkeel, Ravikumar, Malathi Srinivasan, Fei Song, and Mulchand S. Patel. Programming into adulthood of islet adaptations induced by early nutritional intervention in the rat. *Am J Physiol Endocrinol Metab* 281: E640–E648, 2001.—To investigate the influence of a high carbohydrate (HC) intake during the suckling period on pancreatic function in adult life, neonatal rats were artificially reared on an HC milk formula during the preweaning period and then weaned onto lab chow. In the adult HC rat, hyperinsulinemia is sustained by a variety of biochemical and molecular adaptations induced in the HC islets during the suckling period. The adult HC islets showed a distinct left shift in the glucose-stimulated insulin-secretory pattern. HC islets were also able to secrete moderate levels of insulin in the absence of glucose and in the presence of Ca2+ channel inhibitors. In addition, the mRNA levels of preproinsulin, somatostatin, transcription factor-1, upstream stimulatory factor-1, stress-activated protein kinase-2, phosphatidylinositol kinase, and GLUT-2 genes were significantly increased in HC islets. These results show that consumption of a HC formula during the suckling period programs pancreatic islet function in adult rats, resulting in the maintenance of hyperinsulinemia in the postweaning period and eventually leading to the development of obesity in adult life.

neonatal nutrition; metabolic adaptations; insulin secretion; obesity

IT IS NOW INCREASINGLY CLEAR that dietary influences exerted early in life have long-term consequences leading to the development of pathological conditions in adulthood (2). Several studies on animal models and human epidemiological data support the notion that fetal and immediate postnatal nutritional adaptations have permanent effects on cellular structure, physiology, and metabolism in different organs (7). The late fetal and early postnatal periods in the rat have been recognized as being critical for pancreatic islet ontogeny (10). Previous studies from this laboratory have illustrated both the immediate and long-term consequences of a dietary modification in the form of a high-carbohydrate (HC) diet fed to neonatal rats in the preweaning period (8, 32). Artificial rearing of neonatal rat pups on a HC milk formula (56% of total calories from carbohydrate vs. 8% in rat milk) results in the immediate onset of hyperinsulinemia and alterations in lipid metabolism in these rats (8). These changes persist into adulthood with the development of adult-onset obesity (8).

The ability of β-cells to secrete insulin is regulated by nutrient, hormonal, and neuronal stimuli. Glucose metabolism has been widely accepted to play a principal role in insulin secretion by its effects on the ATP-sensitive K+ (KATP) and Ca2+ channels (26). Glucose metabolism coupled to the activation of protein kinase A (PKA) and protein kinase C (PKC) can augment insulin secretion by the Ca2+-independent pathway (12). Long-term regulation of insulin secretion is regulated at the level of transcription of the proinsulin gene and translation of its mRNA. In response to the HC dietary intervention during the suckling period, several adaptations at the molecular, cellular, and biochemical levels take place in islets isolated from 12-day-old HC rats. Molecular adaptations include up-regulation of the specific transcription factor genes, thereby facilitating increased proinsulin gene transcription (29a). In addition, the transcription of several islet-specific transcription factors that modulate cellular development of the pancreas is also increased (29a). Considering the fact that this dietary treatment overlaps the critical window of pancreatic development in rat, the increased gene transcription of these transcription factors may be critical for the altered ontogeny of the HC pancreas during this period. Cellular alterations in 12-day-old HC islets include changes in the number and size of the islets and rate of proliferation and apoptosis (22). Biochemical adaptations in 12-day-old HC islets include extensive changes in both proximal and distal sites in the insulin-secretory pathway (1, 29). Significant among these changes are the ability of the HC islets to secrete insulin in a glucose- and Ca2+-independent manner, a marked left shift in the response to a glucose stimulus, and up-regulation of a glucagon-like peptide-1 (GLP-1)-mediated process in islets (29).

Our hypothesis is that early adaptations in islets from HC neonatal rats (29) are programmed into adulthood and contribute to the hyperinsulinemic state of
100-day-old HC rats (32). Hence, we have analyzed biochemical and molecular changes in islets from 100-day-old male HC rats and have observed that the early events in 12-day-old HC islets are sustained into adulthood, indicating the importance of the consequences of a nutritional stimulus during the critical period of organ development.

MATERIALS AND METHODS

Materials. 2-Deoxyglucose, mannoheptulose, glibenclamide, iodoacetate, collagenase type I, diprotinin, GLP-1, acetylcholine (ACh), kits for assay of triglycerides and glucose, and all other reagent-grade chemicals were from Sigma Chemical (St. Louis, MO). The insulin RIA kit was from Linco Research (St. Louis, MO). The assay kit for the estimation of free fatty acids (FFA) was from Boehringer Mannheim (Indianapolis, IN). Nimodipine was from Calbiochem (San Diego, CA) and 1–2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA) was from Molecular Probes (Eugene, OR). The protein assay kit was from Bio-Rad (Hercules, CA). TRizol reagent, murine leukemia virus reverse transcriptase, and all the primers were from GIBCO-BRL (Grand Island, NY). pGEM-3Z vector was from Promega (Madison, WI).

Animal protocol. The Institutional Animal Care and Use Committee approved all animal protocols used in this study. Timed-pregnant Sprague-Dawley rats were obtained from Zivic Miller Laboratories (Zelienople, PA) and were housed in a temperature- and light-controlled room with free access to laboratory chow and water. Newborns of several mothers were pooled and randomly distributed to nursing mothers (12 pups/dam). On postnatal day 4, pups were randomly assigned to either control or experimental group. In the mother-fed (MF) control group, pups were allowed to be nursed by their dams. The pups in the experimental group were reared artificially on a HC formula wherein 56% of the total daily calories were derived from carbohydrates compared with 8% in rat milk. The artificial rearing technique employed in this study has been described in detail previously (8). In brief, the pups were anesthetized, and intragastric cannulas were introduced. They were housed in Styrofoam cups floating in a 37°C waterbath and were fed HC formula at the rate of 0.45 kcal/g body wt⁻¹·day⁻¹. The pups were stroked in the urogenital region to promote urination and defecation every day. On day 18, the canulas of the pups were cut close to the skin, and the pups were housed in plastic cages and continued on the HC formula provided in feeding tubes until day 24. On postnatal day 24, control and experimental pups were weaned onto laboratory stock diet. Food and water were provided ad libitum, and both groups were killed on day 100. Blood and pancreases were collected at the time of killing and processed as described below.

Plasma levels of insulin, GLP-1, glucose, triglyceride, and free fatty acids. One hundred-day-old HC and MF rats were killed by decapitation, and trunk blood was collected in heparinized tubes (containing 50 μM diprotinin for GLP-1). Plasma was collected by centrifugation at 5,000 rpm for 10 min and stored at −70°C. Plasma insulin was measured by RIA with rat insulin as standard. Plasma glucose, triglycerides, and free fatty acids (FFA) were quantitated using kits according to manufacturers’ instructions. Plasma GLP-1 (active form) was measured by the Assay Services of Linco Research.

Islet isolation and insulin secretion. Pancreatic islets were isolated from 100-day-old HC and MF rats by collagenase digestion (34). The islets were hand picked under a stereomicroscope and were used fresh for studies related to insulin secretion or were stored frozen at −80°C until further use. For insulin secretion experiments, islets (10/tube) were preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing 16 mM HEPES, 5.5 mM glucose, and 0.01% BSA, pH 7.4, for 30 min under an atmosphere of 95% O₂-5% CO₂ in a shaking waterbath at 37°C. At the end of the preincubation period, the entire solution was aspirated, fresh KRB buffer containing the appropriate glucose concentration and the required agonist/antagonist was introduced, and an aliquot of the buffer was removed for determination of insulin levels at time 0, followed by subsequent removal of aliquots at 10 and 60 min for the determination of insulin release. For insulin secretion studies in the absence of glucose, preincubation was also done in the absence of glucose. Similarly, for a stringent Ca²⁺-deprived condition, both preincubation and incubation were carried out under a stringent Ca²⁺-depleted condition. Insulin levels in the samples were determined by RIA with rat insulin as standard. Duplicate islet incubations were performed for each animal. The results are expressed as femtomoles of insulin released per 10 islets per time interval.

Measurement of glucose-phosphorylating activity. Glucose-phosphorylating activity was assayed by a modification of the method described previously (31). Briefly, islets (~200) were sonicated in ice-cold buffer containing (in mM) 20 potassium phosphate (pH 7.4), 1 EDTA, 110 KCl, and 5 dithiothreitol (DTT) (pH 7.4). The homogenate was then centrifuged at 12,000 g at 4°C for 20 min. The pellet was resuspended in the same buffer. Glucose-phosphorylating activity in islet extracts (pellet and supernatant fractions) was measured at 0.5 or 100 mM glucose, as described previously (1). For glucokinase activity, correction for the hexokinase fraction was applied by subtracting the activity measured at 0.5 mM glucose from the activity measured at 100 mM glucose.

RNA isolation and cDNA synthesis. Total RNA was isolated from islets obtained from 100-day-old HC and age-matched MF control rats by use of the TRizol reagent-phenol-chloroform procedure (GIBCO-BRL). cDNA was prepared using 6 μg of total islet RNA and 20 pmol random hexamers in a 30-μl solution containing (in mM) 50 Tris·HCl, pH 8.4, 75 KCl, 4 MgCl₂, 10 DTT, and 0.125 of each dNTP and 200 units of Moloney murine leukemia virus reverse transcriptase. After incubation for 1 h at 42°C, the reaction mixture was heated to 70°C for 15 min to inactivate the reverse transcriptase. The cDNA was stored at −20°C.

The levels of specific mRNAs were determined using a semiquantitative PCR-based assay as described previously (27). A known amount of synthetic competitor DNA was added to each reaction. A semiquantitative RT-PCR assay (9), in which the same amount of competitor template DNA was added to each reaction, was used to compare the levels of specific mRNAs in islets from HC and MF rats. This internal standard was amplified using the same primers as the experimental cDNA target and was designed to generate a PCR product with an internal deletion that was easily distinguished from the cDNA target. All competitor DNAs for measuring the mRNAs for preproinsulin, upstream stimulating factor-1 (USF-1), glucose transporter-2 (GLUT-2), phosphatidylinositol 3-kinase (PI 3-kinase), and stress-activated protein kinase-2 (SAPK-2) were prepared by introducing a fills in by 10.220.33.6 on June 21, 2017 http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/
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Table 1. Sequences of PCR primers and PCR conditions for analysis of specific mRNAs

<table>
<thead>
<tr>
<th>mRNA</th>
<th>GenBank Accession Number</th>
<th>Primer Sequences (5'-3')</th>
<th>PCR Reaction Conditions</th>
<th>Size of PCR Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>J00747</td>
<td>TGCCCGGCTTTTCTCAACAGCAACCTT</td>
<td>95</td>
<td>65</td>
</tr>
<tr>
<td>STF-1</td>
<td>S67435</td>
<td>CTGCCAGGGAAGGTGGCTGAA</td>
<td>95</td>
<td>65</td>
</tr>
<tr>
<td>USF-1</td>
<td>AF026476</td>
<td>CAGTGATCTGACGAAACAC</td>
<td>95</td>
<td>65</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>J03145</td>
<td>GCCAGGTGCGTGGTCGCAAT</td>
<td>95</td>
<td>65</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>AB009636</td>
<td>CAGTGAATATCGGAGTACGCCAC</td>
<td>95</td>
<td>58</td>
</tr>
<tr>
<td>SAPK-2</td>
<td>U91847</td>
<td>GATTCATTTGATGCTGCAAC</td>
<td>95</td>
<td>46</td>
</tr>
</tbody>
</table>

Den, denaturing temperature; Ann, annealing temperature; Ext, extension temperature; Cyc, no. of cycles; STF-1, somatostatin transcription factor-1; PI, phosphatidylinositol; SAPK-2, stress-activated protein kinase-2.

DNAs were cloned into pGEM-SZ vector (Promega). PCR reactions were carried out in a 50-μl volume containing cDNA, competitor DNA, dNTPs, 10 pmol of a pair of oligonucleotide primers (Table 1), 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl2, and 1 unit of Taq DNA polymerase. The PCR products were separated by electrophoresis on a 2% agarose gel, analyzed by Bio-Rad Gel Doc 1000 and Molecular Analyst Software for quantitative analysis, and normalized using competitor controls. The results are expressed as degree of change in HC animals compared with age-matched MF controls.

Protein assay. Protein assays were carried out using a kit from Bio-Rad according to the instructions of the manufacturer.

Statistical analysis. The results are means ± SE. The significance of difference between MF and HC groups was analyzed by use of Student’s t-test. Differences were considered significant at P < 0.05.

RESULTS

Effect of feeding HC diet to neonates on physiological parameters and insulin secretion by islets of adult rats. Earlier (32), we reported that HC rats reared artificially on a HC formula during the suckling period maintained hyperinsulinemia with normoglycemia in the postweaning period and gained body weight at a higher rate starting around day 55 compared with MF controls. The present study (Table 2) indicates that the body weight of 100-day-old HC rats is significantly higher than age-matched MF rats and that they continue to be hyperinsulinemic (~3-fold increase) but are normoglycemic. Because obesity is also associated with the changes in plasma FFA and triglyceride levels in other obesity-related models, we determined their levels in the present study. The results presented in Table 2 show that there are no significant differences in the plasma levels of glucose, FFA, and triglycerides between MF and HC groups. Thus these factors may not be contributing to the maintenance of hyperinsulinemic state in adult HC rats.

To examine the mechanism responsible for hyperinsulinemia, we first studied the insulin-secretory response to a range of glucose concentrations (1–16.7 mM). Pancreatic islets were isolated from 100-day-old MF and HC rats, and the insulin release pattern was studied under static incubation conditions. As is evident from Fig. 1, MF islets did not secrete any measurable amount of insulin at 1 mM glucose (subbasal glucose concentration) at both 10 and 60 min. However, HC islets secreted significant amounts of insulin at both 10 and 60 min under this condition. Progressive increases in glucose concentration (~16.7 mM elicited significant increases (P < 0.001) in insulin secretion at both 10 and 60 min in the HC islets compared with MF islets at corresponding glucose concentration. Taken together, these results clearly indicate a modified insulin-secretory pattern in HC islets, with a marked left shift for glucose sensitivity.

Table 2. Effect of early dietary intervention on body weight and plasma profile in 100-day-old HC rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>423 ± 8.2</td>
<td>603 ± 8*</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>342 ± 8</td>
<td>1195 ± 46*</td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>7.0 ± 0.2</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>Plasma FFA, mM</td>
<td>0.45 ± 0.07</td>
<td>0.45 ± 0.07</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/l</td>
<td>52.1 ± 10.8</td>
<td>54.6 ± 6.3</td>
</tr>
<tr>
<td>Plasma GLP-1, pM</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 rats. MF, mother fed; HC, high-carbohydrate fed; FFA, free fatty acids; GLP-1, glucagon-like peptide-1. Rats were weighed before being killed, trunk blood was collected in heparinized tubes, and plasma was separated for analysis of the various parameters as indicated in MATERIALS AND METHODS. *P < 0.001 vs. MF controls.

Effect of channel inhibitors and modulators on insulin secretion by HC and MF islets. According to the paradigm proposed for glucose-stimulated insulin secretion, there are at least three target sites important for the regulation of insulin release: 1) glucose metabolism via the glycolytic and mitochondrial pathways for ATP production, 2) closing of the KATP channel, and 3) opening of the voltage-sensitive Ca2+ channel (16). In accordance with the above paradigm, we examined the alterations at all of the three target sites by using inhibitors and modulators of various pathways on insulin secretion. In the presence of iodoacetate (an-
hitor of glycolysis), HC islets secreted ~15% (~6 fmol·10 islets⁻¹·60 min⁻¹) of the amount they secreted in the presence of basal glucose concentration (Table 3) and similar to MF islets at basal glucose concentration. Notably, the entire amount (~6 fmol·10 islets⁻¹·60 min⁻¹) was secreted during the late phase of insulin secretion (Table 3). Furthermore, the HC islets secreted a similar amount of insulin in the presence of 2-deoxyglucose (a nonmetabolizable analog of glucose). Under conditions where the K⁺ channels are closed and the membrane is depolarized in the presence of 100 μM glibenclamide, MF islets secreted significantly more insulin compared with their basal values both at 10 and 60 min; however, this treatment had no effect on HC islets (Table 3). The exposure to diazoxide (pharmacological activator of K<sub>ATP</sub> channel) abolished the basal insulin secretion by MF islets, but HC islets secreted ~15% of the amount they had secreted under basal condition at 60 min (Table 3). When the islets were treated with the L-type Ca²⁺ channel blocker nimodipine (1 μM) or BAPTA (1 μM), the same pattern of insulin secretion (~6 fmol·10 islets⁻¹·60 min⁻¹) by the HC islets was observed as with the treatment of diazoxide and as in the complete absence of glucose (Table 3). These results indicate that the altered basal insulin secretion in the HC islets is affected through modifications at all of the three target sites discussed earlier.

To further understand the mechanism supporting insulin secretion (~6.0 fmol·10 islets⁻¹·60 min⁻¹) in the absence of nutrient stimulus, insulin secretion was studied in the presence of iodoacetate (an inhibitor of glycolysis), diazoxide (pharmacological activator of K<sub>ATP</sub> channels), and under stringent Ca²⁺-deprived

**Table 3. Effect of inhibitors and nonnutrient modulators on insulin secretory response by isolated islets from 100-day-old MF and HC rats**

<table>
<thead>
<tr>
<th>Incubation Conditions Addition/Deletion</th>
<th>Glucose, mM</th>
<th>mf mol·10 islets⁻¹·10 min⁻¹</th>
<th>mf mol·10 islets⁻¹·60 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td>HC</td>
</tr>
<tr>
<td>None</td>
<td>5.5</td>
<td>1.3 ± 0.2</td>
<td>10.8 ± 1.9</td>
</tr>
<tr>
<td>Iodoacetate, 1 mM</td>
<td>5.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Deoxyglucose, 11 mM</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glibenclamide, 0.1 mM</td>
<td>5.5</td>
<td>14.0 ± 1.5</td>
<td>10.8 ± 2.0</td>
</tr>
<tr>
<td>Diazoxide, 250 μM</td>
<td>5.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nimodipine, 1 μM</td>
<td>5.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BAPTA, 1 μM</td>
<td>5.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mannohexulose, 11 mM</td>
<td>5.5</td>
<td>1.7 ± 0.2</td>
<td>11.2 ± 2.2</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Diazoxide, 250 μM</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Iodoacetate, 1 mM</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ca²⁺-free condition</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 experiments. BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid. Ten islets from each group were preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing either 0 or 5.5 mM glucose under an atmosphere of 95% O₂-5% CO₂ for 30 min. Islets were then washed, and the incubation was continued in the same medium in the presence of different modulators at the indicated concentrations for an additional 60 min. Aliquots of the incubation medium were withdrawn at 10 and 60 min for analysis of insulin. When indicated, mannohexulose and BAPTA were also present in the preincubation period. For 2-deoxy-D-glucose studies, preincubation and incubation were done in KRB buffer containing 2-deoxy-D-glucose, and for the stringent Ca²⁺-free condition, preincubation and incubation were carried out in KRB buffer devoid of calcium but containing EGTA (500 μM), BAPTA (1 μM), and nimodipine (1 μM). ND, no detectable levels of insulin under experimental condition.
condition in the absence of glucose (Table 3). When insulin secretion was measured in the absence of glucose together with iodoacetate, HC islets secreted amounts of insulin similar to the amounts under conditions when no glucose was available (Table 3). To exclude the possibility of increased influx and availability of Ca\(^{2+}\)-supporting insulin secretion in the absence of glucose, insulin secretion was monitored in the absence of glucose and under a simultaneous stringent Ca\(^{2+}\)-deprived condition (Ca\(^{2+}\)-free buffer in the presence of EGTA, BAPTA, and nimodipine). HC islets secreted ~6 fmol·10 islets\(^{-1}·60 \text{ min}^{-1}\) of insulin under this condition, indicating the ability of these islets to secrete insulin in a glucose- and Ca\(^{2+}\)-independent manner (Table 3). In the presence of diazoxide, the influx of Ca\(^{2+}\) is excluded; hence, this experiment ruled out the possibility of increased activity of the voltage-gated Ca\(^{2+}\) channel's supporting insulin secretion when no glucose was available. These results show that the HC islets are able to secrete insulin in a glucose- and Ca\(^{2+}\)-independent manner.

**Insulin secretion via the Ca\(^{2+}\)-independent augmentation pathway in adult HC rats.** It is known that Ca\(^{2+}\) is required for both the K\(_{\text{ATP}}\) channel-dependent and -independent pathways for insulin secretion. Additionally, there exists a Ca\(^{2+}\)-independent augmentation pathway that is glucose dependent and requires the simultaneous activation of PKA and PKC (11). The possible alteration in this pathway and the contribution of this pathway to the increased insulin secretion was examined in the HC and MF islets under a stringent Ca\(^{2+}\)-deprived condition at three glucose concentrations (0, 5.5, and 16.7 mM). Because the response under this condition was seen only in the late phase, the results of this experiment are reported for the late phase only. As indicated in the previous section, the HC islets secreted a significant amount of insulin (6 fmol·10 islets\(^{-1}·60 \text{ min}^{-1}\)) under glucose-deprived and Ca\(^{2+}\)-free conditions, and this secretion was significantly increased in the presence of GLP-1 plus ACh (Fig. 2A). There was no effect of these agents for MF islets (Fig. 2A). At 5.5 mM glucose, the activators of PKA and PKC caused insulin secretion (~2 fmol) in the MF islets, whereas HC islets secreted 17 fmol of insulin (Fig. 2B). At a higher glucose concentration (16.7 mM), the combined presence of PKA and PKC activators increased HC islet insulin secretion twofold compared with the amount secreted by the MF islets under identical conditions (Fig. 2C).

Because HC islets secreted a significant amount of insulin in the absence of glucose, it was of interest to see whether the normal inhibitory responses were intact. Norepinephrine is an important physiological antagonist of insulin secretion in cultured islets (19). Norepinephrine (10 \(\mu\)M) completely inhibited insulin secretion by both MF and HC islets at 0, 5.5, and 16.7 mM glucose in the presence of PKA and PKC activators (Fig. 2, A–C). These results indicate that the glucose- and calcium-independent insulin secretion seen in HC islets is sensitive to norepinephrine.

**Glucose metabolism in adult HC and MF islets.** In view of the known relationship between glucose metabolism and insulin secretion (20) and also the fact that we have observed increased oxidation and utilization of glucose in adult HC islets (unpublished observation), experiments were carried out to determine whether increased insulin secretion would reflect increases in the activity of glucose-metabolizing enzymes. Glucose is transported via the action of GLUT-2 into the pancreatic \(\beta\)-cell, where it is metabolized via both the glycolytic pathway and the tricarboxylic acid pathway, resulting in ATP production. Glucose is phosphorylated by the glucose-phosphorylating enzymes glucokinase (high Michaelis-Menten constant \(K_m\)) and hexokinase (low \(K_m\)), and this step is considered to be the rate-limiting step in glycolytic flux; hence, these activities were measured in the islet homogenates (supernatant and pellet fractions). Glucokinase activity in

![Fig. 2. Effect of protein kinase A (PKA) and protein kinase C (PKC) activators on insulin secretion by islets from 100-day-old MF and HC rats at 0 (A), 5.5 (B), and 16.7 mM glucose (C) under stringent Ca\(^{2+}\)-deprived condition. Ten islets were incubated in KRB buffer under stringent Ca\(^{2+}\)-deprived condition with varying concentrations of glucose in the presence of glucagon-like peptide (GLP)-1 (100 nM) + ACh (1 \(\mu\)M) for 60 min. The same treatment was repeated in the presence of norepinephrine (NE; 10 \(\mu\)M) during the incubation period. Values are means ± SE of 4 experiments. *P < 0.001, ^P < 0.002, and ^P < 0.009 vs. insulin secretion in the absence of GLP-1 + ACh for each group. *No detectable insulin secretion under the experimental condition.](http://ajpendo.physiology.org/)

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![Figure 2](http://ajpendo.physiology.org/)
the supernatant fraction of HC homogenate showed an increase of 12% compared with that seen in the MF islets (Fig. 3). Hexokinase activities in the supernatant and pellet fractions of the islet homogenate were observed in nearly equal levels for both groups but were increased significantly in both fractions from HC islets compared with MF islets.

**Upregulation of gene expression in HC islets.** In the short term, insulin secretion demands are met by the translation of preexisting mRNA. However, to meet the chronic demand for insulin secretion, appropriate alterations have to take place at the level of gene expression. In keeping with this hypothesis of expression of the preproinsulin gene, several transcriptional factors and related proteins were investigated by measuring mRNA levels using a semiquantitative RT-PCR procedure (27). As depicted in Fig. 4A, the preproinsulin mRNA level was increased about threefold in the HC islets compared with the MF islets. The mRNA level of GLUT-2 was also increased ~3.5-fold in the HC islets compared with the MF islets (Fig. 4B).

Transcription of the preproinsulin gene is regulated by interactions among several transcription factors (e.g., STF-1) and the specific cis-acting elements in the preproinsulin gene promoter. STF-1 mRNA was significantly increased in HC islets compared with MF islets (~3-fold; Fig. 4C). STF-1 gene transcription is, in turn, regulated by binding of the upstream stimulatory factor-1 (USF-1) to its promoter (24). The level of USF-1 mRNA was also significantly increased in HC islets compared with MF islets (Fig. 4D). The binding activity of STF-1 to its cognate sequences in the preproinsulin gene promoter is modulated by glucose via a cascade of events including SAPK-2 and PI 3-kinase (15). SAPK-2 is activated by a phosphorylation cascade involving PI 3-kinase and, upon activation, causes the phosphorylation of STF-1, resulting in its translocation to the nucleus and subsequent activation of insulin promoter activity (33). The mRNA levels of SAPK-2 (Fig. 4E) and PI 3-kinase (Fig. 4F) were increased ~3.5-fold each in the HC islets compared with MF islets.

Fig. 3. Glucose-phosphorylating activities in 100-day-old MF and HC islets. Islets (~200) were sonicated in phosphate buffer and centrifuged at 12,000 g for 20 min to separate the pellet and supernatant fractions. Glucokinase (GK) and hexokinase (HK) activity were measured, and the data are means ± SE of 4 experiments. *P < 0.05 vs. MF.

Fig. 4. Relative expression of mRNA levels of the preproinsulin (A), GLUT-2 (B), somatostatin transcription factor (STF)-1 (C), upstream stimulating factor (USF)-1 (D), stress-activated protein kinase (SAPK)-2 (E), and phosphatidylinositol (PI) 3-kinase (F) genes in islets from 100-day-old MF and HC rats. mRNA levels were quantified by a semiquantitative RT-PCR method. Top: representative ethidium bromide-stained agarose gel(s) separation for specific mRNA levels in MF and HC islets. In each agarose gel(s) separation, the upper band corresponds to the cDNA for the specific mRNA indicated, and the lower band corresponds to the competitor DNA. Bottom: means of relative densitometric values from quantification of specific mRNA levels. Values are means ± SE of 4 experiments. *P < 0.001 vs. MF.
DISCUSSION

Earlier studies on the HC rat model have shown that the mere switch from high-fat-derived calories in rat milk to high-carbohydrate-derived calories in the HC formula causes extensive adaptations at the molecular, cellular, and biochemical levels in islets isolated from 12-day-old HC rats (Refs. 22, 29, 29a). The late fetal and immediate postnatal periods are critical periods in pancreatic ontogeny in the rat. Nutritional experiences early in life as an etiological factor in the development of adulthood diseases have been reported. For example, dietary restriction during pregnancy in rats, particularly of proteins, produces reduction in birth weight and causes the onset of hypertension and hyperglycemia in adult offspring, even in the absence of nutritional insult (13, 14, 23). Our results presented here clearly indicate that several of the adaptations induced in pancreatic islets during the suckling period due to rearing of neonatal pups on the HC milk formula are programmed into adulthood and form the basis for the adult-onset obesity seen in 100-day-old HC rats. Hyperinsulinemia and the associated changes in islets from HC rats are unique to neonatal exposure of islets to the HC diet, because when neonates are fed a formula high in fat, they do not show hyperinsulinemia (32).

Despite the withdrawal of the HC formula at the time of weaning, circulating insulin levels continued to be significantly increased in 100-day-old HC rats compared with age-matched MF rats, indicating persistence of the altered insulin-secretory capacity of the HC islets. In 12-day-old HC rats, the plasma GLP-1 level was markedly higher compared with age-matched MF controls, and the GLP-1-mediated signal for augmented insulin release was shown to be one of the mechanisms supporting the hyperinsulinemic state in these rats (29). However, GLP-1-mediated events appear not to be programmed into adulthood in the adult HC rats, as is evident from the similar circulating GLP-1 level between MF and HC rats (Table 2). Mannoheptulose is reported to competitively block the hexose binding site of glucokinase to produce inhibition of insulin secretion (17). In the present study, neither the MF nor the HC islets showed any susceptibility to inhibition by mannoheptulose (Table 3) due to the basal concentration of glucose employed in the secretion studies. Because the activities of these two crucial enzymes are significantly increased in adult HC islets, it emerges that the overall increase in the glucose-phosphorylating activities contributes significantly to the hyperinsulinemic state in the 100-day-old HC rats. It appears that adaptations with respect to glucose metabolism observed in islets from 12-day-old HC rats (1) are programmed and continued to be expressed in islets from 100-day-old HC rats.

Ca\textsuperscript{2+} is an important element required for metabolically regulated insulin secretion. Although the majority of insulin secretion (~85%) is inhibited in the absence of extracellular Ca\textsuperscript{2+}, a small but significant amount of insulin (~15% of the amount secreted in the basal state by adult HC islets) was secreted in the complete absence of extracellular Ca\textsuperscript{2+} or when the Ca\textsuperscript{2+} channels were blocked (Table 3). Secretion of insulin from Ca\textsuperscript{2+}-depleted islets is indicative of either structural changes in islets or the possibility of the existence of a Ca\textsuperscript{2+}-channel-independent pathway (11). Depolarization of the membrane by glibenclamide did not appear to alter insulin secretion by HC islets, indicating that the Ca\textsuperscript{2+} stores were already elevated in the HC islets and partly contributed to the elevated secretion of basal insulin. Again, these altered insulin-secretory capacity of 100-day-old islets reflects the persistence of the early adaptive responses observed in 12-day-old islets.

Recently, it has been shown that, in islets, a Ca\textsuperscript{2+}-independent augmentation pathway operates that stimulates insulin secretion under stringent calcium-depleted conditions but requires glucose metabolism and simultaneous activation of PKA and PKC (11). In the present study, 100-day-old HC islets released moderate amounts of insulin under stringent glucose- and calcium-deprived conditions in the presence of physiological activators of PKA and PKC (Fig. 2). In the presence of glucose, still higher amounts of insulin...
were secreted, indicating that the Ca\(^{2+}\)-independent augmentation pathway is upregulated in HC islets compared with MF islets and may play an important role in the maintenance of hyperinsulinemia in adult rats.

The ability of the \(\beta\)-cell to hypersecrete insulin presumably results from an altered pattern of preproinsulin gene expression. The chronic hyperinsulinemic state of the HC rats warrants continuous replenishment of insulin stores on a minute-to-minute basis by compensation at the molecular level by increases in the rates of translation of the preproinsulin mRNA and transcription of the preproinsulin gene. In 100-day-old HC islets, the level of preproinsulin mRNA was markedly increased compared with the MF islets. That preproinsulin gene expression is modulated according to demand is illustrated by decreases in mRNA levels associated with a decreased insulin content in Zucker diabetic rats (30) and increased mRNA levels in corticosterone-induced insulin resistance (6, 21).

Transcription of the preproinsulin gene is dependent on the specific cis-acting sequences located within the proximal promoter (up to \(-350\) bp from the transcription start site) (4). Specifically, the E boxes located at \(-104\) and \(-232\) and the A boxes located at \(-76\), \(-130\), \(-210\) and \(-312\) are important (5). The E1 box binds insulin enhancer factor-1, and the A boxes bind a number of homeodomain transcription factors, of which STF-1 is the most abundant in the \(\beta\)-cells (25). In the present study, the level of STF-1 mRNA is increased about threefold in the HC rat islets compared with MF islets, and this increase may contribute to the increased transcription of the preproinsulin gene. In addition to its effect on preproinsulin gene transcription, STF-1 transactivates several other islet genes, including the GLUT-2 gene, which were also significantly increased in HC islets. The altered expression of other genes may also explain the coordinated upregulation of insulin gene transcription. For example, the enhanced level of USF-1 mRNA may account for the increased expression of STF-1, because USF-1 specifically binds to the STF-1 promoter and can thereby function as an upstream regulator of STF-1 gene expression in HC islets (24).

Glucose can modulate the STF-1 DNA binding activity via a phosphorylation cascade involving SAPK-2 (15). In pancreatic \(\beta\)-cells, high glucose is shown to activate SAPK-2 via PI 3-kinase (15). Activation of SAPK-2 leads to phosphorylation in the cytoplasm of an inactive 31-kDa form of STF-1 that is modified to a 46-kDa active form that is translocated to the nucleus and causes increased preproinsulin gene transcription (33). In the present study, the mRNA levels of both PI 3-kinase and SAPK-2 were increased significantly in the HC islets compared with MF islets. Considering the fact that these two play an important role in the phosphorylation cascade leading to the activation of STF-1 and its binding to its cognate DNA binding site, it is reasonable to conclude that their increased levels do contribute to the overall increase in preproinsulin gene transcription observed in this study.

In conclusion, the results from this study on islets from 100-day-old HC rats clearly indicate that the adaptive responses induced in 12-day-old HC islets are sustained in 100-day-old islets. For example, the altered insulin-secretory pattern with leftward shift in response to a glucose stimulus, the increased glucose-phosphorylating activities, the ability to secrete insulin in a calcium and glucose-independent manner, the upregulation of the calcium-independent augmentation pathway, and the increased preproinsulin gene expression supported by upregulation of immediate upstream events are all programmed into adulthood. The question is raised how the augmented expression of each of the mRNAs studied might relate to the observed phenotypic change. It is possible that a common molecular mechanism may be responsible for these alterations such as the increased expression of a key transcription factor(s) that is required for normal expression of \(\beta\)-cell genes. The expression of this factor is probably regulated by glucose-derived metabolite(s) in neonatal rats as the phenotypic change observed in adult HC rats is induced by a mere switch in the type of calories consumed (from fat to carbohydrates) during the suckling period.

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