Programming of Islet Functions in the Progeny of Hyperinsulinemic/Obese Rats
Malathi Srinivasan, Ravikumar Aalinkeel, Fei Song, and Mulchand S. Patel

Neonatal female rat pups that were raised artificially on a high-carbohydrate (HC) milk formula during their suckling period developed hyperinsulinemia immediately, maintained chronic hyperinsulinemia in the postweaning period on laboratory diet, and developed obesity in adulthood. Pups (second-generation HC [2-HC]) born to such female rats (first-generation HC [1-HC]) spontaneously developed chronic hyperinsulinemia and adult-onset obesity (HC phenotype) without the requirement for any dietary intervention in their suckling period. Leftward shift in the insulin secretory response to a glucose stimulus, increase in hexokinase activity, and increased preproinsulin gene transcription were observed in islets from 28-day-old 2-HC rats, and these adaptations are similar to those reported for islets from 12-day-old and 100-day-old 1-HC rats. Unlike 1-HC islets, the ability to secrete moderate amounts of insulin in the absence of glucose and calcium and the incretin input for augmentation of insulin secretion were not observed in 2-HC islets. These results show that a dietary modification in the early postnatal life of the 1-HC female rat sets up a vicious cycle of spontaneous transfer of the HC phenotype to its progeny, implicating a new component to the growing list of factors that contribute to the fetal origins of adult-onset diseases. Diabetes 52:984–990, 2003
Research Design and Methods

Animal protocol. All animal protocols were approved by the Institutional Animal Care and Use Committee. 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 diet and water. The 1-HC female rats in this study were reared by an artificial rearing technique described in detail previously (10,11). Briefly, intragastric cannulas were introduced into 4-day-old pups under mild anesthesia, and they were fed the HC formula at the rate of 0.45 kcal·g body wt−1·day−1. Nutrient composition (percentage of caloric content) of the HC formula was 56% carbohydrates, 24% proteins, and 20% fat (11). On postnatal day 24, the pups from both the groups (mother fed [MF] and 1-HC) were weaned onto laboratory diet and water ad libitum. On day 60, female rats from the MF and 1-HC groups were bred with normal adult (MF) male rats. Foster mothers nursed the resulting progenies (2-HC) until day 24, when they were weaned on laboratory diet and water ad libitum.

For verifying that the artificial rearing protocol per se had no effects on the pups, neonatal rat pups were artificially reared on a high-fat milk formula, the macronutrient composition of which is identical to that of rat milk. The rats that were fed a high-fat milk formula did not develop hyperinsulinemia or adult-onset obesity, suggesting that the HC phenotype is induced as a result of the HC dietary intervention (10,11).

Plasma levels of insulin, glucagon-like peptide-1, glucose, triglycerides, and free fatty acids. Trunk blood was collected in heparinized tubes from 2-HC and age-matched MF rats. Plasma insulin was measured using a radioimmunossay kit (Linco Research, St. Louis, MO). Plasma glucose, triglycerides, and free fatty acids (FFAs) were quantified using kits according to manufacturer’s instructions (Sigma, St. Louis, MO, and Boehringer-Mannheim, Indianapolis, IN). Plasma glucagon-like peptide-1 (GLP-1; active form) was measured by the Assay Services of Linco Research.

Islet isolation and insulin secretion. Pancreatic islets were isolated from 2-HC and age-matched MF rats (on postnatal days as indicated in the legends to the figures) by collagenase digestion as described previously (22). For insulin secretion experiments, islets were preincubated in Krebs-Ringer bicarbonate buffer containing 2.8 mmol/l glucose for 30 min at 37°C (22). Islets (duplicates for each animal) were further incubated in fresh Krebs-Ringer bicarbonate buffer containing the appropriate glucose concentration, and the required agonist/antagonist and aliquots were withdrawn at 0, 10, and 60 min for the determination of insulin. For insulin secretion studies in the absence of glucose or a stringent Ca2+-deprived condition, preincubation was also done under the same conditions. Insulin levels in the samples were determined by radioimmunoassay.

Measurement of hexokinase and glucokinase activities. These activities were assayed by a modification of the method described previously (23). Briefly, islets (~200) were homogenized and centrifuged and the glucose-phosphorylating activities in the crude mitochondrial pellet and supernatant fractions were measured at 0.5 or 100 mmol/l glucose as described previously (15). Protein assays were carried out using a kit from Bio-Rad.

Insulin biosynthesis. Insulin biosynthesis was quantified as described by Halben et al. (24). Briefly, islets were incubated in the presence of 25 μg of L-[4,5-3H]leucine in minimum essential medium lacking L-leucine for 1 h at 37°C. The newly synthesized proinsulin was immunoprecipitated using an anti-rat insulin antibody and Protein A-Sepharose and counted in a scintillation counter.

RNA isolation and cDNA synthesis. Total RNA was isolated from islets obtained from 28-day-old 2-HC and age-matched MF control rats, and cDNA was prepared using Moloney murine leukemia virus reverse transcriptase as described earlier (18). A semiquantitative RT-PCR–based assay in which a known amount of competitor template with an internal deletion was added to each reaction was used to compare the levels of specific mRNAs in islets from 2-HC and MF rats (18). Preparation of competitor DNAs for preproinsulin, pancreatic duodenal homeobox transcription factor-1 (PDX-1), upstream stimulatory factor-1 (USF-1), β2/NeuroD, regenerating factor 3 (RegIII), hepatocyte nuclear factor 3β (HNF3β), stress-activated protein kinase 2 (SAPK2), and GLUT2 and PCR for determination of mRNA levels were carried out as described previously (18,19). The products separated by electrophoresis on a 2% agarose gel were analyzed using Bio-Rad Gel Doc 1000 and Molecular Analyst Software. The results are expressed as fold change in HC animals compared with age-matched MF controls.

Statistical analysis. The results are means ± SE. The significance of difference between the MF and 2-HC groups was analyzed by use of Student’s t test. Differences were considered significant at P < 0.05.

Results

Biochemical characteristics. The 1-HC pregnancy was characterized by hyperinsulinemia and increased body weight in the 1-HC female rat. Plasma insulin levels of 1-HC female rats on day 21 of gestation were significantly higher compared with age-matched pregnant MF female rats (138 ± 21 pmol/l for MF compared with 423 ± 46 pmol/l for 1-HC). The 1-HC female rats during pregnancy were ~10% heavier compared with age-matched MF pregnant rats. There was no significant difference in the plasma glucose levels between the two groups of pregnant rats on day 21 of gestation (98 mg/dl for MF compared with 106 mg/dl for 1-HC).

As seen in Fig. 1, there was no significant difference in plasma insulin levels between the two 2-HC and MF rats from postnatal day 4 to 24, which corresponds to the suckling period. Upon weaning to laboratory diet on day 24, there was a significant increase in the plasma insulin levels of the 2-HC rats on day 26 compared with age-matched MF rats with an ~2.5-fold increase on day 35. It is interesting that there were no significant differences in the body weight and plasma levels of glucose and the active form of GLP-1 between the two groups of rats. In contrast, plasma FFA levels of 28-day-old 2-HC rats were significantly increased compared with MF rats.

Glucose-induced insulin secretion. From day 12 to day 24, the insulin secretory responses to three glucose concentrations (2.8, 5.5, and 16.7 mmol/l) ranged from almost undetectable to very minimal and were nearly similar for islets from both groups of rats (Fig. 2). A striking difference in the insulin secretory response by isolated islets from the two groups was, however, evident only after day 24. On postnatal days 26 and 28, whereas MF islets did not secrete any measurable amount of insulin at 2.8 mmol/l glucose at both 10 and 60 min, 2-HC islets secreted increased amounts at both 10 and 60 min (Fig. 2). At 5.5
mmol/l glucose, whereas MF islets did not secrete any detectable amount of insulin at 10 min and very low amounts at 60 min. 2-HC islets secreted significant amounts of insulin at both time points on postnatal days 26 and 28 (Fig. 2). At 16.7 mmol/l glucose, two insulin secretory responses were markedly higher for islets from 2-HC rats compared with islets from age-matched MF islets (Fig. 2). 2-HC islets from day 26 onward demonstrated a distinct leftward shift in the insulin secretory response to a glucose stimulus and secreted more insulin compared with MF islets at all of the glucose concentrations tested.

Hexokinase and glucokinase activities. To evaluate the contribution of glucose phosphorylation to basal hyperinsulinemia in 2-HC rats, we measured both hexokinase and glucokinase activities in islet cellular fractions. Although glucokinase activity was similar between the two groups of rats on day 28, the hexokinase activity was significantly increased in both the supernatant and the pellet fractions of islet homogenates from the 2-HC group compared with the MF group (Fig. 3).

Effect of FFA on glucose-induced insulin secretion from 2-HC rat islets. Because circulating plasma FFA levels were higher in 28-day-old 2-HC rats (Table 1), we investigated the acute effects of sodium palmitate on insulin secretion by islets from these rats. The inclusion of sodium palmitate (at 0.125 or 0.250 mmol/l) in both the preincubation and incubation buffers did not alter insulin secretion by MF islets (Table 2). In the case of 2-HC islets, the early phase of insulin secretion (at 10 min) was not affected by sodium palmitate, but the insulin secretion at 60 min was significantly increased in a dose-dependent manner. Furthermore, whereas the inhibitor of fatty acid oxidation, bromopalmitate, did not affect insulin secretion by MF islets at either time points, it reduced insulin secretion by 2-HC islets at 60 min by ~40%.

Glucose- and calcium-independent insulin secretion in 2-HC rat islets. The responses of 2-HC islets to the absence of glucose and in the presence of basal glucose to iodoacetate (condition of inhibition of glucose metabolism), diazoxide (condition of restriction of entry of extracellular calcium into islets as a result of opening of potassium channel), and glibenclamide (closure of potassium channel) are shown in Table 3. Both 2-HC and MF islets did not secrete any insulin in the absence of glucose or when glucose metabolism was inhibited by iodoacetate. When potassium channels were kept open by the inclusion of diazoxide, insulin secretion by both 2-HC and MF islets was completely inhibited, suggesting that 2-HC islets did not have the ability to secrete insulin in the absence of calcium. The inclusion of glibenclamide augments insulin secretion by 28-day-old MF islets in the presence of 5.5 mmol/l glucose to levels observed for 2-HC islets in the presence of 5.5 mmol/l glucose alone. The addition of glibenclamide had no effect on insulin secretion by 2-HC islets at 5.5 mmol/l glucose.

TABLE 1
Biochemical characteristics of 28-day-old 2-HC and age-matched MF rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MF</th>
<th>2-HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>102 ± 4.7</td>
<td>101 ± 5.2</td>
</tr>
<tr>
<td>GLP-1 (active form; pmol/l)</td>
<td>2.60 ± 0.01</td>
<td>2.66 ± 0.01</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.46 ± 0.15</td>
<td>6.96 ± 0.48</td>
</tr>
<tr>
<td>FFAs (mmol/l)</td>
<td>0.30 ± 0.03</td>
<td>0.40 ± 0.02*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>41.7 ± 5.6</td>
<td>55.6 ± 10.5</td>
</tr>
</tbody>
</table>

Values are means ± SE of four independent experiments. *P < 0.05 compared with MF.
presumably because the insulin secretion was already elevated compared with age-matched MF islets (Table 3).

**Insulin biosynthesis in 2-HC rat islets.** To determine whether the basal hyperinsulinemia in 28-day-old 2-HC rats was supported by increased insulin biosynthesis, we measured the incorporation of $^3$H-leucine into newly synthesized insulin. Figure 4 shows an increased incorporation of $^3$H-leucine into insulin by 2-HC islets, suggesting an increase in insulin biosynthesis by these islets compared with age-matched MF islets.

**Regulation of preproinsulin gene transcription in 2-HC islets.** It is to be anticipated that the chronic hyperinsulinemia in the 2-HC rat would necessitate increased preproinsulin gene transcription. As seen in Fig. 5, there was an increase in preproinsulin mRNA levels in 2-HC islets compared with increased insulin biosynthesis. PDX-1 regulates pancreatic ontogeny as well as preproinsulin gene transcription (25). The mRNA level of PDX-1 was also substantially increased in 2-HC islet (Fig. 5). USF-1 augments PDX-1 gene expression (26), and its mRNA level was significantly higher in 28-day-old 2-HC rats. Transcription factors such as $\beta 2$/NeuroD, HNF3$\beta$, and RegIII are implicated in the development of pancreas in the rat (27,28). Although the mRNA levels of $\beta 2$/NeuroD and GLUT2 were significantly higher in 2-HC islets, the mRNA levels of HNF3$\beta$, RegIII, and SAPK2 were not significantly altered.

**DISCUSSION**

Recent research provides compelling evidence that health in adulthood is determined to a large extent by the conditions under which the organism develops in the womb. The fetal origins of adult-onset diseases hypothesis is now widely accepted as an important determinant for the steadily increasing incidence of the metabolic syndrome worldwide. The environment in which the fetus develops modulates the expression of its genetic makeup, suggesting that we are products of both nature (genes) and nurture (intrauterine environment).

The results from this study emphasize the importance of adequate nutrition in the immediate postnatal period of the mother in her infancy in the context of adverse effects for the progeny. 1-HC female rats that received an isocaloric HC dietary intervention (with only a switch in the nature of calories from high fat to high carbohydrate) not only acquired the HC phenotype of chronic hyperinsulinemia and adult-onset obesity in their own lifetime but also transmitted these characteristics to their progeny (21). Mere fetal development in the 1-HC (hyperinsulinemic) intrauterine environment enabled the progeny to acquire the HC phenotype without the need for any dietary treatment in their immediate postnatal period. In a diabetic or a gestational diabetic pregnancy, plasma glucose levels are significantly higher compared with normal pregnancy, resulting in the increased supply of glucose (and other nutrients) to the fetus and concomitant adaptations in the fetus (4). In the 1-HC pregnant rats, plasma glucose levels were not significantly different compared with age-matched MF pregnant rats, suggesting that in the 1-HC pregnancy, the mechanism of transmission of the HC phenotype was not glucose-mediated as is the case in a diabetic or a gestational diabetic pregnancy. In the protein- or calorie-restricted pregnancy in the rat, malnutrition in the female rat during gestation and/or lactation restricted pancreatic development of the progeny, resulting in the onset of type 2 diabetes in adulthood (6,7). Human epidemiological studies also indicate that a malnourished pregnancy has several adverse effects on the progeny that are expressed as adult-onset diseases (9). The HC rat model provides a unique situation wherein there is neither limitation nor excess of nutrients during pregnancy or the early postnatal period but a redistribution of calories favoring carbohydrate-derived calories in the immediate postnatal period of the 1-HC mother, resulting in an intrauterine environment characterized by chronic hyperinsulinemia and moderate increase in body weight (10% increase) (21). Our results indicate that fetal development in such a 1-HC intrauterine environment...

**TABLE 2**

<table>
<thead>
<tr>
<th>Glucose (mmol/l)</th>
<th>Additions</th>
<th>MF insulin (fmol/20 islets) 10 min</th>
<th>60 min</th>
<th>2-HC insulin (fmol/20 islets) 10 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>None</td>
<td>0.45 ± 0.02</td>
<td>1.95 ± 0.02</td>
<td>2.10 ± 0.05</td>
<td>3.71 ± 0.18</td>
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<tr>
<td>5.5</td>
<td>Palmitate (0.125 mmol/l)</td>
<td>0.64 ± 0.08</td>
<td>1.95 ± 0.05</td>
<td>2.10 ± 0.01</td>
<td>6.68 ± 0.15*</td>
</tr>
<tr>
<td>5.5</td>
<td>Palmitate (0.250 mmol/l)</td>
<td>0.38 ± 0.03</td>
<td>2.03 ± 0.03</td>
<td>2.06 ± 0.04</td>
<td>11.33 ± 0.49*</td>
</tr>
<tr>
<td>5.5</td>
<td>Bromopalmitate (0.3 mmol/l)</td>
<td>0.38 ± 0.03</td>
<td>1.95 ± 0.05</td>
<td>2.14 ± 0.03</td>
<td>2.25 ± 0.02**</td>
</tr>
</tbody>
</table>

Values are means ± SE of four independent experiments. *P < 0.001 compared with MF and compared with 5.5 mmol/l glucose; **P < 0.01 compared with 5.5 mmol/l glucose.

**TABLE 3**

<table>
<thead>
<tr>
<th>Glucose (mmol/l)</th>
<th>Additions</th>
<th>MF insulin (fmol/20 islets) 10 min</th>
<th>60 min</th>
<th>2-HC insulin (fmol/20 islets) 10 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>None</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5.5</td>
<td>None</td>
<td>0.13 ± 0.06</td>
<td>1.00 ± 0.10</td>
<td>2.31 ± 0.22*</td>
<td>5.42 ± 0.64*</td>
</tr>
<tr>
<td>5.5</td>
<td>Iodoacetate (1 mmol/l)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5.5</td>
<td>Diazoxide (250 µmol/l)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5.5</td>
<td>Glibenclamide (100 µmol/l)</td>
<td>2.21 ± 0.36</td>
<td>4.74 ± 0.44</td>
<td>2.51 ± 0.22</td>
<td>5.54 ± 0.38</td>
</tr>
</tbody>
</table>

Values are means ± SE of four independent experiments. *P < 0.001 compared with MF. ND, not detectable.
results in the spontaneous programming and expression of the HC phenotype.

Earlier we demonstrated that several adaptations at the molecular, cellular, and biochemical levels in islets of 1-HC rats supported the immediate onset of hyperinsulinemia and its persistence throughout the period of dietary intervention and maintenance in adulthood even after withdrawal of the dietary intervention (15–20). Unlike the 1-HC rats in which hyperinsulinemia was evident even on postnatal day 5 (1 day after initiation on the HC formula) (10), the HC progeny (2-HC rats) demonstrated hyperinsulinemia only immediately upon weaning (day 26; Fig. 1). The ability to hypersecrete insulin was probably suppressed during the suckling period in these rats as a result of the high fat content of the rat milk.

Investigation of adaptations that supported basal hyperinsulinemia in 28-day-old 2-HC islets indicated that there were both similarities and dissimilarities compared with the adaptations observed in 1-HC islets. Islets from 28-day-old 2-HC rats demonstrated a distinct leftward shift in their response to a glucose stimulus for both the early and late phases of insulin secretion compared with age-matched MF islets. It seems that these islets were programmed to recognize and respond to basal and sub-basal glucose concentrations as if they were high glucose levels.

On the basis of the report that different subpopulations of \( \beta \)-cells exist within islets with different degrees of readiness to secrete insulin (29), it is possible that in 2-HC islets there is a predominance of \( \beta \)-cells with an impaired recognition of glucose threshold. This leftward shift to a glucose stimulus is supported by increased hexokinase activity in these islets, which enables phosphorylation of glucose at lower glucose concentrations. The increased expression of the GLUT2 gene supports increased glucose uptake by these islets and consequently increased glucose metabolism. All of these features were also expressed by 12-day-old 1-HC rats (15).

Both preproinsulin gene transcription and insulin biosynthesis were markedly increased in islets from 28-day-old 2-HC rats compared with controls. PDX-1 has important roles in pancreatic organogenesis and in the transactivation of the preproinsulin gene in islets (25,27,30). The increase in the gene expression of PDX-1 correlated well with the increase in the mRNA level of USF-1, a transcription factor regulating PDX-1 gene transcription (26). In addition to PDX-1, \( \beta 2 \) regulates preproinsulin gene expression (30). The increased mRNA levels of these transcription factors in 28-day-old 2-HC islets via increased transcription of preproinsulin gene in 2-HC islets may have implications for the hyperinsulinemia in 2-HC rats. Transcription factors HNF3\( \beta \) and RegIII modulate the development of the pancreatic islets (27,28). The mRNA levels of these transcription factors are not significantly altered in 2-HC islets, suggesting that at least in 28-day-old 2-HC rats there may not be extensive structural adaptations.

Twelve-day-old 1-HC islets secreted moderate amounts of insulin in the absence of glucose and in the absence of calcium (threefold more than the amount secreted by age-matched MF islets in the presence of 5.5 mmol/l glucose at 60 min) (16). 2-HC islets did not possess this ability (Table 3). In 1-HC rats, there was a significant increase in the circulating levels of GLP-1, and the incretin-mediated signals for augmentation of insulin secretion were important for maintaining the hyperinsulinemic condition in these rats (16). The incretin input for augmentation of insulin secretion by islets was absent in 2-HC rats as there was no difference in the circulating levels of GLP-1 in these rats. In these aspects, 2-HC islets behaved differently from 1-HC islets, suggesting that the entire metabolic program in 1-HC islets (from the 1-HC mother) was not transferred to the progeny.

An interesting observation in the present study is that plasma FFA levels were ~30% higher in 28-day-old 2-HC rats compared with age-matched MF rats (Table 1), suggesting that signals derived from FFAs could modulate...
insulin secretion by 2-HC rats. The dose-dependent increase in insulin secretion by 2-HC islets in the presence of palmitic acid at 60 min suggested that in 28-day-old 2-HC rats fatty acids augmented the effect of basal glucose (Table 2), indicating a positive role for FFAs on insulin secretion by 2-HC islets. This observation is further substantiated by the inhibition of insulin secretion at basal glucose by ~40% in the presence of bromopalmitate, an inhibitor of FFA oxidation (Table 2), suggesting that ~40% of insulin secreted at 5.5 mmol/l glucose at 60 min was FFA-mediated in 2-HC islets.

Milburn et al. (31) suggested that increased plasma FFAs may facilitate the hypersecretion of insulin by islets from obese Zucker rats. The augmentary effect of FFAs on insulin secretion by islets is indicated by the observation that lowering of plasma FFA levels with nicotinic acid in patients with type 2 diabetes and normal volunteers lowered insulin secretory response and peripheral insulin concentrations in both groups of subjects (32). The increase in the long-chain acyl-CoA pool by the carnitine palmitoyltransferase-1/malonyl-CoA axis enhances insulin secretion (33). The augmenting effect of FFAs on insulin secretion at basal glucose by 2-HC islets is in concurrence with these reports. In 12-day-old 1-HC rats, the plasma FFA levels were significantly reduced compared with age-matched MF rats; hence, no role for FFAs on insulin secretion could be implicated.

The only difference between 2-HC rats and age-matched MF controls is fetal development in the 1-HC intrauterine environment in the case of 2-HC rats as both groups of rats were nursed naturally after birth (by foster MF mothers) and were weaned onto laboratory diet on day 24. Obviously, the capacity for basal hyperinsulinemia was most likely acquired in utero, but the expression of this ability is stifled by the high fat content of rat milk during the suckling period and that this repression was relieved by exposure to laboratory diet, which is a high-carbohydrate diet.

The prevalence of type 2 diabetes has tripled in the past 30 years, and much of it is attributed to a surge in Western populations, it is to be expected that complications associated with such pregnancies will also be on the rise. In this context, as a result of limitations of human experimentation, this rat model is ideal for studying the role of fetal origins of adult diseases associated with a hyperinsulinemic/obese pregnancy (uncomplicated with genetic determinants associated with genetic animal models for diabetes and obesity).

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