Maternal hyperinsulinemia predisposes rat fetuses for hyperinsulinemia, and adult-onset obesity and maternal mild food restriction reverses this phenotype

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Srinivasan, Malathi, Ravikumar Aalinkeel, Fei Song, Paul Mitran, Jignesh D. Pandya, Brenda Strutt, David J. Hill, and Mulchand S. Patel. Maternal hyperinsulinemia predisposes rat fetuses for hyperinsulinemia, and adult-onset obesity (HC phenotype) and that the maternal HC phenotype was transmitted to their progeny (2-HC rats) because of fetal development in the HC female rat. The aims of this study were to investigate 1) the fetal adaptations that predisposed the progeny for the expression of the HC phenotype in adulthood and 2) whether the transfer of the HC phenotype to the progeny could be reversed by maternal food restriction. Fetal parameters such as plasma insulin and glucose levels, mRNA level of preproinsulin gene, pancreatic insulin content, and islet insulin secretory response in vitro were determined. On gestational day 21, 2-HC fetuses were hyperinsulinemic, had increased insulin content and mRNA level of the preproinsulin gene in their pancreata and demonstrated an altered glucose-stimulated insulin secretory response by isolated islets. Modification of the intrauterine environment in HC female rats was achieved by pair feeding them to the amount of diet consumed by age-matched control rats from the time of their weaning. This mild dietary restriction reversed their HC phenotype and also prevented the development of the HC phenotype in their progeny. These findings show that malprogramming of the progeny of the hyperinsulinemic-obese HC female for the expression of the HC phenotype is initiated in utero and that normalization of the maternal environment in HC female rats by mild food restriction resulted in the normal phenotype in their progeny.

maternal intrauterine environment; fetal programming; fetal hyperinsulinemia; obesity; pair feeding

EPIDEMIOLOGICAL DATA AND RESULTS from animal models indicate that the nutritional status of the mother during pregnancy impacts on the expression of metabolic diseases in adulthood of the progeny (2, 3, 7, 11, 12, 18, 21, 30). Extensive organ development continues to occur in the immediate postnatal life of the rat, suggesting that this period, too, is vulnerable for metabolic programming effects. Earlier, we showed that the overlap of an altered nutritional experience in the form of a high-carbohydrate (HC) milk formula with the critical window of postnatal organ development in neonatal rat pups resulted in the establishment of the HC phenotype (chronic hyperinsulinemia and adult-onset obesity) in these rats (19, 26). Because of fetal development in HC female rats, adult progeny demonstrated the same HC phenotype (29). Whether the adaptations that predispose the progeny for expression of the HC phenotype in adult life occur during fetal development in the HC intrauterine environment was not explored in the earlier study.

The suckling period in the rat is not easily amenable to dietary modifications, since it is difficult to rear rat pups away from their natural dams. This difficulty was overcome by adapting the artificial rearing technique described by Hall (8). We have developed a rat model for adult-onset obesity by rearing 4-day-old rat pups on a HC milk formula (56% of the calories from carbohydrate compared with 8% in rat milk) up to postnatal day 24 when they are weaned on a standard rodent diet (19, 26). The mere switch in the major source of calories from fat in rat milk to carbohydrate in the HC milk formula without alterations in total caloric intake results in the immediate onset of hyperinsulinemia, its persistence even after its withdrawal on postnatal day 24, and adult-onset obesity occurring in both male and female rats (9, 10, 28).

A significant observation from these studies was that first-generation female rats (1-HC) that were artificially reared on the HC milk formula in their immediate postnatal period spontaneously transmitted the HC phenotype of chronic hyperinsulinemia and adult-onset obesity to their progeny (2nd generation HC; 2-HC) without the necessity for any dietary intervention in 2-HC rats (29). Cross-breeding experiments showed that only 1-HC female rats and not 1-HC male rats could effect this transfer to the progeny, indicating that it is the fetal development in the intrauterine environment of the 1-HC female rat that predisposes them for metabolic malprogramming (S. Vadlamudi, M. Srinivasan, and M. S. Patel, unpublished observations). Additionally, the postnatal rearing of 2-HC rat pups by foster dams does not prevent the establishment of the HC phenotype in these rats (S. Vadlamudi, M. Srinivasan, and M. S. Patel, unpublished observations), which further reinforces the hypothesis that it is fetal development in the 1-HC female rat that predisposes the progeny for the expression of the HC phenotype. Therefore, in this study, we have investigated fetal metabolic adaptations in pancreatic islets that prime them for the development of chronic hyperinsulinemia and adult-onset obesity.

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E129
Additionally, we have also investigated if reversal of the transfer of the maternal phenotype to the progeny could be achieved. Earlier, it was observed that adult 1-HC rats consumed 10–15% more food on a daily basis compared with age-matched mother-fed (MF) control rats (S. Vadlamudi and M. S. Patel, unpublished observations). Caloric restriction, the consumption of fewer calories while avoiding malnutrition, has been reported to have beneficial effects, such as extension of life span and delay in the onset and reduction in the severity of age-related diseases in several species (32, 33). Our hypothesis is that dietary restriction in 1-HC female rats would normalize the intrauterine environment, thereby preventing the transmission of the maternal HC phenotype to the progeny. On the basis of the beneficial effects of dietary restriction, we have pair-fed (PF) 1-HC female rats to the quantity of laboratory chow consumed by age-matched MF female rats during both prepregnancy and pregnancy and investigated the consequences in the progeny (2-HC/PF) during fetal and in adult life.

**MATERIALS AND METHODS**

All chemicals used were of reagent grade and obtained from Sigma (St. Louis, MO). TRIzol reagent and murine leukemia virus transcriptase were from Invitrogen (San Jose, CA). RPMI 1640, heat-inactivated FBS, antibiotic solution, and all primers were from Invitrogen (Grand Island, NY). The RIA kit for insulin was from Linco Research (St. Louis, MO). The kits for assay of plasma glucose and triglycerides were obtained from Sigma, and the kit for the assay of plasma levels of free fatty acid (FFA) was from Boehringer Mannheim (Indianapolis, IN).

**Animal protocol.** The Institutional Animal Care and Use Committee approved all animal protocols. Pregnant Sprague-Dawley rats obtained from Zivic Miller Laboratories (Zelienople, PA) were provided with water and a standard laboratory chow (16% Protein Rodent Diet; Harlan Teklad, Madison, WI; approximate weight profile: 16% protein, 4% fat, and 61% carbohydrate) ad libitum and housed under controlled conditions of temperature (25 ± 2°C) and a 12:12-h (6:00 AM–6:00 PM) light-dark cycle. Female rat pups naturally reared by their own dams (11 pups/dam) and weaned on rodent diet and water ad libitum on postnatal day 24 constituted the control MF group. 1-HC female rats used in this study were raised by the artificial rearing technique described in detail elsewhere (9, 10). Briefly, intragastric cannulas were introduced in 4-day-old female rat pups under mild anesthesia, and these pups were reared on a HC milk formula until postnatal day 24 when they were weaned on rodent diet and water ad libitum. The percentages of caloric content of macronutrients in the HC milk formula was 56% carbohydrate, 24% protein, and 20% fat compared with 8% carbohydrate, 24% protein, and 68% fat in rat milk. The HC milk formula was delivered to the pups at the rate of 0.45 kcal·g body wt·day⁻¹. Our earlier studies revealed that, when neonatal rat pups were reared on a high-fat milk formula, the macronutrient composition of which was identical to that of rat milk, they did not acquire the HC phenotype, suggesting that the artificial protocol per se does not contribute to the pathogenesis associated with the HC rat (10).

For pair-feeding studies, 1-HC female rats were given the same quantity of diet eaten by age-matched MF female rats on a daily basis, starting from postnatal day 24 (time of weaning). This regimen was continued during gestation and lactation periods. Body weights (every 10 days) and plasma insulin levels (on postnatal days 40 and 60) were monitored periodically in the pair-fed 1-HC female (1-HC/PF) and MF rats.

MF, 1-HC, and 1-HC/PF adult female rats were bred with adult MF male rats after postnatal day 65. MF, 1-HC, and 1-HC/PF pregnant dams were killed on gestational day 21 between 9:00 and 10:00 AM, and trunk blood was collected in heparinized tubes. After centrifugation, plasma was separated and stored at −20°C. The trunk blood from all the fetuses of the same litter was pooled in a heparinized tube and centrifuged, and plasma was stored at −20°C until used. To determine the pancreatic insulin content, the pancreas from one fetus of each mother was weighed and homogenized in acid-ethanol (75 ml ethanol, 1.5 ml 12 N HCl, and 23.6 ml water). The pancreatic extracts were centrifuged and the supernatants stored at −20°C until assayed for insulin. For studies on insulin secretion by islets isolated from fetal pancreas, the protocol described by Cherif et al. (5), with some modifications, was used. RPMI 1640 supplemented with 11 mM glucose, 10% heat-inactivated fetal bovine serum, and antibiotics (2,000 U/l penicillin, 0.3 g/l streptomycin) was used for the isolation and culture of fetal islets, and all steps were carried out under aseptic conditions. Pancreata from fetuses of the same mother were pooled, minced, and digested with collagenase (Sigma type V; 1.5 mg in 3 ml RPMI 1640) at 37°C in a shaking water bath at 120 rpm for 6–8 min. The enzyme reaction was stopped by the addition of ice-cold RPMI 1640 medium. The tissue digestate was washed three times with ice-cold medium, resuspended in 10 ml of medium, and stirred at low speed at room temperature for 30 min. After a brief centrifugation at low speed, the islets were resuspended in 10 ml of RPMI 1640 medium, distributed to 60-mm culture dishes, and incubated for up to 7 days in a humidified atmosphere of 5% CO₂ in air at 37°C. After the first 48 h, the medium was replaced every 24 h. The islets were hand picked using an inverted stereomicroscope, and the insulin secretory response of these islets to 5.5 (basal) and 16.7 (high) mM glucose and 5.5 mM glucose plus either 10 mM arginine or 10 mM leucine at 60 min was determined as described earlier (31).

For quantitation of mRNA levels in pancreata from MF and 2-HC fetuses on gestational day 21, isolation of total RNA and preparation of cDNA were carried out as described previously (27). 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 from 21-day-old MF and 2-HC fetal pancreata (14). Preparation of competitor DNAs for preproinsulin, pancreatic duodenal homeobox transcription factor-1 (PDX-1), β/NeuroD, hepatocyte nuclear factor 3β (HNF3β), and the sequences of the PCR primers and PCR conditions for analysis of specific mRNAs were as described by us earlier (24, 27). The PCR products were separated by electrophoresis and analyzed using BioRad Gel Doc 1000 and Molecular Analyst software for quantitation of mRNA levels. For each sample, mRNA levels were first normalized using the level of expression of its competitor control, and the final results are expressed as the degree of change in the 2-HC group compared with the MF control group.

For studies on the adult progeny, IHC and MF female rats were raised in their immediate postnatal life as described above. MF and 1-HC rats were weaned on rodent diet and water ad libitum on postnatal day 24. The 1-HC/PF group of female rats (raised as IHC rats from postnatal day 4–24) was pair-fed the same amount of diet consumed by age-matched MF rats from the time of weaning until the end of the lactation period. 1-HC, 1-HC/PF, and MF female rats were bred with normal male rats on approximately postnatal day 65. After delivery of the pups, the litter size was adjusted to 11 pups/dam, and they were reared by their natural dams. On postnatal day 24, male rats were weaned on rodent diet and water ad libitum. Their body weights were recorded on postnatal days 24 and 30 and then every 10 days thereafter up to postnatal day 100. Tail blood was collected on postnatal days 28 and 55 for measurement of plasma insulin levels between 9:00 and 10:00 A.M. On postnatal day 150, the progeny (2-HC, 2-HC/PF, and MF) rats were killed, and trunk blood was collected. Plasma was separated and stored at −20°C until assayed.

All assays using commercial kits were carried out per instructions from the manufacturers, and the values were within the linear range recommended by the manufacturers. The values of duplicate assays varied less than 5%.
RESULTS

Earlier (29), we reported that 1-HC female rats were significantly heavier compared with age-matched MF female rats during pregnancy and that their plasma insulin levels were significantly higher during both pre- and postpregnancy. Also in the present study, we observed that both their body weights and plasma insulin levels were significantly higher in the 1-HC pregnant rats compared with the MF pregnant rats on gestational day 21 (Table 1). The moderate degree of diet restriction (pair feeding) applied to the 1-HC female rats resulted in dramatic improvement in body weights and in maternal plasma characteristics (Table 1). The mean body weight and plasma insulin levels of the 1-HC/PF female rats on gestational day 21 were significantly reduced compared with 1-HC female rats. Both body weight and plasma insulin levels in 1-HC/PF female rats on gestational day 21 were similar to the values observed for age-matched MF rats (Table 1). There were no significant changes in the plasma levels of glucose among the three groups of rats (Table 1). Plasma FFA levels were significantly reduced in the 1-HC female rat compared with MF rat and significantly higher in 1-HC/PF female rats compared with the levels in 1-HC rats. Plasma triglyceride levels were not significantly different among the three groups of rats (Table 1). Table 2 shows physiological characteristics of 21-day-old fetuses of MF, 2-HC, and 2-HC/PF female rats. There were no significant differences in body weights between the three groups of rats (Table 2). The placental weights of these three groups of rats (521 ± 8 mg for MF, 514 ± 2 for 2-HC, and 489 ± 2 for 2-HC/PF) were not significantly different from one another on gestational day 21. Plasma insulin levels in 2-HC fetuses were significantly higher compared with MF fetuses on gestational day 21 (Table 2). Compared with 2-HC fetuses, plasma insulin levels were significantly decreased in 2-HC/PF fetuses on gestational day 21 and were comparable to the plasma insulin levels in age-matched MF fetuses (Table 2). A greater than twofold increase in pancreatic insulin content was observed for 2-HC fetuses compared with MF fetuses (Table 2). No significant differences were seen in the plasma concentrations of glucose, FFA, and triglycerides among MF, 2-HC, and 2-HC/PF fetuses on gestational day 21 (Table 2).

It was of interest to investigate whether islets from 2-HC fetuses demonstrated an altered insulin secretory capacity during fetal life. Figure 1 depicts the insulin secretory response of fetal islets isolated from MF, 2-HC, and 2-HC/PF fetuses on gestational day 21 to 5.5 (basal) and 16.7 (high) mM glucose as well as to 5.5 mM glucose plus either 10 mM arginine or leucine. In islets, the insulin secretory response depends on ambient glucose concentrations; hence, glucose levels were used at 5.5 mM (as basal concentration) and 16.7 mM (as high glucose concentration) for insulin secretion studies. Leucine and arginine are also known insulin secretagogues; hence, their

Table 2. Biochemical characteristics of fetuses of MF, 1-HC, and 1-HC/PF female rats on gestational day 21

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MF</th>
<th>1-HC</th>
<th>1-HC/PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>444 ± 3</td>
<td>499 ± 13*</td>
<td>426 ± 26†</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>131 ± 13</td>
<td>362 ± 40*</td>
<td>119 ± 12†</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>116 ± 4</td>
<td>119 ± 4</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>Plasma free fatty acids, μM</td>
<td>680 ± 90</td>
<td>577 ± 30*</td>
<td>692 ± 260†</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>184 ± 28</td>
<td>352 ± 38</td>
<td>190 ± 33</td>
</tr>
</tbody>
</table>

Values are means ± SE of fetuses from 6 rats. MF, mother fed; 1-HC, 1st-generation high carbohydrate; 1-HC/PF, 1st-generation high carbohydrate and pair fed. Data were analyzed by one-way ANOVA followed by post hoc analysis using the Tukey’s test to evaluate the significance of the differences between MF and 1-HC and 1-HC/PF rats. P < 0.05, 1-HC vs. MF ( * ) and 1-HC/PF vs. 1-HC ( † ).

Fig. 1. Insulin secretory response of islets isolated from maternal-fed (MF), 2nd-generation high-carbohydrate (2-HC), and 2-HC/pair-fed (PF) fetuses on gestational day 21 in response to 5.5 mM glucose (basal), 16.7 mM glucose, 5.5 mM glucose + 10 mM arginine (Arg), or 5.5 mM glucose + 10 mM leucine (Leu) at 60 min. Results are means ± SE of 5 rats. The significance of differences between MF and 2-HC and 2-HC/PF fetuses was analyzed by 1-way ANOVA followed by Tukey’s test for post hoc analysis of the significance of the differences between MF and 2-HC fetuses and 2-HC and 2-HC/PF fetuses. P < 0.05, 2-HC vs. MF ( a ) and 2-HC/PF vs. 2-HC ( b ).

Table 1. Maternal biochemical characteristics on gestational day 21 in MF, 1-HC, and 1-HC/PF rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MF</th>
<th>1-HC</th>
<th>1-HC/PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>5.41 ± 0.12</td>
<td>5.7 ± 0.11</td>
<td>5.48 ± 0.14</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>469 ± 18</td>
<td>842 ± 77*</td>
<td>499 ± 25†</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>67 ± 2</td>
<td>67 ± 2</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Plasma free fatty acids, μM</td>
<td>77 ± 7</td>
<td>56 ± 10</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>44.5 ± 2.4</td>
<td>34.6 ± 1.8</td>
<td>38.3 ± 1.5</td>
</tr>
<tr>
<td>Pancreatic insulin content, μg/g</td>
<td>18.4 ± 1.5</td>
<td>49.9 ± 5.1*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE of fetuses from 6 rats. The significance of the differences between the three groups was analyzed by one-way ANOVA followed by post hoc analysis using the Tukey’s test to evaluate the significance of the difference between MF and 2-HC and 2-HC and 2-HC/PF fetuses. ND, not determined. P < 0.05, 2-HC vs. MF ( *) and 2-HC/PF vs 2-HC ( † ).

Pancreatic insulin content, μg/g 18.4 ± 1.5 49.9 ± 5.1* ND
Plasma glucose, mg/dl 67 ± 2 67 ± 2 67 ± 5
Plasma free fatty acids, μM 77 ± 7 56 ± 10 47 ± 4
Plasma triglycerides, mg/dl 44.5 ± 2.4 34.6 ± 1.8 38.3 ± 1.5
Pancreatic insulin content, μg/g 18.4 ± 1.5 49.9 ± 5.1* ND

Fig. 1. Insulin secretory response of islets isolated from maternal-fed (MF), 2nd-generation high-carbohydrate (2-HC), and 2-HC/pair-fed (PF) fetuses on gestational day 21 in response to 5.5 mM glucose (basal), 16.7 mM glucose, 5.5 mM glucose + 10 mM arginine (Arg), or 5.5 mM glucose + 10 mM leucine (Leu) at 60 min. Results are means ± SE of 5 rats. The significance of differences between MF and 2-HC and 2-HC/PF fetuses was analyzed by 1-way ANOVA followed by Tukey’s test for post hoc analysis of the significance of the differences between MF and 2-HC fetuses and 2-HC and 2-HC/PF fetuses. P < 0.05, 2-HC vs. MF ( a ) and 2-HC/PF vs. 2-HC ( b ).
The truncated competitor DNA for the same gene. Corresponds to the cDNA for the gene and the band on /H9252 and hepatocyte nuclear factor 3 on /H11011 and MF fetuses on /H11006.

To decipher if the positive outcome observed in 2-HC/PF fetuses persists in the postweaning period, we monitored the body weight gains and plasma insulin levels in 2-HC/PF rats in the postweaning period. The beneficial effects of PF in the 1-HC female rats for the body weight gains in the progeny are seen in Fig. 3. The body weight gains of 2-HC/PF rats were not significantly different compared with the body weight gains of MF control rats from postnatal day 24 up to postnatal day 100, suggesting that PF in the 1-HC females reversed the increased body weight gain observed in 2-HC rats (Fig. 3). For the purpose of comparison, the graph for the body weight gain of 2-HC rats is adapted from our earlier report (29). Plasma insulin levels on postnatal days 28, 55, and 150 were significantly lower in 2-HC/PF rats compared with age-matched 2-HC rats and not significantly different compared with the plasma insulin levels in age-matched MF rats (Fig. 4). On postnatal day 150, it was observed that plasma triglyceride (143 ± 11 mg/dl for 2-HC/PF males compared with 215 ± 13 mg/dl for 2-HC males) and FFA (0.35 ± 0.03 mM for 2-HC/PF compared with 0.42 ± 0.01 mM for 2-HC males) levels were significantly decreased compared with age-matched 2-HC rats and were similar to the levels observed in age-matched MF rats (triglyceride levels 137 ± 15 mg/dl and FFA levels 0.35 ± 0.03 mM). Plasma glucose levels were not different among MF, 2-HC, and 2-HC/PF rats on postnatal day 150, indicating that normalization of plasma insulin levels in the 2-HC/PF rats did not result in compensatory increased plasma glucose levels (127 ± 3 mg/dl for 2-HC/PF, 130 ± 3 mg/dl for 2-HC, and 130 ± 4 mg/dl for age-matched MF males).

To explore if molecular adaptations support the observed hyperinsulinemia in the 2-HC fetuses, mRNA levels of the preproinsulin gene and some transcription factors that augment its transcription were quantified in the pancreas from 2-HC and MF fetuses on gestational day 21. As seen in Fig. 2, on gestational day 21, the mRNA level of preproinsulin gene was increased (~50%) in the 2-HC pancreas compared with its level in the MF pancreas. Additionally, the mRNA levels of both PDX-1 and β2/NeuroD were also increased in the 2-HC fetal pancreas. There was no significant change in the mRNA levels of HNF3β.

Fig. 2. Expression of the mRNA levels of the preproinsulin (insulin), pancreatic duodenal homeobox transcription factor-1 (PDX-1), β2/NeuroD (Beta2), and hepatocyte nuclear factor 3β (HNF3β) genes in MF and 2-HC pancreata on gestational day 21. Top: representative gel wherein the band on top corresponds to the cDNA for the gene and the band on bottom corresponds to the truncated competitor DNA for the same gene. Bottom: representation of the means of the relative densitometric values from quantification of the mRNA levels. Results are means ± SE of 6 rats. The significance of the differences in the mRNA levels in pancreases of MF and 2-HC fetuses on gestational day 21 was analyzed using Student’s t-test. *P < 0.05, 2-HC vs. MF.

Fig. 3. Body weight gain of 2-HC/PF progeny from postnatal day 24 to postnatal day 100 compared with the body weight gain of age-matched MF rats. Both groups of rats had access to laboratory chow and water ad libitum (ad lib). The solid line representing the body weight gain of 2-HC rats is taken from an earlier publication (29). Results are means ± SE of 6 rats. The significance of the differences in the body weights of MF and 2-HC/PF rats were analyzed using Student’s t-test. No comparisons were made for the body weight gain of 2-HC/PF rats with the body weight gain of 2-HC rats.
21-HC fetuses demonstrated hyperinsulinemia, which was associated with increased mRNA levels of preproinsulin and PDX-1 genes in these islets (25). In this study, we provide evidence to show significantly higher insulin content and a blunted response to a glucose stimulus by fetal islets (30). On the other hand, a severe diabetic pregnancy results in reduced pancreatic insulin content and a blunted response to a glucose stimulus by fetal islets (30).

Pregnancy itself elicits increased levels of plasma insulin compared with the age-matched nonpregnant rat. However, in pregnant 1-HC females, the plasma insulin levels were markedly higher compared with the levels in age-matched pregnant MF rats (Table 1). This could be indicative of the severity of insulin resistance during pregnancy in the 1-HC females compared with age-matched pregnant MF rats. The body weights of 21-day-old 2-HC fetuses were similar to age-matched MF fetuses, indicating normal growth unlike a malnourished or diabetic rat pregnancy wherein fetal growth is altered. Although the placenta is an important determinant of fetal growth and its postnatal development, placental weights were not significantly different between 2-HC and MF rats on gestational day 21. Hence, in our HC rat model, fetal body weight and placental weight did not provide any indication of the eventual onset of obesity in adulthood of 2-HC rats.

Pancreatic organogenesis begins around gestational day 15 in the rat, and thus an abnormal intrauterine environment could structurally and functionally alter the fetal pancreas, the consequences of which manifest later in life (13). This has been demonstrated in several studies using animal models (1, 4, 6, 15, 22). There were no significant changes in the total number of islets, percent distribution of large- and small-sized islets, or number of insulin-positive cells in pancreata from 2-HC fetuses (data not shown). Unlike the above studies (1, 4, 6, 15, 22) and as observed in pancreata of 12-day-old 1-HC rats (20), the observed hyperinsulinemia in 2-HC fetuses is not because of structural changes at the level of number and size of islets.

Caloric restriction and intermittent fasting have been shown to extend the life span and decrease susceptibility to age-related diseases in rats and monkeys and to improve the health of overweight humans (17). They enhance cardiovascular and brain functions, reduce hypertension, and improve insulin sensitivity in target organs (17). In our studies, a drastic reduction in the availability of food was not applied to the 1-HC female rat, as is the norm in caloric restriction studies. 1-HC rats consume ~10–15% more food compared with age-matched MF rats (S. Vadlamudi and M. S. Patel, unpublished observations). In our studies, instead of providing food ad libitum to the 1-HC female rat, we pair-fed them to the diet consumed by age-matched MF rats on a daily basis. Even this marginal reduction in food consumption from the time of

**DISCUSSION**

Several of the reported animal models for metabolic programming of adult-onset diseases are concerned with an altered intrauterine environment during fetal development because of manipulation of the maternal nutritional status during pregnancy. In contrast to these studies, the HC rat model as reported here investigates the consequences of an altered dietary experience in the immediate postnatal life of the female rat. A HC dietary intervention in female rat pups during the suckling period results in the metabolic malprogramming of chronic hyperinsulinemia and onset of obesity in its adulthood as well as in the spontaneous transmission of this phenotype to its progeny.

Because the period of fetal development is the only difference between 1-HC progeny and control MF rats, we postulated that metabolic malprogramming for expression of the HC phenotype might be evident during fetal life. Earlier, we had shown that, during the suckling period, 2-HC rats did not demonstrate hyperinsulinemia, but immediately upon weaning to laboratory chow their plasma insulin levels were significantly higher compared with age-matched MF rats (25). Additionally, we observed, on gestational day 28, alterations in islet functions, such as a leftward shift to a glucose stimulus and increased mRNA levels of preproinsulin and PDX-1 genes in 2-HC islets (25). In this study, we provide evidence to show that, in 2-HC rats, metabolic malprogramming effects are observed in fetuses on gestational day 21. On gestational day 21, 2-HC fetuses demonstrated hyperinsulinemia, which was supported by the observed increases in 1) mRNA levels of preproinsulin gene and its upstream regulators, such as PDX-1 and B2/NeuroD, 2) pancreatic insulin content, and 3) insulin secretory response by 2-HC fetal islets to basal glucose (5.5 mM), high glucose (16.7 mM), and basal glucose plus leucine. The altered in vitro insulin secretory response of 2-HC fetal islets suggests increased responsiveness of these islets to these secretogogues. The increased insulin secretory response of fetal 2-HC islets to basal and high glucose is preserved in the immediate postweaning period (postnatal day 28) and in adulthood (16, 25). Aberrations in islet functions that prime them for adult-onset disorders have been reported for fetuses of dams fed a low-protein diet and fetuses of a diabetic pregnancy. Fetuses of dams fed a low-protein diet during pregnancy demonstrated alterations in pancreatic functions, including reduction in islet size, insulin content, islet cell multiplicity in vivo, and vascularization of fetal islets (23). Additionally, an impairment of insulin secretion was observed after stimulation of fetal islets from low-protein-fed dams by various metabolic or nonmetabolic secretogogues (5). A mild diabetic pregnancy induced an increase in pancreatic insulin content and an exaggerated insulin secretory response to a glucose stimulus by fetal islets (30). On the other hand, a severe diabetic pregnancy results in reduced pancreatic insulin content and a blunted response to a glucose stimulus by fetal islets (30).

Fig. 4. Plasma insulin levels in MF, 2-HC, and 2-HC/PF rats on gestational day 21 (GD 21) and postnatal days 28, 55, and 150. Results are means ± SE of 6 rats. The significance of differences between MF, 2-HC, and 2-HC/PF rats was analyzed by 1-way ANOVA followed by post hoc analyses using Tukey’s test to compare the significance of the differences between MF and 2-HC and 2-HC/PF rats. P < 0.05, 2-HC vs. MF (a) and 2-HC/PF vs. 2-HC (b).
weaning by 1-HC female rats had a positive outcome for its pregnancy. The plasma insulin levels and body weights of 1-HC/PF female rats were significantly reduced compared with ad libitum-fed 1-HC females and resulted in a near-normal maternal intrauterine environment in 1-HC/PF females. Fetuses of 1-HC/PF females on gestational day 21 had significantly reduced plasma insulin levels compared with age-matched fetuses of 1-HC females without any alterations in plasma glucose levels. Interestingly, these effects were maintained into adulthood, since the 2-HC/PF progeny were neither hyperinsulinemic nor obese in adulthood.

Our study indicates that nutritional challenges applied only during pregnancy, as observed in malnourished or diabetic pregnancies, are not the only means for metabolic malprogramming of the fetuses leading to adult-onset diseases. In the 1-HC pregnant female, there is no nutritional stress applied during pregnancy, yet there is perpetuation of the HC phenotype. The hyperinsulinemic-obese intrauterine environment in 1-HC female rats resulting from ingestion of increased carbohydrate-derived calories in its immediate postnatal life malprograms its fetuses for the onset of the metabolic syndrome in adulthood. The specific factors and mechanisms responsible for this transmission are not clear at the present time. Our results also indicate that moderate dietary restriction enforced early in life in female rats susceptible to chronic hyperinsulinemia and adult-onset obesity significantly improves their intrauterine environment such that the progeny of these rats do not spontaneously acquire the maternal phenotype. These observations may have important implications for the human obesity scenario involving maternal transfer of the obesity trait to their progeny.

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