A Long-Term High-Carbohydrate Diet Causes an Altered Ontogeny of Pancreatic Islets of Langerhans in the Neonatal Rat

JAMES PETRIK, MALATHI SRINIVASAN, RAVIKUMAR AALINKEEL, STEPHEN COUKELL, EDITH ARANY, MULCHEND S. PATEL, AND DAVID J. HILL

Lawson Research Institute, St. Joseph's Health Centre, London, Ontario N6A 4V2, Canada [J.P., S.C., E.A., D.J.H.]; Departments of Physiology [J.P., D.J.H.], Medicine [E.A., D.J.H.], and Paediatrics [D.J.H.], University of Western Ontario, London, Ontario, N6A 5A5, Canada; and Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, 3435 Main Street, Buffalo, New York 14214, U.S.A. [M.S., R.A., M.S.P.]

ABSTRACT

Neonatal rats fed a high-carbohydrate (HC) formula by gastrotomy are hyperinsulinemic but normoglycemic. We determined whether HC formula altered pancreatic islet cell ontogeny. Rats were reared from d 4 on an HC formula or a high-fat formula, or were allowed to suckle naturally, and the pancreata were examined histologically from animals ≤ 24 d of age. The mean area of individual islets was reduced, but islet number was increased in HC rats compared with mother-fed or high fat-fed animals, which were similar. Islets from HC animals were relatively deficient in α cells and had a greater incidence of islet cells with fragmented DNA, indicative of apoptosis. Ductal epithelium, a source of new islets by neogenesis, had a greater incidence of cells staining immunopositive for proliferating cell nuclear antigen, a marker of cell replication, and a lower inci-

dence of apoptosis. The islet cell mitogen and survival factor, IGF-II, had a reduced mRNA expression in whole pancreas from HC animals. The relative area of islet cells demonstrating IGF-II immunoreactivity was reduced in HC-fed rats *versus* controls, although a greater percentage of ductal epithelial cells were immunopositive. HC formula alters islet cell ontogeny by affecting islet size and number, which may be linked to an altered IGF-II expression. (*Pediatr Res* 49: 84–92, 2001)

Abbreviations

HC, high carbohydrate
MF, mother fed
HF, high fat
PCNA, proliferating cell nuclear antigen

Hyperinsulinemia in obesity is associated with pancreatic islet hyperplasia and hypertrophy (1, 2). Additionally, both genetic and nutritionally induced obesity in animal models cause impaired glucose tolerance and increased insulin resistance (1–3). Such predisposition to obesity and diabetes may be related to nutritional experiences early in fetal life or childhood during critical windows of endocrine pancreatic development. We have previously reported that feeding an HC formula to rat pups by an artificial rearing technique induces hyperinsulinemia, and in the long-term affects lipogenesis, leading to obesity (4). When HC formula was commenced on postnatal d 4, β -cell mass and pancreatic insulin content were increased compared with MF control rats by 12 d of age (5). The hyperinsulinemia, obesity, and glucose intolerance that

were observed in the first generation of rats fed an HC diet during their early postnatal life were transmitted, *via* the females, into the next generation, who were fed a normal diet, leading to hyperinsulinemia, obesity, and impaired glucose tolerance in the next generation of animals also (6, 7).

The rapid increase in islet cell mass that occurs in late fetal and neonatal life in the rat may explain why pancreatic morphology is so sensitive to nutritional insult at this time. In the 2 d before term, the cellular area staining immunopositive for insulin increases 2-fold because of both β -cell replication and the neogenesis of new β cells from the pancreatic ductal epithelium (8, 9). In the adult animal, the β -cell replication rate is low, approximately 3% per day (10). Development of the endocrine pancreas is associated with a balance between islet cell replication and neogenesis and programmed cell death. We and others have shown previously that a transient wave of apoptosis occurs in the developing rat islets between 1 and 2 wk of postnatal age (10–12). The mechanisms controlling islet cell neogenesis and apoptosis are not well understood, but there is considerable evidence that IGFs are major contributors

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Correspondence and reprint requests: Dr. D.J. Hill, Lawson Research Institute, St. Joseph's Health Centre, 268 Grosvenor Street, London, Ontario N6A 4V2, Canada.

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to β -cell growth, maturation, and function, and are expressed by β cells throughout life. We have shown previously that the levels of IGF-II peptide and mRNA are relatively high in fetal and early neonatal life and then decline sharply toward adulthood (11, 13). We recently demonstrated that the wave of β -cell apoptosis during rat pancreatic development is temporally associated with a loss of expression of IGF-II within pancreatic islets and that the IGFs can act as survival factors, protecting β cells against cytokine-induced apoptosis (11).

The purpose of this study was to examine the impact of feeding an HC formula during early postnatal life on pancreatic islet cell ontogeny, and to determine whether the long-term changes seen in insulin release could result from an altered neonatal pancreatic development. A second objective was to determine whether any ontological changes seen were related to an altered local expression of IGF-II.

METHODS

Animal studies. All animal protocols were approved by the Institutional Animal Care and Use Committee. Timed pregnant Sprague-Dawley rats (Zivic Miller Labs., Zellenople, PA, U.S.A.) were given free access to a stock laboratory diet (Formulab 5008, Purina Mills, St. Louis, MO, U.S.A.) and water *ad libitum*. Offspring of both sexes were removed from their mothers on postnatal d 4 and assigned randomly to either control or experimental diet groups. Pups were either assigned to the MF control group, where they were allowed to nurse from their foster mother as normal, or to an HC or HF formula. Nutrient composition (percent caloric content) of the HC formula was 24% protein, 56% carbohydrates, and 20% fat as described in detail previously (4). The HF formula was designed to be similar in composition to rat milk to isolate the effects of the artificial rearing technique *per se*, and consisted of 24% protein, 8% carbohydrate, and 68% fat (4). The formulae were prepared as described previously (4) and frozen for several days before use. The animals in this study were artificially reared using the technique developed by Hall (14) in which a chronic intragastric cannula was inserted *via* the mouth under mild anesthesia. The pups were kept in styrofoam cups at a constant temperature of 37°C, and were fed the HC or HF formula at a rate of 0.45 kcal/g body wt/d. Pups from the three groups were killed at postnatal d 6, 12, 14, 18, and 24. On postnatal d 18, the pups from the HC and HF groups had the cannulae cut and were allowed free access to their formula until postnatal d 24. Pancreata were then removed and either fixed in ice-cold 0.2% glutaraldehyde/4% paraformaldehyde buffered with 70 mM phosphate buffer (pH 7.4) or immediately immersed in liquid nitrogen and stored at -80°C. After fixation, the pancreata were washed in four changes of PBS during a 48-h period before dehydration in 70% (vol/vol) ethanol and embedding in paraffin. Blood (300 μ L) was collected from each pup for RIA of circulating levels of insulin, IGF-I, and IGF-II.

Northern blot analysis. Total RNA was extracted from pancreata as described previously (13). Briefly, frozen pancreata (approximately 0.25 g) were homogenized in 4 M guanidine isothiocyanate (GIBCO BRL, Burlington, Ontario, Can-

ada) and RNA was separated by centrifugation at 36,000 rpm overnight over a cushion of 5.7 M cesium chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.). The RNA was precipitated and redissolved in diethyl pyrocarbonate (DEPC)-treated water (Sigma Chemical Co.). Before hybridization, the integrity and relative amounts of RNA from each pancreas were assessed by size separation on 2% agarose TAE (Tris HCl-EDTA) gels with ethidium bromide. Pancreata in which ribosomal RNA showed degradation were not used. Northern blot hybridization was performed as described previously (13) using 20 μ g of total RNA after separation by size on 1% agarose gels with 2.2 M deionized formaldehyde. The separated RNA was transferred to Zetaprobe membranes (BioRad Inc., Richmond, CA, U.S.A.) by capillary transfer. The blots were prehybridized for 4 h in hybridization buffer containing 1 $\times$ SSPE (150 mM NaCl, 10 mM sodium phosphate monobasic, 1 mM EDTA), 7% (vol/vol) SDS, 100 μ g of salmon sperm DNA, and 50% deionized formamide. The blots were then hybridized in a change of the same buffer at 42°C overnight in a hybridization oven (TEK-star, BioCan Scientific, Mississauga, Ontario, Canada) with 2 $\times$ 10⁶ cpm/mL radiolabeled cDNA probe for IGF-II or 1 $\times$ 10⁶ cpm/mL for cDNA encoding 18S ribosomal RNA. After hybridization overnight, blots were washed with 1 $\times$ standard saline citrate (SSC, 0.75 M NaCl and 0.075 M sodium citrate) with 0.1% SDS (wt/vol) for 30 min at 42°C, followed by two final 30-min washes at 42°C in 0.1 $\times$ SSC with 0.1% (wt/vol) SDS in a shaking water bath. Blots were air-dried and exposed to x-ray film (Kodak XAR-5, Eastman Kodak Inc., Rochester, NY, U.S.A.) at -80°C with intensifying screens for $\leq$ 7 d. Blots were hybridized consecutively with cDNAs for IGF-II followed by 18S ribosomal RNA. Between hybridizations, the blots were stripped with two changes of 0.01 $\times$ SSC with 0.5% (wt/vol) SDS at 90°C for 30 min each wash to remove the previous labeled cDNA probe.

Radiolabeling of cDNA probes with [³²P]dCTP (ICN Biomedicals Inc., Irvine, CA, U.S.A.) was performed by random priming using a Pharmacia oligolabelling kit (Pharmacia LKB Biotechnology, Uppsala, Sweden), to specific radioactivities of 1–2 $\times$ 10⁹ dpm/ μ g. Separation of radiolabeled cDNA from unincorporated [³²P]dCTP was performed using ProbeQuant G-50 microcolumns (Pharmacia). Complementary DNA probes were kindly provided by the following investigators: an 807-bp mouse IGF-II in pGEM 4z by Dr. G. Bell, University of Chicago, Chicago, IL, U.S.A.; and a rat 18S ribosomal RNA cDNA by Dr. D. Denhardt, Rutgers University, Piscataway, NJ, U.S.A. The 18S ribosomal RNA cDNA was used to determine equality of RNA loading and transfer.

Immunohistochemistry. Sections of pancreas (5 μ m) were cut from paraffin blocks with a rotary microtome and mounted on Superfrost Plus glass slides (Fisher Scientific, Nepean, Ontario, Canada). Immunohistochemistry was performed to localize IGF-II, PCNA, insulin, glucagon, and somatostatin within islets by a modified avidin-biotin peroxidase method (15) as described by us previously for pancreas (11). Sections were deparaffinized in xylene, rehydrated in a descending alcohol series (100%, 90%, 70%, vol/vol), and washed in PBS. Sections were then incubated in 1% (vol/vol) hydrogen perox-

ide to block endogenous peroxidase activity, followed by a 15-min incubation in 5% (wt/vol) BSA, 0.02% (wt/vol) sodium azide in PBS to reduce nonspecific binding. Slides were incubated with either rabbit anti-human IGF-II (1:300 dilution; GroPep Ltd., Adelaide, Australia); mouse anti-PCNA (1:750 dilution; Sigma Chemical Co.); guinea pig anti-insulin (1:15 dilution; provided by Dr. T.J. McDonald, University of Western Ontario, London, Ontario, Canada); rabbit anti-porcine glucagon (1:100 dilution; C-terminal-specific O4A antiserum kindly provided by Dr. R. Ungar, Dallas, TX, U.S.A.); or rabbit anti-rat somatostatin (1:100 dilution; Dako Laboratories, Mississauga, Ontario, Canada). All antisera were diluted in 0.01 M PBS (pH 7.5) containing 1% (wt/vol) BSA and 0.02% (wt/vol) sodium azide (100 μ L per slide). All subsequent incubations were performed at room temperature. Biotinylated goat anti-rabbit IgG (1:30 dilution), goat anti-mouse IgG (1:100 dilution), or mouse anti-guinea pig IgG (1:500 dilution; all from Vector Laboratories, Burlingame, CA, U.S.A.), were diluted in the same buffer and applied to the tissue for 2 h in a humidified chamber. The slides were then washed in PBS and incubated with avidin and horseradish peroxidase for 1 h. Peptide immunoreactivity was visualized by incubation with fresh 1.89 mM diaminobenzidine tetrahydrochloride (Fast DAB tablets, Sigma Chemical Co.) for 2 min. Tissue sections were counterstained with Carrazi's hematoxylin, dehydrated in ascending series of alcohols (50%, 70%, 90%, 100%, vol/vol), cleared in xylene, and mounted under glass coverslip with Permount (Fisher Scientific).

To establish specificity of staining, the primary antisera for IGF-II was preadsorbed overnight at 4°C with excess homologous antigen before application to the sections, resulting in an abolition of staining. Further controls included substitution of primary antisera with nonimmune serum and omission of the secondary antiserum. Dual staining for PCNA and insulin, or PCNA and glucagon, was performed by first performing immunohistochemistry for PCNA as described above using diaminobenzidine as the chromogen. Before counterstaining and dehydration, the sections were then subjected to immunohistochemistry for insulin or glucagon as described above, using alkaline phosphatase (blue) (alkaline phosphatase substrate kit III, Vector) as the chromogen. After incubation with antisera against insulin or glucagon, anti-guinea pig or anti-porcine alkaline phosphatase conjugate (Sigma Chemical Co.) was applied to the sections for 1 h, followed by incubation with alkaline phosphatase substrate for 20 min before washing and counterstaining with Mayer's hemalum. Sections were mounted under glass coverslips with an aqueous mounting solution (Aquamount, Polysciences, Warminster, PA, U.S.A.).

To demonstrate that pancreatic ductal tissue and acinar tissue could be appropriately identified for morphometric analysis, immunohistochemistry was performed with mouse anti-human cytokeratin 20 (1:50; Dako Corporation, Santa Barbara, CA, U.S.A.), or rabbit anti-human α amylase (1:2000; Sigma Chemical Co.), respectively. For the visualization of cytokeratin, tissues were first incubated with Bacto-Trypsin (0.015% wt/vol in Trizma buffer, pH 7.6; Difco Laboratories, Detroit, MI, U.S.A.) for 45 min at 37°C.

Visualization of apoptosis. Immunohistochemistry was performed to localize apoptotic nuclei within histologic sections of pancreas (16) using the Apoptag *in situ* apoptosis detection kit (Oncor Inc., Gaithersburg, MD, U.S.A.), as described by us in detail (11). Staining was performed according to the manufacturer's protocol. Histologic sections (5 μ L) were deparaffinized in xylene, rehydrated in a descending alcohol series (100%, 90%, 70%, vol/vol), and washed in PBS before incubation with proteinase K (20 μ g/mL; Boehringer-Mannheim, Dorval, Quebec, Canada) for 15 min at room temperature. After proteinase K digestion, sections were incubated with 2% (vol/vol) hydrogen peroxide for 5 min to quench endogenous peroxidase activity, followed by application of the terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h at 37°C. Apoptotic nuclei were visualized with 1.89 mM diaminobenzidine (Sigma Chemical Co.) as described for immunohistochemistry, followed by counterstaining with methyl green for 1 min. Tissues were dehydrated in butanol, cleared in xylene, and mounted under glass coverslips with aqueous mounting solution (Aquamount, BDH, Poole, Dorset, U.K.).

Radioimmunoassays. The insulin, IGF-I, and IGF-II contents of serum obtained from MF, HF, and HC animals were measured by RIAs. Circulating levels of insulin were measured with a rat insulin RIA Kit (Linco Research Inc., St. Louis, MO, U.S.A.) with rat insulin used for the standard curve. Sensitivity of 0.1 ng/mL was achieved with overnight equilibrium using a 100- μ L serum sample. IGF-I and -II RIAs were also performed as previously described (17) after separation from binding proteins by incubation with 8 M formic acid in a 1 M Tris base.

Morphometric and statistical analysis. Morphometric analysis was performed using a Zeiss transmitted light microscope at a magnification of $\times 250$ or $\times 400$. Analyses were performed with Northern Eclipse (v2.0) morphometric analysis software (Empix Imaging Co., Mississauga, Ontario, Canada). An islet was defined as being a unit of six insulin-positive immunoreactive cells or greater, thus excluding the newly formed endocrine cells resulting from neogenesis at the ductal epithelium. The percentage of islet, ductal, or exocrine cells staining immunopositive for IGF-II or PCNA, or demonstrating apoptotic nuclei, was calculated for each dietary condition at each age from five tissue sections obtained from each pancreas. Sections were taken every 50 μ m representing predominantly the head region of the pancreas. Pancreata from five animals were examined for each age and variable. To calculate islet area, or the percentage islet area staining immunopositive for insulin, glucagon, or somatostatin, individual islets were circled for image analysis and selected by RGB threshold. For Northern blot analysis, the ratio of hybridization signal for IGF-II compared with 18S rRNA was calculated at each age for each of three separate pancreata after scanning densitometry. Differences among mean values for variables within individual experiments were compared statistically by two-way ANOVA followed by a Scheffe's test. No differences between MF and HF groups of animals were found for each of the variables measured. Data for the MF group are therefore presented as a control group *versus* HC.

RESULTS

Pancreata from offspring chronically fed HC formula contained islets that were significantly ($p < 0.05$) smaller than age-matched MF and HF controls. Islets in the MF group had a mean area of $8,965 \pm 1,326 \mu\text{m}^2$ (mean $\pm$ SD) at postnatal d 6, rising to a mean area of $17,058 \pm 2,652 \mu\text{m}^2$ at d 24. At 6 d of age, the mean islet area for HF-fed animals was $8,482 \pm 966 \mu\text{m}^2$, and at 24 d was $16,250 \pm 2,827 \mu\text{m}^2$, similar to that in MF rats and showing that intragastric feeding did not alter islet cell area by the time of weaning. The HC animals, however, had islets with a mean area of $4,014 \pm 856 \mu\text{m}^2$ at postnatal d 6, increasing to $8,309 \pm 2,119 \mu\text{m}^2$ at postnatal d 24; a reduction in area of approximately 50 to 60% compared with controls. Although the mean islet size in the HC group was significantly smaller than controls, there was significant ($p < 0.05$) increase in the number of islets per unit area of pancreas in HC compared with MF controls at each age between d 12 and 24 (Table 1). Further analysis of the size distribution of small ($<10,000 \mu\text{m}^2$) or larger ($>10,000 \mu\text{m}^2$) islets showed that rats fed HC formula demonstrated a much greater percent distribution of small islets compared with MF animals, many of these being small endocrine cell clusters with between 6 and 20 β cells (Table 2). The mean area of individual islet cells did not differ among diet groups, or with age (data not shown).

Immunohistochemistry was used to determine the number of islet cells staining immunopositive for the pancreatic hormones insulin, glucagon, and somatostatin. Islets from HC animals had a significantly reduced relative area occupied by glucagon-positive α cells at ages 12–18 d, whereas the area occupied by insulin-positive β cells was significantly greater at 18 d, but not at other ages. The β/α cell ratio, assessed by these criteria, was significantly greater at each age in HC rats (Table 3). The mean

($\pm$ SD) percentage area of islets demonstrating immunoreactive somatostatin was 3.3 ± 1.0 , and did not differ with age or diet. More than 94% of islet cells could be accounted for, the balance probably reflecting pancreatic polypeptide-containing cells and endothelial cells, which were not analyzed. Therefore, pancreata from animals fed HC formula contained smaller, but more numerous, islets that contained a relatively higher proportion of β cells relative to α cells through early development.

As an indicator of islet cell replication, sections of pancreata were examined for the presence of PCNA by immunohistochemistry. The percentage of islet cells that stained for PCNA did not significantly change in MF rats with age (Table 4), but was greater in HC rats than in control animals on d 12 and 18. When insulin or glucagon were each colocalized immunohistochemically with PCNA, it was found that approximately 70 to 80% of the islet cells staining for PCNA were β cells, whereas between 16 and 26% were α cells (Fig. 1). This did not change significantly with age or diet. The histomorphological identity of pancreatic ductal epithelium was confirmed from the immunohistochemical localization of cytokeratin 20. In MF controls the PCNA labeling index within ductal epithelial cells was approximately 10% on d 6 and did not change at subsequent ages (Fig. 1 and Table 4). However, in HC animals significantly more ductal epithelial cells contained immunoreactive PCNA as early as d 6, and on d 18 and 24. The identity of acinar tissue was confirmed by immunohistochemical staining for amylase. The PCNA labeling of exocrine acinar cells varied between 4 and 8% but did not differ with age or diet.

The occurrence of apoptotic cell death within islet cells at each age was examined by molecular histochemistry using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method. Such cells were compacted, with little cytoplasm and immunoreactive nuclei (Fig. 2). They were not restricted to any particular region of the islets. In islets of control animals, the incidence of apoptosis was low at postnatal d 6 and 12, with approximately 5 to 8% of cells undergoing programmed cell death. The incidence of cell death in the control groups peaked at 12% at postnatal d 18, before falling back to $8 \pm 1\%$ at d 24 (Table 5). At all ages studied, islets from the HC group had a higher percentage of cells undergoing apoptosis. The presence of apoptosis was also determined in cells within the adjacent ductal epithelium from which islets derive by neogenesis. In contrast to the findings in control islets, a decreased ($p < 0.05$) number of apoptotic cells were found in the ductal epithelium within pancreata from rats fed HC formula on d 14 and 24 (Table 5). The rate of apoptosis within acinar tissue was relatively low (3 to 6%) and did not change with age or diet.

To determine whether the altered proliferation and survival of islet cells seen in rats fed a long-term HC diet were associated with alterations in the expression or presence of IGF-II, pancreatic sections from HC and control groups were examined using immunohistochemistry. When the percentage area of islet cells positive for IGF-II was assessed, there was a significant ($p < 0.05$) decrease with age in both the HC and MF groups, but values were consistently reduced in the HC animals (Figs. 3 and 4A). Immunoreactivity for IGF-II was

Table 1. Changes with age in the number of islets per square millimeter of pancreas for animals receiving either HC diet or being fed by the mothers*

Age (d)	HC	MF
6	9.1 ± 4.2	6.4 ± 2.6
12	$13.0 \pm 3.8^\dagger$	7.7 ± 3.0
14	$14.8 \pm 3.8^\dagger$	8.0 ± 2.9
18	$16.9 \pm 3.0^\dagger$	10.0 ± 3.1
24	$17.8 \pm 1.3^\dagger$	10.2 ± 3.9

* Results represent mean $\pm$ SD ($n = 5$). $^\dagger p < 0.05$ vs MF controls.

Table 2. Percent distribution of number of islets according to size in histologic sections of pancreas from rats of 6–24 d of age after feeding with an HC formula or continued feeding by the mother. Islets were arbitrarily classified as small ($<10,000 \mu\text{m}^2$) or large ($>10,000 \mu\text{m}^2$)*

Age (d)	Small		Large	
	HC	MF	HC	MF
6	95	55	5	45
12	95	60	5	40
14	100	60	0	40
18	100	50	0	50
24	95	35	5	65

* Results are derived from five animals at each age.

Table 3. Changes in the proportional area (%) of islet cells containing immunoreactive insulin or glucagon, or in the insulin/glucagon ratio in pancreata between 6 and 24 d of age after feeding an HC formula or continued MF since d 4*

Age (d)	Insulin		Glucagon		I/G ratio	
	HC	MF	HC	MF	HC	MF
6	69.9 ± 5.3	65.4 ± 7.5	19.8 ± 4.6	24.7 ± 2.6	3.5 ± 0.2 [†]	2.6 ± 0.1
12	72.3 ± 12.0	66.3 ± 7.8	16.1 ± 1.9 [‡]	21.5 ± 2.2	4.4 ± 0.1 [‡]	3.0 ± 0.1
14	79.5 ± 5.7	74.7 ± 5.8	15.8 ± 2.1 [‡]	21.7 ± 2.1	5.0 ± 0.2 [‡]	3.4 ± 0.1
18	79.7 ± 3.1 [†]	71.8 ± 6.8	15.9 ± 1.9 [‡]	21.6 ± 2.2	4.9 ± 0.2 [‡]	3.3 ± 2.0
24	71.8 ± 6.8	73.4 ± 4.4	21.6 ± 2.2	21.7 ± 2.1	5.1 ± 0.2 [‡]	3.3 ± 0.1

* Results represent mean ± SD ($n = 5$). [†] $p < 0.05$, [‡] $p < 0.01$ vs MF group for same age and hormone.

Abbreviation: I/G, insulin/glucagon.

Table 4. Changes with age in the percentage of cells demonstrating immunoreactivity for PCNA in islets or ductal epithelium of the pancreas for animals receiving either HC diet or being fed by the mothers*

Age (d)	Islets		Ductal epithelium	
	HC	MF	HC	MF
6	5.8 ± 1.7	4.9 ± 1.8	18.7 ± 6.4 [†]	10.3 ± 4.2
12	5.2 ± 1.5 [†]	3.3 ± 0.7	18.8 ± 5.7	9.6 ± 4.6
14	5.1 ± 1.6	3.5 ± 0.7	17.5 ± 6.7	10.1 ± 4.1
18	10.5 ± 2.2 [‡]	6.0 ± 1.0	26.0 ± 8.6 [‡]	9.3 ± 4.4
24	4.6 ± 0.6	3.6 ± 0.7	20.7 ± 5.9 [‡]	10.8 ± 3.8

* Results represent mean ± SD ($n = 5$). [†] $p < 0.05$, [‡] $p < 0.01$ vs MF controls.

localized to all regions of the islets. Cells staining immunopositive for IGF-II were also quantified in the pancreatic ductal epithelium. In contrast to islets, the percentage of IGF-II-positive ductal epithelium cells was significantly greater in the HC group than in MF controls (Figs. 3 and 4B). The abundance of mRNA for IGF-II was determined in whole pancreata using Northern blot hybridization. Three main mRNA transcripts for IGF-II were detected of 7.4 kb, 4.6 kb, and 2.4 kb in pancreata of both HC and MF rats (Fig. 5A). In both groups, IGF-II expression declined with age ($p < 0.01$), but was relatively lower in the HC animals. When IGF-II mRNA expression was expressed relative to 18S ribosomal RNA in the same samples for three replicate animals at each age, this was significantly lower in the HC group *versus* MF controls between postnatal d 6 and 18 (Fig. 5B).

Circulating levels of IGF-I, IGF-II, and insulin were measured with RIA in serum samples from HC and control animals. Serum IGF-II levels in MF animals decreased approximately 7-fold between 6 and 24 d of age, from 208 ± 132 ng/mL to 27 ± 2 ng/mL (mean ± SD, $n = 5$), but did not significantly differ with diet except at d 14 (HC, 58 ± 18 ; MF, 94 ± 26 ; $p < 0.05$). During the same period, serum IGF-I values increased approximately 4-fold, from 47 ± 6 ng/mL to 151 ± 28 ng/mL, but did not differ with diet at any age. Serum insulin levels were significantly elevated in HC animals on d 12, 14, and 24 (Fig. 6).

DISCUSSION

This study was designed to investigate the effects of an HC formula on rat islet ontogeny in the immediate postnatal period. The model of delivering HC nutrition *via* an intragastric cannula used here in early postnatal life has been shown

previously to result in obesity by 60–100 d of age, probably secondary to hyperinsulinemia (4–7). This model differs from those of diet-induced obesity in which overnourishment was used to induce obesity, often associated with hyperinsulinemia and glucose intolerance (18–21).

We and others have shown that a developmental window exists in the ontogeny of the rat pancreas between 1 and 2 wk after birth in which islets are depleted of β cells by a transient wave of apoptosis, and β cells are replaced by neogenesis from the pancreatic ductal tissue (11, 12). The present study suggests that this ontogeny is substantially altered by feeding the animals an HC formula. We previously found that at 12 d of age, animals had a slightly greater mean islet diameter and more insulin-positive cells per pancreas (5). A more detailed analysis now shows that within 8 d of commencing HC diet, at 12 d age there is a fundamental redistribution of islets with increased numbers of, on average, smaller islets per unit pancreas area. The different interpretation in the present study is because of the consideration of all islets, rather than larger, mature islets alone (5). These differences are maintained at least until d 24. The smaller islets in HC animals showed a selective reduction in their proportional content of α cells, leading to an increased β -cell to α -cell proportional ratio. Hence, given that islet number is increased, it is likely that β -cell mass per pancreas is greater in HC rats despite a smaller mean islet size, as we previously reported (5). Circulating insulin levels were increased on d 12, 14, and 24. In separate studies we showed recently that there was an increase in glucose sensitivity of insulin release for islets isolated from HC animals, associated with an increase in the activities of glucokinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate dehydrogenase complex, and an increased abundance of the glucose transporter, GLUT 2 (22). The likely increase in islet generation coupled with the greater loss of islet cells by apoptosis after HC would suggest a greater population of relatively immature β cells, which might be expected to have a lesser responsiveness to glucose. This supports a change in the functional phenotype of β cells that develop in the HC environment.

We hypothesized that the changes seen in islet morphology were caused by alterations in cell proliferation rate or developmental apoptosis in the endocrine pancreas and within precursor cell populations in the surrounding ductal epithelium. PCNA is an auxiliary protein of DNA polymerase, which begins to accumulate in the nucleus during G1 of the cell cycle, is most abundant in S phase, and declines during G2/M (23).

Figure 1. Dual chromogen immunohistochemistry for PCNA (brown) and either insulin (*A* and *B*, blue), or glucagon (*C* and *D*, blue) in islets within sections of rat pancreas from animals at 14 d postnatal age. Animals either continued to be suckled by the mothers (*A* and *C*) or were fed an HC formula by gastrotomy (*B* and *D*). Staining for PCNA is seen within islet (*i*) cells (arrows), within the ductal epithelium (*d*), and within acinar tissue (*a*). Magnification bar 10 μ m.

As reported previously (24), we found that most islet cells in the neonatal rat pancreas containing immunoreactive PCNA were β cells. Despite the mean islet size being smaller, the proliferative rate of islet cells was higher in islets from HC animals at d 12 and 18. This was mainly because of β -cell proliferation as the relative proportion of α cells was reduced in HC-fed rats. However, the rate of islet cell apoptosis was also increased at every age examined in pancreata from HC

versus MF rats, suggesting that the net effect was to limit islet cell number, and thereby mean islet growth rate. A transient peak of islet cell apoptosis occurred at postnatal d 14–18, as shown previously (11). The timing of this event was not altered in HC rats, although the amplitude of the apoptosis was increased. It was difficult to identify from which endocrine cell population apoptotic cells were predominantly derived from because, by definition, they were condensed with little cyto-

Figure 2. Immunohistochemical localization of apoptotic cells (arrows) using molecular histochemistry in a pancreatic islet from an HC formula-fed rat of 14 d age. Apoptotic cells have condensed nuclei with little cytoplasm. Magnification bar 10 μ m.

Table 5. Changes with age in the percentage of cells demonstrating apoptosis in islets or ductal epithelium of the pancreas for animals receiving either HC diet or being fed by the mothers*

Age (d)	Islets		Ductal epithelium	
	HC	MF	HC	MF
6	9.2 $\pm$ 2.4 [†]	5.7 $\pm$ 1.2	6.6 $\pm$ 3.1	8.5 $\pm$ 3.1
12	8.9 $\pm$ 1.9 [†]	6.8 $\pm$ 2.1	7.6 $\pm$ 2.8	8.6 $\pm$ 3.2
14	13.0 $\pm$ 2.5 [‡]	9.1 $\pm$ 0.7	8.5 $\pm$ 3.6 [†]	11.5 $\pm$ 2.7
18	14.7 $\pm$ 2.4 [†]	12.1 $\pm$ 1.1	8.3 $\pm$ 3.6	10.9 $\pm$ 2.4
24	9.2 $\pm$ 1.1 [†]	7.9 $\pm$ 0.8	6.8 $\pm$ 2.1 [†]	11.9 $\pm$ 2.4

* Results represent mean $\pm$ SD ($n = 5$). [†] $p < 0.05$, [‡] $p < 0.01$ vs MF controls.

plasm and no longer stained for any hormone by immunocytochemistry. However, apoptotic cells were located in all regions of the islets and likely represented both α and β cells.

The increased number of smaller islets within HC rats suggests that the rate of endocrine cell neogenesis from the ductal epithelium may have been higher. This would be supported by a greater percentage of ductal epithelial cells showing immunoreactivity for PCNA in HC animals, with a lower incidence of apoptosis. In MF animals 8 to 12% of ductal epithelial cells showed evidence of apoptosis at any age, which suggests that substantial remodelling occurs in this tissue in early postnatal life. The changes in ductal and islet cell morphology that accompany an HC diet were not accompanied by similar changes in acinar tissue, in which the incidence of PCNA immunoreactivity and apoptosis were unaltered by diet. An increased β -cell neogenesis from ductal epithelium, rather than islet cell replication, was recently shown to be the major mechanism responsible for an increase in β -cell mass in rats after infusion of glucose to normal animals or those depleted of β cells by the administration of low-dose streptozotocin (25).

The IGFs are mitogens for islet endocrine cells in fetal and neonatal life (26–28). Exogenous IGFs have been shown to prevent apoptosis in a variety of cell types (29, 30), and we have shown previously that IGF-I and IGF-II can protect islet cells against apoptosis induced by cytotoxic cytokines (11).

Figure 3. Immunohistochemical localization of IGF-II in islets within sections of rat pancreas from animals at 12 d postnatal age. Animals either continued to be suckled by the mothers (A) or were fed an HC formula by gastrostomy (B). Staining for IGF-II is seen within islet (i) cells (arrows) and within the ductal epithelium (d). Magnification bar 10 μ m.

Figure 4. Quantification of IGF-II immunoreactivity (% cell number, mean $\pm$ SD) in (A) islets of Langerhans and (B) ductal epithelia from animals of 6–24 d age after MF (open bars) or administration of an HC formula by gastroscopy (closed bars). Results are derived from five animals for each age. * $p < 0.05$, ** $p < 0.01$, [†] $p < 0.001$ vs MF.

The transient increase in islet cell apoptosis in the neonatal rat is temporally associated with a rapid loss of expression of IGF-II mRNA from pancreatic islet cells (11, 13). We have shown that IGF-II transgenic mice have a dramatic increase in islet size at birth related to both an increased islet cell proliferation and a reduction in islet cell apoptosis (31). When we examined the pancreatic expression of IGF-II mRNA in HC

Figure 5. *A*, Northern blot hybridization of mRNAs for IGF-II for total RNA extracted from representative whole rat pancreata for animals of 6 or 12 d age after MF or administration of an HC formula by gastrostomy. Three major mRNA transcripts were seen of 7.4, 4.6, and 2.4 kb. The hybridization signal for IGF-II was reduced at both days for animals given HC formula relative to MF controls. The same filters were rehybridized with a cDNA for 18S rRNA to estimate the efficiency of loading and transfer of total RNA. mRNA transcript sizes are shown to the left. *B*, quantification of mRNA for IGF-II (mean $\pm$ SEM) from Northern blot hybridization, expressed as a ratio of the hybridization signals for the 7.4-kb mRNA transcripts, relative to the signal for 18S rRNA in the same sample, in pancreata from animals of 6–24 d age after MF (open bars) or administration of an HC formula by gastrostomy (closed bars). Results are derived from three separate hybridizations for each age, each using tissues from different animals. * $p < 0.05$ or better vs MF.

Figure 6. Changes in circulating insulin (ng/mL) (mean $\pm$ SD) in animals of 6–24 d age after MF (open bars) or administration of an HC formula by gastrostomy (closed bars). Results are derived from five animals for each age. * $p < 0.05$ vs MF.

rats, this was shown to decrease with postnatal age, as noted previously (13), but was further decreased at each age after HC feeding. We previously showed using *in situ* hybridization that the major site of IGF-II mRNA synthesis was the pancreatic islets (32). Immunohistochemistry confirmed that the proportional area of islet cells containing immunoreactive IGF-II decreased with age and was lower in HC animals. This association suggests that the mechanism of action by which an HC diet leads to altered mean islet size might include a reduction in IGF-II presence in islets, impairing islet cell survival. The finding that β -cell labeling with PCNA was increased at some ages in the face of increased apoptosis suggests an attempt to maintain islet size through increased β -cell replication, and that other locally acting growth factors may be altered in addition to IGF-II. These might include fibroblast growth factors 1 and 2, which we have shown to be expressed in islets at this time (33), or glucagon-like polypeptide 1-related molecules, which are potent β -cell mitogens (34).

Conversely, the presence of IGF-II immunoreactivity within the pancreatic ductal epithelium was increased in HC animals, and may contribute to the associated higher rate of cell replication and reduced apoptotic rate. The ability of the same HC diet to result in divergent changes in IGF-II peptide presence in different tissue compartments of the pancreas suggests that the relationship is probably not direct, and will involve additional paracrine trophic control elements. Endocrine cell neogenesis from pancreatic ductal epithelium is known to involve the expression of transcription factors including Pdx-1, hepatocyte nuclear factor-3 β , and BETA-2 (35). Pdx-1 expression is modulated by both glucose and FFA *in vitro* (36, 37). Although both IGF-I and IGF-II are present in the circulation in the neonatal rat, their levels did not differ between HC and control animals, suggesting that local changes in pancreatic expression are more relevant to islet ontogeny.

In summary, these experiments suggest that a long-term HC diet in early postnatal life induced fundamental changes in pancreatic islet ontogeny. These are likely to have long-lasting effects on glucose homeostasis and may contribute to the abnormal insulin secretion seen in these animals as adults (5).

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