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# Gestational high-fat programming impairs insulin release and reduces Pdx-1 and glucokinase immunoreactivity in neonatal Wistar rats

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**Abstract**

Hyperglycemia and compromised β-cell development were demonstrated in neonatal rats programmed with a gestational high-fat diet. The aim of this study was to determine whether these changes were attributed to impaired insulin release and altered immunoreactivity of Pdx-1, glucokinase (GK), and glucose transporter (GLUT)–2 in high-fat–programmed neonates. Fetuses were maintained, via maternal nutrition, on either a standard laboratory diet (control) or a high-fat diet throughout gestation (HFG). Pancreata from 1-day–old neonates were excised for islet isolation and the subsequent measurement of insulin release at 2.8, 6.5, 13, and 22 mmol/L glucose. Other pancreata were either snap frozen for quantitative polymerase chain reaction or formalin fixed for immunohistochemistry followed by image analysis. The HFG neonates had reduced insulin release at 13- and 22-mmol/L glucose concentrations. No significant differences were found in Pdx-1, GK, or GLUT-2 messenger RNA expression. In HFG neonates, immunoreactivity of both Pdx-1 and GK was significantly reduced, with a nonsignificant reduction in GLUT-2. Gestational high-fat programming impairs insulin release and reduces Pdx-1 and GK immunoreactivity.


## 1. Introduction

The developing fetus is plastic because of its capability of following many diverse routes and forms, with the intrauterine environment programming it along certain pathways to aid fetal survival and development [1]. *Developmental programming* is defined as a stimulus or insult in utero or in early postnatal life that induces long-lasting changes in progeny physiology and metabolism. Although developmental programming refers to long-term metabolic and physiologic effects, several of these changes are already evident as early as birth and weaning [2-9]. Furthermore, the related concept of developmental origins of health and disease proposes that maternal characteristics before and during pregnancy influence fetal survival, growth, size, and body composition and the function of various systems, with some of these effects already demonstrable at birth [1]. Maternal high-fat consumption during pregnancy and/or lactation leads to developmental programming [6-8,10,11]. High-fat programming is induced by maternal high–saturated-fat intake during defined periods of gestation and/or lactation and programs the physiology and metabolism of the offspring in early life [12].

Intrauterine nutrition may regulate β-cell differentiation in prenatal life [13]. Pdx-1 is believed to be one of the most important transcription factors involved in the regulation of β-cell development, differentiation, and function and has been reported to regulate the glucose transporter GLUT-2 [14] and insulin [15] genes. In the mature β-cell, glucose is first transported into the β-cell by GLUT-2 and is then phosphorylated to glucose-6-phosphate by glucokinase (GK) to initiate glycolysis. This ultimately leads to insulin secretion. A high-fat diet (HFD) is known to compromise glucose sensing and insulin signaling, evident by reduced expression of GLUT-2, GK, Pdx-1, and insulin after high-fat feeding or exposure to free fatty acids [16-19]. BDF1 mice fed an HFD develop obesity-dependent diabetes presumably caused by dysfunction of pancreatic islets for producing and/or secreting insulin [20]. Diabetes in HFD-fed BDF1 mice is partly based on impaired insulin secretion in response to glucose to compensate for insulin resistance characterized by

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reduced insulin content along with impaired insulin release by isolated pancreatic islets [20].

Previous studies, in rodents, have shown that a protein-deficient diet in utero results in low birth weights and reduces pancreatic islet size, islet vascularization, β-cell number, and insulin content [3,21]. We have recently shown that neonatal rats maintained on an HFD in utero were hyperglycemic and displayed compromised β-cell development demonstrated by their reduced β-cell volume and number [6]. This investigation sought to determine whether these adverse effects on β-cell development and function were attributed to impaired insulin release and reduced expression of Pdx-1, GK, and GLUT-2 after programming with a gestational HFD.

## 2. Methods

### 2.1. Study design

Animals were maintained as previously described [6]. Principles of laboratory animal care were adhered to, and ethical approval was obtained from the institution's ethics committee. Before mating, female Wistar rats were fed a control diet. After pregnancy was confirmed by the presence of vaginal plugs, female rats were assigned to either a standard laboratory diet (control) or an HFD for all 3 weeks of gestation. At birth, control neonates were generated from dams maintained on a standard laboratory diet; and HFG neonates, from dams fed an HFD throughout gestation. The standard laboratory diet (2.6 kcal/g; in pellet form) constituted 10% fat (as energy), 15% protein, and 75% carbohydrate and was obtained commercially. In contrast, the HFD (2.06 kcal/g) contained 40% fat (as energy), 14% protein, and 46% carbohydrate. The HFD (in patty form) was designed by dieticians to mimic human HFDs. The fat in the HFD was mainly derived from saturated animal fat.

### 2.2. Phenotype of HFG mothers and neonates

High-fat feeding induces hyperphagia in HFG mothers concomitant with an increase in body weight and hyperglycemia on days 7 and 14 of gestation and on the day of delivery, with no significant changes in circulating insulin concentrations [6]. Furthermore, HFG mothers consumed more food over the entire duration of pregnancy compared with control mothers [6]. The HFG neonates are hyperglycemic, displaying reduced β-cell volume and numbers, with augmented α-cell volume, numbers, and sizes [6]. The compromised β-cell development in HFG neonates is suggested to be attributed to the HFD either by inhibiting β-cell replication pathways or by inducing apoptosis [6]. In addition, the HFD appeared to have a stimulatory effect on α-cell development [6]. However, HFG neonates have normal birth weights, with no significant changes in insulinemia or β-cell size [6].

### 2.3. Islet isolation

For the islet studies, 3 dams were used for each group. Neonates were fasted for 3 hours and decapitated, and the carcasses were dipped in 70% ethanol before the pancreata were excised. Pancreata (n = 30-39 per group) of each litter of neonates per dam were pooled. For islet isolation, 1 mg/mL collagenase XI (Sigma-Aldrich, St Louis, MO) was added for digestion in a water bath at 37°C for 20 minutes. Hanks buffered salt solution (HBSS; Lonza, Walkersville, MD) and fetal calf serum (Gibco, Invitrogen, Paisley, United Kingdom) were added to the collagenase to stop the reaction. After washing and centrifugation with HBSS, the pellet was resuspended in 10 mL Histopaque 1.119 g/L (Sigma) and layered with HBSS. After centrifugation at 4°C for 20 minutes at 2000 rpm, the top interphase was pipetted into a new tube and washed in HBSS, followed by the addition of Dulbecco modified Eagle medium and incubation for 45 to 60 minutes.

For determination of glucose-stimulated insulin release, islets were carefully isolated with a pipette under a compact inverted microscope (Olympus CKX31, Hertfordshire, United Kingdom). A total of 10 islets of approximately equal size were isolated per group for each glucose concentration. The experiments for insulin secretion were performed in quadruplicate (4 wells of 10 islets of similar size were used for each glucose concentration; ie, n = 40 islets per glucose concentration per group). All of the freshly isolated islets were first preincubated for 1 hour at 37°C in an oven in 2.8-mmol/L glucose medium. Afterward, the islets were incubated, in quadruplicate, for 2 hours at 37°C in media (1 g/L Dulbecco modified Eagle medium + 10% fetal calf serum) at the following glucose concentrations: 2.8 mmol/L, to evaluate non–glucose-stimulated insulin release; 6.5 mmol/L, which is the basal glucose concentration in rats; 13 mmol/L, which is the upper physiologic glucose level in rats; and 22 mmol/L, which is the maximum stimulatory concentration. An aliquot of 200 to 250 μL of medium was removed for insulin radioimmunoassay (Linco Research, St Charles, MO).

### 2.4. Quantitative LightCycler polymerase chain reaction (Roche Diagnostics, Mannheim, Germany)

RNA was isolated from neonatal pancreata (n = 10 per group), and complementary DNA was synthesized for reverse transcriptase polymerase chain reaction (PCR) experiments [7]. Primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were produced and purified by polyacrylamide gel electrophoresis/high-performance liquid chromatography (IDT, Coralville, IA). Gene expression was determined for Pdx-1, GK, GLUT-2, and porphobilinogen deaminase (PBGD), a housekeeping gene recommended by the manufacturer. The primer sequences were as follows:

Pdx-1 forward: 5′-GCT GGA GCT GGA GAA GGA AT-3′
Pdx-1 reverse: 5′-CGT TGT CCC GCT ACT ACG TT-3′
GK forward: 5′-CAG TGG AGC GTG AAG ACA AA-3′

GK reverse: 5′-AGG GAA GGA GAA GGT GAA GC-3′
GLUT-2 forward: 5′-CAG TGG AGC GTG AAG ACA AA-3′
GLUT-2 reverse: 5′-AGG GAA GGA GAA GGT GAA GC-3′
PBGD forward: 5′-GCA TAC AGA CCG ACA CTG TGG-3′
PBGD reverse: 5′-CTC TGG CAA GGT TTC CAG GG-3′.

The PCR products were detected using SYBR green (Stratagene, Amsterdam, the Netherlands). Relative quantification of the genes was determined using the RelQuant software (Roche Diagnostics, Mannheim, Germany). Coefficient files and standard curves were generated for gene analysis. Porphobilinogen deaminase was used as a reference for the expression level of the target genes (Pdx-1, GK, and GLUT-2). The second derivative maximum method was used to determine the crossing points. The relative amount of the target gene was calculated as a ratio of the target concentration to the reference concentration (PBGD). Afterward, this ratio of target to reference of the sample was divided by the target to reference ratio of the calibrator. The final result was expressed as a normalized ratio. The messenger RNA (mRNA) expression of the control group was taken as 1, with sample readings greater than 1 indicative of overexpression and those less than 1 representing underexpression of a particular gene. In Table 1, mRNA levels were expressed as normalized units per total pancreas.

### 2.5. Tissue preparation and sectioning

In other 1-day–old neonatal rats, pancreata were excised and placed in 4% paraformaldehyde overnight and processed in an automated tissue processor (Citadel, Shandon, Cheshire, United Kingdom) through ascending concentrations of ethanol from 70% to 100%, followed by xylene. The tissue was embedded in paraffin wax (Paraplast Plus; Monoject Scientific, St Louis, MO). Sections, 4 $\mu$m thick, were cut on a rotary microtome and mounted on slides coated with 3-aminopropyltriethoxysilane.

### 2.6. Immunohistochemistry and image analysis

Pancreatic sections (n = 6 per group) were immunostained separately with 1:1500 Pdx-1 antibody (a kind gift from Prof C Wright, Department of Cell Biology, Vanderbilt University Medical Center, Nashville, TN), 1:1000 GK antibody (kindly donated by Dr H Vertigan, CVGI Discovery Department, AstraZeneca, Cheshire, United Kingdom), or 1:100 GLUT-2 antibody (WAK-Chemie, Bad Soden, Germany) [7]. Immunolabeled sections were viewed, and micrographs were captured for morphometric islet analysis on a Zeiss Axioskop2 light microscope (Carl Zeiss, Jena, Germany) linked to a Zeiss Axiocam digital camera system (Carl Zeiss). The image analysis system uses specific macros to semiquantitatively measure protein levels, which are optimally configured for measuring each target protein. All of the pancreas sections were scanned for the specific protein (Pdx-1, GK, or GLUT-2) immunoreactivity; and the levels of immunolabeled protein in the control were taken to equal 1, with immunoreactivity of HFG neonates expressed as a ratio of the control levels. In Table 2, immunoreactivity was expressed as stained area (Pdx-1, GK, or GLUT-2) per total islet area.

Table 1
Pdx-1, GK, and GLUT-2 mRNA expression in neonatal offspring from dams fed an HFD during gestation

| | Pdx-1 mRNA | GK mRNA | GLUT-2 mRNA |
|---|---|---|---|
| Control | 1 ± 0.58 | 1 ± 0.10 | 1 ± 0.59 |
| HFG | 2.81 ± 0.67 | 0.98 ± 0.07 | 1.48 ± 0.70 |

Neonatal offspring were obtained from dams fed an HFD throughout gestation (HFG). Neonates from dams maintained on a standard laboratory diet throughout gestation represented the control group. Data are means ± SEM. n = 10 neonates per group. The mRNA levels are expressed as normalized units per pancreas.

Table 2
Immunoreactivity for Pdx-1, GK, and GLUT-2 in neonatal offspring from dams fed an HFD during gestation

| | Pdx-1 immunoreactivity | GK immunoreactivity | GLUT-2 immunoreactivity |
|---|---|---|---|
| Control | 1 ± 0.09 | 1 ± 0.07 | 1 ± 0.22 |
| HFG | 0.69 ± 0.05* | 0.65 ± 0.16* | 0.56 ± 0.11 |

Neonatal offspring were obtained from dams fed an HFD throughout gestation (HFG). Neonates from dams maintained on a standard laboratory diet throughout gestation represented the control group. Data are means ± SEM. n = 6 neonates per group. Immunoreactivity is expressed as stained area (Pdx-1, GK, or GLUT-2) per total islet area.

* $P < .05$.

### 2.7. Statistical analysis

The data of the HFG group were compared with the control data and reported as means ± SEM. Comparisons between the 2 groups were analyzed using Student $t$ tests. However, for analysis of the insulin release data, a 1-way analysis of variance was performed followed by Bonferroni multiple comparisons to determine differences between the groups at the varying glucose concentrations. Significance was established at $P < .05$.

## 3. Results

### 3.1. Insulin release

In control neonates, insulin release was higher at 22 mmol/L glucose compared with insulin release at 2.8 mmol/L, which is the nonstimulatory glucose concentration (Fig. 1). As expected, the greatest insulin release from control neonatal islets occurred at the maximum stimulatory glucose concentration of 22 mmol/L (Fig. 1). This is indicative of a normal insulin secretory response to glucose. Furthermore, there were no differences in insulin release at 6.5 and 13 mmol/L glucose

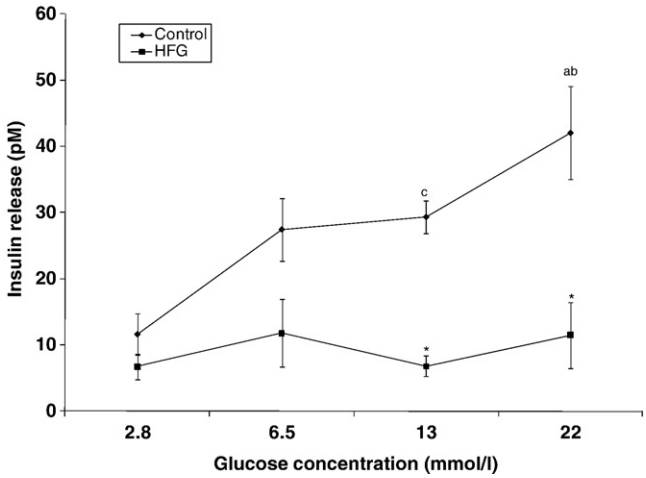


Fig. 1. Insulin release at 2.8-, 6.5-, 13-, and 22-mmol/L glucose concentration in HFG neonates. Neonatal offspring were obtained from dams fed an HFD throughout gestation (HFG). Neonates from dams maintained on a standard laboratory diet throughout gestation represented the control group. Data are means ± SEM. n = 10 islets per group at each glucose concentration. [*]HFG vs control at 13 and 22 mmol/L. [a]Control at 22 mmol/L vs control at 2.8 mmol/L. [b]Control at 22 mmol/L vs HFG at 2.8, 6.5, and 13 mmol/L. [c]Control at 13 mmol/L vs HFG at 2.8 mmol/L. For [*], [a], [b], and [c], $P < .05$.

compared with either 2.8 or 22 mmol/L glucose. In contrast, in HFG neonates, the insulin release at the higher glucose concentrations did not differ compared with levels at 2.8 mmol/L (Fig. 1). Importantly, when compared with the control, HFG neonates had significantly reduced insulin release at 13 and 22 mmol/L glucose (Fig. 1).

Insulin release from control islets at 22 mmol/L glucose was significantly higher when compared with insulin release from HFG islets at all glucose concentrations, namely, 2.8, 6.5, 13, and 22 mmol/L (Fig. 1). In addition, insulin release from control islets at 13 mmol/L glucose was elevated compared with insulin release from HFG islets at 2.8 mmol/L (Fig. 1).

### *3.2. Pdx-1, GK, and GLUT-2 mRNA expression*

No significant differences were found in mRNA expression of Pdx-1, GK, or GLUT-2 (Table 1). In the HFG neonates, there was a trend for approximately 2.8-fold and 1.5-fold increases in Pdx-1 and GLUT-2 mRNA expression, respectively, whereas GK mRNA expression remained unchanged.

### *3.3. Pdx-1, GK, and GLUT-2 immunoreactivity*

Pdx-1 and GK immunoreactivity was reduced in HFG neonates (Table 2). A nonsignificant reduction in GLUT-2 immunoreactivity was found in HFG neonates (Table 2).

## 4. Discussion

Adult β-cells show robust, biphasic insulin secretion [22,23] and consistently respond to varying glucose concentrations, secreting different amounts of insulin [24]. The optimal stimulatory glucose level for the assessment of insulin-releasing function is between 16.5 and 19.3 mmol/L in adult rats [25]. Fetal and neonatal islets have reduced insulin secretory capacity compared with adult islets, as the early postnatal period also represents a critical period for islet development. In the study presented here, control neonates showed a normal insulin secretory response to glucose stimulation, a function that was absent in HFG neonates—HFG neonates released reduced insulin at stimulatory 13- and 22-mmol/L glucose concentrations. Chronic hyperglycemia adversely affects insulin secretion [26] and decreases β-cell mass by inducing apoptosis [27,28]. As HFG neonates display reduced β-cell volume and numbers [6] and immunoreactivity of both Pdx-1 and GK is reduced, the functional capacity of the HFG β-cells is compromised. These effects, concomitant with the reduced insulin release from HFG islets at stimulatory glucose concentrations, indicate that programming with an HFD during gestation impairs β-cell function. The β-cell compensation to insulin resistance can temporarily occur by increasing insulin secretion when β-cell mass is reduced [29]. However, the increase in insulin secretion strains replication-defective β-cells and accelerates β-cell failure [29]. In HFG neonates, it appears that β-cell failure may ultimately ensue because of their reduced β-cell volume and impaired insulin secretion, placing these offspring in a compromised metabolic and physiologic state.

In the hyperglycemic HFG neonates [6], immunoreactivity of both Pdx-1 and GK was reduced. Pdx-1 regulates the insulin gene [15] and is required for normal β-cell development. Gestational high-fat programming reduced Pdx-1 immunoreactivity in the neonates. The reduction of this major β-cell transcription factor could adversely affect insulin gene expression and, in addition, appears to have contributed to the reduction in β-cell volume and number in HFG neonates. A study using a clonal β-cell line to study the impact of glucose levels on Pdx-1 expression in pancreatic β-cells showed that chronic hyperglycemia inhibited Pdx-1 mRNA and protein expression [30]. A gestational HFD induced maternal hyperglycemia and programmed neonatal offspring to be hyperglycemic, with no significant changes in insulinemia in both the mothers and neonates [6]. Essentially, these hyperglycemic HFG neonates were exposed to high circulating glucose concentrations in utero [6], suggesting that, apart from the HFD, exposure of the fetuses to maternal hyperglycemia may also play a role in the reduced Pdx-1 protein expression and subsequent impaired β-cell development. Lowered Pdx-1 expression or activity resulting in impaired expression of both GLUT-2 and insulin could cause hyperglycemia that may progress to type 2 diabetes mellitus [31].

The glucolipotoxicity theory states that simultaneous elevation of glucose and lipids results in intracellular accumulation of lipids and lipid metabolites, which are ultimately detrimental to β-cell function and survival [32]. It

is likely that the HFG neonates may be exposed to elevated circulating free fatty acids derived from metabolism of the HFD. The potential dual exposure of the fetus to simultaneous elevated circulating glucose (because of maternal hyperglycemia) and free fatty acid concentrations may therefore implicate glucolipotoxicity in the impairment of its β-cell function.

Glucokinase is considered the rate-limiting step in glycolysis and is thus critical in regulating insulin secretion from β-cells [33,34]. Therefore, the possibility exists that reduced GK immunoreactivity may contribute to the hyperglycemic state of these neonates. This could be verified by assays measuring GK activity.

The HFG neonates were studied at weaning (3 weeks of age); after birth, the mothers were either switched to a standard laboratory diet (to yield HFG weanlings) or continued on the HFD (to yield HFGL weanlings) [7]. Pdx-1, GK, and GLUT-2 mRNA expression was not significantly altered in HFG neonates similar to HFG weanlings [7], whereas Pdx-1 mRNA was overexpressed (11.3 fold) and both GK and GLUT-2 mRNA was underexpressed in HFGL weanlings [7]. This illustrates that continuous high-fat feeding over developmental periods (viz, gestation and lactation) alters the expression of the key β-cell gene, Pdx-1, and the glucose-sensing genes, GLUT-2 and GK. In addition, HFD exposure, throughout gestation only, is not sufficient to induce altered Pdx-1, GK, and GLUT-2 gene expression in both HFG neonates and HFG weanlings, suggesting that this single period of exposure may be too brief to induce changes in gene expression.

Furthermore, HFG neonates had reduced Pdx-1 and GK immunoreactivity, whereas GLUT-2 immunoreactivity was not significantly reduced. However, in both HFG and HFGL weanlings, Pdx-1 immunoreactivity was normal, whereas GK immunoreactivity was normal in HFG weanlings but reduced in HFGL weanlings; and GLUT-2 immunoreactivity was increased (4-fold) in both groups [7]. Because both Pdx-1 and GK immunoreactivity normalized in HFG weanlings, improved diet (switching from an HFD to a standard laboratory or control diet) during the lactational period appears to ameliorate both Pdx-1 and GK immunoreactivity. The reduced GK immunoreactivity persisted in HFGL weanlings, demonstrating that continuous high-fat feeding over both critical developmental periods of gestation and lactation results in a reduction of GK immunoreactivity. In both HFG and HFGL weanlings, GLUT-2 immunoreactivity was increased [7] (compared with a nonsignificant reduction in HFG neonates), which appears to be a compensatory mechanism to the HFD insult.

Interestingly, overexpression of Pdx-1 mRNA evident in HFG neonates persists in HFGL weanlings. In HFG neonates, the 2.8-fold overexpression of Pdx-1 mRNA is not sufficient to maintain normal Pdx-1 immunoreactivity. In contrast, in HFGL weanlings, the 11.3-fold increase in Pdx-1 mRNA expression seems to play a role in the normalization of Pdx-1 immunoreactivity. It appears that substantial overexpression of Pdx-1 mRNA is required to maintain normal Pdx-1 protein profiles, with a threshold approximately between 3- and 11-fold.

There was a lack of correlation for mRNA and protein (immunoreactivity) expression for Pdx-1 evident by a 2.8-fold increase in Pdx-1 mRNA expression (albeit nonsignificant) and a significant reduction in Pdx-1 immunoreactivity in HFG neonates. Protein concentrations correlate with corresponding mRNA levels by only 20% to 40%, making mRNA abundances weak surrogates for the corresponding protein concentration [35,36]. Biological reasons for poor correlation include posttranscriptional and posttranslational modifications and varying protein half-lives [37,38]. Furthermore, gene expression analysis (such as quantitative LightCycler PCR) is far more sensitive than immunohistochemistry and image analysis; genes may also be expressed at low levels that are not adequate for translated protein expression [39]. The increased Pdx-1 mRNA expression may be interpreted as an attempt to compensate for the reduced β-cell function [40].

## 5. Conclusions

High-fat programming during gestation impaired insulin release in neonatal rats. Furthermore, these neonates display reduced Pdx-1 and GK immunoreactivity. This suggests that gestational high-fat programming reduces insulin release by a mechanism that involves reduced Pdx-1 and GK immunoreactivity.

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