Neonatal Exendin-4 Prevents the Development of Diabetes in the Intrauterine Growth Retarded Rat

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Uteroplacental insufficiency resulting in fetal growth retardation is a common complication of pregnancy and a significant cause of perinatal morbidity and mortality. Epidemiological studies show an increased incidence of type 2 diabetes in humans who were growth retarded at birth. The mechanisms by which an abnormal intrauterine milieu leads to the development of diabetes in adulthood are not known. Therefore, a rat model of uteroplacental insufficiency was developed; intrauterine growth-retarded (IUGR) rats develop diabetes with a phenotype similar to that observed in the human with type 2 diabetes. We show here that administration of a pancreatic β-cell trophic factor, exendin-4 (Ex-4), during the prediabetic neonatal period dramatically prevents the development of diabetes in this model. This occurs because neonatal Ex-4 prevents the progressive reduction in insulin-producing β-cell mass that is observed in IUGR rats over time. Expression of PDX, a critical regulator of pancreas development and islet differentiation, is restored to normal levels, and islet β-cell proliferation rates are normalized by the neonatal Ex-4 treatment. These results indicate that exposure to Ex-4 in the newborn period reverses the adverse consequences of fetal programming and prevents the development of diabetes in adulthood. Diabetes 52: 734–740, 2003

Epidiological studies in a large number of populations worldwide have revealed strong statistical links between poor fetal growth and the subsequent development of type 2 diabetes in adulthood (1–6). Subjects with low birth weight have defects in insulin secretion (7–9) as well as in insulin action (10,11). Intrauterine growth restriction also results in a reduced population of pancreatic β-cells in the human (12). Uteroplacental insufficiency limits the availability of substrates, growth factors, and hormones to the fetus and retards growth during gestation. This abnormal intrauterine milieu modifies gene expression in pluripotential and terminally differentiated cells resulting in permanent structural and functional changes in key organs such as the pancreas, liver, and muscle (13–17). We have developed a rat model of uteroplacental insufficiency, hereafter designated as IUGR for intra-uterine growth retardation induced by bilateral uterine artery ligation at 19 days of gestation (term is 22 days). The unique feature of this model is its ability to induce diabetes in adult animals at ~15–26 weeks of age with underlying β-cell secretory defects and insulin resistance, the salient features of most forms of type 2 diabetes in the human (17). β-Cell mass is normal during the first few weeks of life in IUGR rats; however, by 7 weeks of age, β-cell mass is reduced compared with controls. Most importantly, the progressive decline in β-cell mass occurs weeks before the onset of hyperglycemia. Whereas insulin resistance is a critical component of human type 2 diabetes, it is the failure of β-cell function and growth that determines progression to the diabetic phenotype (18). Thus, efforts to prevent the reduction in β-cell mass associated with diabetes could potentially prevent the development of the disease.

During the newborn period there is a high rate of replication, neogenesis, and apoptosis resulting in extensive remodeling of the endocrine pancreas (19). This appears to be followed by a second wave of neogenesis around the time of weaning. After weaning, levels of neogenesis and replication fall to very low levels but do continue throughout life (20). Therefore, the fetal and neonatal period represent a critical window of opportunity for therapies designed to enhance β-cell mass.

The incretin hormone glucagon-like peptide-1 (GLP-1) promotes the expansion of pancreatic β-cell mass by stimulating neogenesis as well as proliferation of existing β-cells (21–26). Administration of the long-acting GLP-1 analog Exendin-4 (Ex-4) during regeneration after 90% partial pancreatectomy (Ppx) in rats results in a sustained improvement in glucose homeostasis associated with a 40% increase in β-cell mass due to increases in both neogenesis and replication (27). Further, chronic treatment of adult diabetic mice with either GLP-1 or Ex-4 also improves glucose tolerance, increases islet size, and stimulates pancreatic duodenal homeobox (PDX) protein expression in the pancreas (28). These studies suggest that one of the mechanisms by which Ex-4 stimulates β-cell development may be through its action on PDX.

PDX is a pancreatic homeoprotein that is critical for the early development of both the endocrine and exocrine pancreas, and it mediates glucose-responsive stimulation of insulin gene transcription (29). A role for PDX in adult islet neogenesis is suggested by the marked up-regulation
of PDX in the ductal epithelium during regeneration after 90% Ppx in rats (30). Transgenic overexpression of PDX in the IRS2 \textsuperscript{-/-} knockout mouse rescues the \textbeta-cell mass defect and prevents the development of diabetes (31). In the rat IUGR model, PDX mRNA expression levels are already reduced in the fetus and continue to decline progressively with age (32).

Thus, GLP-1 and its analogs are promising agents for the treatment of diabetes, both because of their glucose-dependent insulinotropic effects as well as their exciting potential to regulate PDX expression and expand the mass of insulin-producing \textbeta-cells. In this study, we treated IUGR rats with Ex-4 during the early postnatal period and discovered that a brief course of Ex-4 completely prevents the development of adult-onset diabetes in this model.

**RESULTS**

**Metabolic parameters.** IUGR rats were treated with Ex-4 on postnatal days 1–6 (Fig. 1). The dose of Ex-4 (1 nmol \textcdot kg body wt \textsuperscript{-1 \cdot day} \textsuperscript{-1}) was previously demonstrated to augment \textbeta-cell regeneration in the 90% Ppx rat (27). Weights, plasma glucose, and insulin levels were determined at the beginning and end of Ex-4 treatment. Glucose and insulin concentrations did not vary among the four treatment groups early in life. Weights of the IUGR vehicle-treated and IUGR Ex-4–treated animals were significantly lower than those of control vehicle and control Ex-4 rats throughout the treatment period (days 1–6 of life) (data not shown). As expected, IUGR vehicle rats remained significantly lighter than control vehicle rats at 2 weeks (Table 1). Neonatal Ex-4 treatment significantly decreased weight in both IUGR and control pups at 2 weeks. This effect persisted into adulthood. As previously reported, IUGR rats are significantly heavier than control rats by 3 months of age (17). Further, neonatal Ex-4 reduced body weight in both control and IUGR rats.

**Glucose homeostasis and prevention of adult-onset diabetes in Ex-4–treated IUGR rats.** We have previously determined that glucose tolerance is impaired in IUGR rat pups and that they show a progressive loss in the ability to handle a glucose load as they age (17). To determine whether Ex-4 treatment improves glucose tol-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>2 weeks (g) (n = 9)</th>
<th>3 months (g) (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vehicle</td>
<td>27.7 ± 0.3</td>
<td>331.7 ± 7.0</td>
</tr>
<tr>
<td>Control Ex-4</td>
<td>22.2 ± 0.6*</td>
<td>305.3 ± 12.7*</td>
</tr>
<tr>
<td>IUGR Ex-4</td>
<td>13.8 ± 0.7†</td>
<td>311.0 ± 4.0†</td>
</tr>
<tr>
<td>IUGR vehicle</td>
<td>17.2 ± 0.7‡</td>
<td>351.7 ± 26.2‡</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 control Ex-4 vs. control vehicle; †P < 0.05 IUGR Ex-4 vs. IUGR vehicle; ‡P < 0.05 control vehicle vs. IUGR vehicle.

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erance in IUGR animals, intraperitoneal glucose tolerance testing was performed in nonfasted pups and after an overnight fast in adult animals. At day 14, Ex-4 treatment improved glucose tolerance in IUGR rats such that there was no significant difference when compared with control rats (Fig. 2A). Ex-4 normalization of impaired glucose tolerance in IUGR rats was maintained at 7 weeks of age (Fig. 2B). When analyzed as area under the curve, Ex-4–treated IUGR animals had significantly improved glycemic excursion compared with IUGR vehicle-treated animals at 2 weeks (231.56 ± 23.28 vs. 282.25 ± 7.19 mg/dl, *P < 0.05 for IUGR Ex-4 vs. IUGR vehicle rats, respectively) and 7 weeks (282.17 ± 14.94 vs. 355 ± 12.65 mg/dl, *P < 0.05 for IUGR Ex-4 vs. IUGR vehicle rats, respectively).

At 3 months of age, vehicle-treated IUGR rats were already diabetic (fasting glucose 332 ± 45 mg/dl), whereas Ex-4–treated IUGR rats had normal glucose tolerance indistinguishable from vehicle– and Ex-4–treated control rats (Fig. 2C; Table 2). At 8 months of age, IUGR vehicle–treated rats were overtly diabetic (fasting glucose 425 ± 10 mg/dl), with two deaths, yet Ex-4 IUGR rats remained normoglycemic, as demonstrated by normal fasting glucose levels (Table 2). At 18 months of age, Ex-4–treated IUGR rats remained normoglycemic, and all vehicle–treated IUGR rats had died.

**Maintenance of normal β-cell mass and normalization of β-cell replication rates.** IUGR rats manifest a progressive decline in the mass of insulin-producing pancreatic β-cells that becomes significantly different than that of control rats by 7 weeks of age. This decline in β-cell mass occurs weeks before the onset of hyperglycemia and is not associated with increased β-cell apoptosis. To examine β-cell dynamics in the Ex-4–treated IUGR rat, we performed point-counting morphometry. At 2 weeks of age, both vehicle– and Ex-4–treated IUGR rats possessed a normal mass of β-cells (Fig. 3A). By 3 months, when vehicle-treated IUGR rats were diabetic, β-cell mass had declined by ~80% compared with vehicle–treated control rats (1.73 ± 0.84 vs. 9.33 ± 0.54 mg, *P = 0.006) (Fig. 3B and C). In contrast, Ex-4–treated IUGR rats had a normal mass of β-cells (10.68 ± 1.47 mg, *P = 0.0017 for Ex-4 IUGR vs. vehicle IUGR rats).

β-Cell mass reflects the balance among rates of β-cell replication, neogenesis from β-cell precursors, and cell death due to apoptosis. To determine which mechanism is responsible for β-cell mass loss in IUGR rats, we assessed β-cell replication and apoptosis rates. As previously reported, at postnatal day (PD) 14, IUGR rats exhibited reduced β-cell proliferation (1.69 ± 0.21 vs. 2.54 ± 0.19%, *P = 0.016 for vehicle IUGR vs. vehicle control rats). Neonatal Ex-4 treatment normalized the rate of β-cell proliferation (3.29 ± 0.33%, *P = 0.005 for vehicle IUGR vs. Ex-4 IUGR rats; *P = NS for Ex-4 IUGR vs. vehicle control rats) (Fig. 4). Interestingly, β-cell proliferation in the Ex-4 control group was not elevated. A similar observation has been reported in rats treated with Ex-4 after 90% partial pancreatectomy, in which the already elevated β-cell replication rate was not further stimulated by Ex-4 despite the significant stimulation observed in Sham-operated control rats (27). This may indicate that β-cell proliferation rates are already maximal during the neonatal period. In contrast, rates of apoptosis at postnatal day 14 were unaffected by neonatal Ex-4 treatment (vehicle control 2.16 ± 0.24, Ex-4 control 0.21 ± 0.05 vs. control vehicle, control Ex-4, and IUGR Ex-4).

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>3 months</th>
<th>8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vehicle</td>
<td>4</td>
<td>126 ± 13</td>
<td>156 ± 20</td>
</tr>
<tr>
<td>Control Ex-4</td>
<td>4</td>
<td>139 ± 12</td>
<td>148 ± 24</td>
</tr>
<tr>
<td>IUGR Ex-4</td>
<td>5</td>
<td>115 ± 15</td>
<td>149 ± 18</td>
</tr>
<tr>
<td>IUGR vehicle</td>
<td>4</td>
<td>332 ± 45*</td>
<td>425 ± 10*</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. control vehicle, control Ex-4, and IUGR Ex-4.
2.57 ± 0.84, vehicle IUGR 1.95 ± 0.55, and Ex-4 IUGR 2.77 ± 0.87% of β-cell nuclei; P = NS). Restoration of Pdx-1 expression. Pdx-1 plays critical dual roles in islet β-cell development and differentiation. Because IUGR rats have markedly reduced Pdx-1 expression and Ex-4 has previously been shown to regulate Pdx-1 expression, we sought to determine whether Ex-4 treatment of newborn IUGR rats would enhance Pdx-1 expression. At 14 days of age, Pdx-1 mRNA levels were reduced by 60% in IUGR vehicle-treated rats (Fig. 5A). It is important to note that β-cell mass remains normal at 14 days in the IUGR rat (Fig. 4A), indicating that the reduction in Pdx-1 mRNA level at this age is not due to decreased β-cell mass. Neonatal Ex-4 treatment led to a restoration of Pdx-1 mRNA levels in IUGR rats at 14 days, an effect that persisted at 3 months (Fig. 5A and B).

**DISCUSSION**

The major finding of our study was that a short treatment course of the GLP-1 analog, Exendin-4, in the newborn period completely prevented the development of diabetes in the IUGR rat. The effect of Ex-4 on glucose homeostasis was permanent, with a resultant increase in the life span of IUGR animals. Interestingly, a previous study using Ex-4 treatment of streptozotocin (STZ)-induced diabetic newborn rats resulted in only a modest improvement in β-cell mass in adulthood and no improvement in glucose-to-insulin ratios. In addition, there was no improvement in glucose-stimulated insulin secretion and glucose homeostasis remained impaired (26). The contrasting results compared with the present study may be explained by the limitations of STZ-induced diabetes as a model for type 2 diabetes.

The early normalization of glucose tolerance was observed on PD 14, when IUGR β-cell mass was still normal, suggesting that Ex-4 exerts an effect on β-cell function of the IUGR rat that is independent of its effects on β-cell mass. GLP-1 and Ex-4 are well-established insulinotropic agents. GLP-1 stimulates insulin biosynthesis and glucose-dependent insulin secretion via increases in intracellular cAMP and calcium (21). Ex-4 stimulation of insulin secretion may be mediated through stimulation of Pdx-1 levels. Pdx-1 regulates the early development of both endocrine and exocrine pancreas and then the later differentiation of the β-cell. Recently, it has been demonstrated that a modest reduction in Pdx-1 impairs mitochondrial function and generation of NADH resulting in blunted glucose-stimulated insulin secretion (35). This is particularly relevant, as mitochondrial function is markedly abnormal in islets of IUGR animals (R.A.S., unpublished data). mRNA levels of Pdx-1 are reduced in the IUGR fetus, and expression progressively declined over time.
Similar to our previous studies of Ex-4 treatment of normal and diabetic mice, Ex-4 increased Pdx-1 expression in IUGR pancreas such that levels were similar to those of controls. Remarkably, only 6 days of treatment with this long acting GLP-1 analog led to a permanent recovery of Pdx-1 expression in IUGR animals. Between days 18 and 22 of gestation in the rat, β-cell mass increases nearly 14-fold. This rapid expansion of β-cells does not appear to be impaired in the IUGR fetus, as β-cell mass is normal at this age. Thus, despite a 50% reduction of Pdx-1 levels in IUGR pancreas, β-cell mass is not affected. This is consistent with the observation that milder reductions in Pdx-1 protein levels, as occurs in the Pdx-1+/− mouse, allow for the development of a normal mass of β-cells.

Glucose, amino acids, and oxygen levels are markedly reduced in the IUGR fetus and may contribute to the reduction in Pdx-1 levels. Glucose appears to regulate Pdx-1 at several levels, including transactivation (8), phosphorylation (37), and subcellular distribution (38,39). Pdx-1 autoregulates its own promoter, suggesting that reductions in circulating glucose could impair Pdx-1 gene transcription via an autoregulatory effect on Pdx-1 function (40). Most interestingly, the Pdx-1 promoter transcriptional regulators, USF, Sp1, Sp3, and HNF-1α are also regulated by nutrient availability (40–43). Thus, it is possible that Ex-4 treatment increases Pdx-1 mRNA expression in IUGR rats via one of these nutritionally sensitive upstream transcriptional regulators.

In addition to a life-long normalization of glucose tolerance, we observed a complete rescue of the progressive decline in β-cell mass that is normally observed in IUGR rats. The brief period of neonatal Ex-4 normalized β-cell replication rate in IUGR animals measured at PD 14. In the normal rat, replication of existing β-cells and formation of new β-cells are substantially greater during this period than at any other time in postnatal life. Therefore, even a modest reduction in neonatal β-cell proliferation rates will result in a long-term reduction in β-cell mass. It is likely that increased neogenesis from islet progenitor cells also contributes to the maintenance of β-cell mass in Ex-4–treated IUGR rats (D.A.S. and R.A.S., unpublished data).

Recent studies have demonstrated that Ex-4 exerts anti-apoptotic actions on the β-cell in various animal models of diabetes (24,44,45). Apoptosis plays an important role in pancreatic remodeling during the juvenile period. Similar to other laboratories, we also observed a low rate of apoptosis at this age, and neonatal Ex-4 did not decrease this rate further. Consistent with our previous observations that apoptosis does not play a major role in the decline of β-cell mass in IUGR animals,
apoptosis was similar in vehicle-treated control and IUGR rats. Thus, in the IUGR model, the effect of Ex-4 to expand β-cell mass is mediated by its ability to stimulate β-cell proliferation and possibly neogenesis.

The long-term reduction in body weight induced by the brief neonatal Ex-4 treatment suggests that peripheral actions of Ex-4 also contribute to the normalization of glucose homeostasis in this model. Glucagon-like peptides modify food intake, increase satiety, delay gastric emptying, and suppress glucagon release. GLP-1 is also contributes to improved glucose homeostasis through effects on glucose clearance independent of insulin secretion (21). Future studies will address the mechanisms underlying this long-lasting reduction in body weight.

The permanent improvement in the long-term maintenance of β-cell mass induced by neonatal Ex-4 in the IUGR model suggests that there may be a unique opportunity to influence the development of adult-onset diabetes in humans by intervening during the prediabetic period in at-risk individuals. The newborn period in particular may represent a critical window in which therapies designed to enhance β-cell mass should be initiated. Refining the window for therapeutic intervention will be a subject of great interest in future studies.

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REFERENCES
EX-4 PREVENTS DIABETES DUE TO IUGR


