Glucagon-Like Peptide-1 and Exendin-4 Stimulate β-Cell Neogenesis in Streptozotocin-Treated Newborn Rats Resulting in Persistently Improved Glucose Homeostasis at Adult Age

Cécile Tourrel,¹,² Danielle Bailbé,¹ Marie-Jo Meile,¹ Micheline Kergoat,² and Bernard Portha¹

In neonatal Wistar rats injected with streptozotocin (STZ) at birth (n0-STZ model), a recognized model of β-cell regeneration, we investigated the capacity of early treatment with glucagon-like peptide 1 (GLP-1) or exendin-4 to promote β-cell regeneration and thereby improve islet function in the long term, when animals become adults. To this end, n0-STZ rats were submitted to GLP-1 or exendin-4 from postnatal day 2 to day 6 only, and their β-cell mass and pancreatic functions were tested on day 7 and at 2 months. On day 7, both treatments increased body weight, decreased basal plasma glucose, decreased insulinemia, and increased pancreatic insulin content in n0-STZ rats. At the same age, the β-cell mass, measured by immunocytochemistry and morphometry methods, was strongly increased in n0-STZ/GLP-1 and n0-STZ/Ex rats compared with n0-STZ rats, representing 51 and 71%, respectively, of the β-cell mass in Wistar rats, whereas n0-STZ β-cell mass represented only 21% of the Wistar control value. Despite such early improved β-cell mass, which is maintained at adult age, the basal and glucose-stimulated insulin secretion (in vivo after intravenous glucose load or in vitro using perfused pancreas) were not improved in the 2-month-old n0-STZ rats previously treated with GLP-1 or exendin-4 compared with untreated n0-STZ rats. However, both treated groups significantly exhibited a decreased basal plasma glucose level and an increased plasma glucose clearance rate compared with the 2-month-old untreated n0-STZ group at adult age. These findings in the n0-STZ model indicate for the first time that GLP-1 or exendin-4 applied during the neonatal diabetic period exert both short- and long-term beneficial effects on β-cell mass recovery and glucose homeostasis. However, the increase in β-cell mass, which is still present in the adult n0-STZ rats previously treated, contrasts with the poor β-cell responsiveness to glucose. Further studies are needed to understand the dissociation between β-cell regeneration and the lack of improvement in β-cell function.

Diabetes 50:1562–1570, 2001

One determinant in the development of diabetes is an inadequate mass of β-cells in the pancreas, either absolute in type 1 diabetes or relative in type 2 diabetes (1,2). A better understanding of growth and regeneration could lead to new therapeutic strategies, such as expansion of islet tissue before or after transplantation or stimulation of β-cell regeneration in the pancreas of diabetic patients.

One of the experimental models useful to study the regeneration of β-cell is the neonatal rat with diabetes induced by streptozotocin (STZ) (n0-STZ model). We have previously reported (3,4) that damage to β-cells caused by STZ injection in newborn rats is followed by a rapid remission from neonatal diabetes related to a partial spontaneous β-cell regeneration and a recovery of insulin stores starting from 3–5 days after birth. We and others (5–7) have reported that the β-cell regeneration occurs both through differentiation from precursor cells and increased replication of surviving β-cells. Because β-cell regeneration is incomplete in this model (3,5,8,9), adult rats exhibit a decreased β-cell mass and a chronic pathological pattern that presents functional similarities to type 2 diabetes (3,10): glucose intolerance and low insulin response to glucose.

The aim of the present study was to determine whether glucagon-like peptide 1 (GLP-1) and its long-acting analog exendin-4 would favor regeneration of β-cell mass in n0-STZ rats treated during the first postnatal week. GLP-1 is secreted by the enteroendocrine L-cells in response to fat meals and carbohydrates (11,12). When acutely administered, it is known to enhance glucose-stimulated insulin release and glucose disposal in peripheral tissues, to suppress glucagon release and promote satiety, and to inhibit gastric emptying (11–13). More recently, it was demonstrated that both GLP-1 and exendin-4 stimulate the proliferation of INS-1 cells in vitro (14) and increase β-cell mass in adult rodents in vivo (15–17). These observations raise the question of what is the long-term impact of GLP-1–induced β-cell proliferation in terms of 1) duration of the β-cell mass enlargement, 2) improvement of the β-cell function, and 3) improvement of glucose homeostasis. To our knowledge, the answer to this important question has not been documented.

To address this issue, we investigated the capacity of

From the ¹Laboratory of Physiopathology of Nutrition, Centre National de la Recherche Scientifique ESA 7059, Université Paris, Paris; and ²MERCK-LIPHA, Chilly-Mazarin, France.

Address correspondence and reprint requests to C. Tourrel, Lab. Physiopathology of Nutrition, CNRS ESA 7059, Université Paris7/Denis Diderot, 2 place Jussieu, 75251 Paris Cedex 05, France. E-mail: tourrel@paris7.jussieu.fr.

Received for publication 14 September 2000 and accepted in revised form 21 March 2001.

BrdU, 5'-bromo-2'-deoxyuridine; DAB, 3,3'-diaminobenzidine-tetra-hydrochloride; ΔG, integrated change in plasma glucose; ΔI, incremental change in plasma insulin; GLP-1, glucagon-like peptide 1; STZ, streptozotocin.
GLP-1 or exendin-4 treatment to promote β-cell regeneration in the neonatal period and thereby to improve islet function in the n0-STZ model and in the long term, when animals become adults. To this end, n0-STZ rats were administered GLP-1 or exendin-4 injections from postnatal day 2 to day 6 only, and their β-cell mass and pancreatic functions were tested on day 7; at 2 months, the mechanism of β-cells was tested.

RESEARCH DESIGN AND METHODS

Animals. Wistar rats were fed ad libitum with pelleted food (UAB, Villemois-son-sur-Orge, France). Females were caged with a male for one night, and pregnancy was detected by abdominal palpation 14 days later. Natural birth occurred 22 days after mating.

At birth, newborns received a single intraperitoneal injection of 100 μg/g body wt of STZ (Sigma, St. Quentin-Fallavier, France) freshly dissolved in citrate buffer (0.05 mol/l, pH 4.5). The number of animals per litter was kept at 10, and the pups were left with their mothers. All the neonates were tested on day 2 for glycosuria with Clinitest (Bayer Diagnostics, Puteaux, France), and animals were included in the study only if they were glycosuric (β+ value with Clinitest test) on day 2 after birth.

Four experimental groups were studied: the control group (Wistar rats), in which newborns received a single intraperitoneal injection of citrate buffer; the n0-STZ group; and the n0-STZ/GLP-1 and n0-STZ/Ex groups. n0-STZ rats received a daily subcutaneous injection of GLP-1 (400 μg/kg body wt) (Fraga, Sigma, St. Quentin-Fallavier, France) or exendin-4 (3 μg/kg body wt) (Bachem, Voisins-le-Bretonnes, France) for 5 days (from days 2–6) after their birth. All animals responded to GLP-1 or exendin-4 treatment.

Animals were killed by decapitation at 7 days or at 2 months after birth. Blood samples were collected after decapitation in 7-day-old rats or from caudal vessels in 2-month-old rats and immediately centrifuged at 4°C and stored at –20°C until assayed.

Pancreas removal and treatment. After excision, pancreases from four to five rats were removed and weighed. For measurement of insulin content, pancreases were homogenized and centrifuged in acid-alcohol solution (75% ethanol, 1.5% HCl 12N, 23.5% distilled water), and the supernatant was stored at –20°C. For immunohistochemical study, other pancreases were fixed in aqueous Bouin’s solution for 24 h and embedded in Paraplast (Labonord, Templemars, France).

Immunocytochemistry and morphometry. Each pancreatic block was serially sectioned (7 μm) throughout its length. Ten sections were randomly chosen every 35th section throughout the block and were immunostained for insulin using a peroxidase indirect labeling technique. Sections were incubated for 1 h with primary antibodies (guinea pig anti-porcine insulin; ICN Pharmaceuticals, Orsay, France). Thereafter, peroxidase-conjugated second antibodies were applied for 45 min (rabbit anti–guinea pig immunoglobulin G [IgG]; Dako, Trappes, France). Staining was visualized by incubation with 3,3′-diaminobenzidine-tetra-hydrochloride (DAB) (kit DAB; Valbiotech, Compiègne, France). Sections were mounted in Eukitt (Labonord). Quantitative evaluation of total β-cell area was performed using a computer-assisted image analysis procedure based on an Olympus BX 20 microscope connected via a video camera to a PC computer and using the Visiolab 1000 software (Biocom, Les Ulis, France). The areas of insulin-positive cells, as well as those of total pancreatic sections, were evaluated in each stained section. The relative volume of β-cells was determined by stereological morphometric methods, calculated from the ratio between the area occupied by immunoreactive cells and that occupied by total pancreatic cells. Total β-cell mass per pancreas was derived by multiplying this ratio by the total pancreatic weight.

Individual β-cell area. β-Cell size was measured on the insulin-stained sections. β-Cell nuclei on a random section were counted, and the area of β-cell tissue in that section was measured by planimetry as described above. The individual β-cell size was determined by dividing the β-cell area by the number of nuclei. By using this technique, it is possible that the actual number of β-cells is higher than the number counted because not all β-cells are sectioned across their nuclei. Therefore, the size of the β-cells may be overestimated.

β-Cell replication. Pancreatic sections that had not already been used for morphometric studies were used to measure the β-cell replication rate. 5′-Bromodeoxyuridine (BrdU) was incorporated into the S phase of the cell cycle, and therefore it labeled replicating cells. In each group, animals were injected with BrdU (50 mg/kg body wt i.p.) (Sigma) 1 h before death. Sections were double-stained for BrdU (using a cell proliferation kit [Amersham International, Amersham, U.K.] for insulin. Sections were incubated with a mouse monoclonal antibody anti-BrdU diluted in a nuclease solution (according to the kit protocol) for 1 h at room temperature and washed with Tris 0.05 mol/l, pH 7.6. Thereafter, sections were incubated with an affinity-purified peroxidase anti-mouse IgG and stained with DAB using a peroxidase substrate kit DAB. Sections were then incubated with an alkaline phosphatase antibody-alkaline phosphatase complex was revealed with an alkaline phosphatase substrate kit. Sections were counterstained with light green and embedded in Paraplast (Labonord, Templemars, France).

Analytical techniques. Plasma glucose was determined using a glucose analyzer (Beckman, Palo Alto, CA). Plasma insulin and pancreatic insulin content were determined by radioimmunoassay (19). Immunoreactive insulin was separated from proinsulin and insulin using affinity-purified rabbit anti-insulin antibodies (C.D. Montrouge, France), antibody to insulin, and rat IgG moniodiiodinated insulin (Dian- sorin, Saluggia, Italy) as tracer. Charcoal (Sigma) was used to separate free from bound hormone. This method allows the determination of 0.1 ng/ml with a coefficient of variation within and among an assay of 10%.

DIABETES, VOL. 50, JULY 2001 1563
NEONATAL GLP-1 TREATMENT AND β-CELL REGENERATION

TABLE 1

<table>
<thead>
<tr>
<th>Rats</th>
<th>Body weight (g)</th>
<th>Pancreas weight (mg)</th>
<th>Plasma glucose (mmol/L)</th>
<th>Plasma insulin (pmol/L)</th>
<th>Pancreatic insulin</th>
<th>β-Cell mass</th>
<th>mg/pancreas</th>
<th>μg/pancreas</th>
<th>β-Cell mass</th>
<th>mg/pancreas</th>
<th>μg/pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>16.2 ± 0.3 (15)</td>
<td>40.1 ± 1.5 (15)</td>
<td>7.1 ± 0.16 (15)</td>
<td>195 ± 15 (15)</td>
<td>20.2 ± 0.6 (11)</td>
<td>531 ± 23 (11)</td>
<td>0.76 ± 0.06 (4)</td>
<td>17.2 ± 1.1 (4)</td>
<td>12.6 ± 0.9* (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n0-STZ</td>
<td>10.9 ± 0.3 (19)</td>
<td>38.2 ± 1.4 (19)</td>
<td>12.8 ± 1.43 (19)</td>
<td>270 ± 30 (19)</td>
<td>2.1 ± 0.3 (14)</td>
<td>47 ± 7.1 (14)</td>
<td>0.16 ± 0.02 (5)</td>
<td>3.8 ± 0.4 (5)</td>
<td>0.6* (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n0-STZ/GLP-1</td>
<td>12.7 ± 0.3* (24)</td>
<td>39 ± 1.1 (24)</td>
<td>7.5 ± 0.24* (24)</td>
<td>135 ± 15* (24)</td>
<td>8.1 ± 0.8* (18)</td>
<td>208 ± 21* (18)</td>
<td>0.39 ± 0.03* (4)</td>
<td>10.1 ± 0.6* (4)</td>
<td>0.39* (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n0-STZ/Ex</td>
<td>13.3 ± 0.77 (17)</td>
<td>42 ± 1.5 (17)</td>
<td>9.3 ± 0.10* (17)</td>
<td>300 ± 34 (17)</td>
<td>3.7 ± 0.3* (11)</td>
<td>106 ± 11* (11)</td>
<td>0.54 ± 0.03* (4)</td>
<td>12.6 ± 0.9* (4)</td>
<td>0.54* (4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE (n). Animals were in the nonfasted state. *P < 0.001; †P < 0.01; ‡P < 0.05 compared with the untreated n0-STZ group.

Statistical analysis. Values are expressed as means ± SE. The significance of differences between mean values was evaluated by one-way analysis of variance followed by a Fisher’s test.

RESULTS

Effects of GLP-1 and exendin-4 treatments from day 2 to day 6 after birth: studies on day 7. The characteristics of 7-day-old Wistar rats, diabetic n0-STZ, n0-STZ/GLP-1, and n0-STZ/Ex rats are shown in Table 1. n0-STZ/GLP-1 and n0-STZ/Ex rats exhibited increased body weight (P < 0.001 and P < 0.01, respectively) but no change in their pancreas weight compared with untreated n0-STZ rats. In both groups, basal plasma glucose level was significantly decreased (P < 0.001 and P < 0.05); in n0-STZ/GLP-1 rats, it was no longer different from values in Wistar rats (Table 1). The plasma insulin level was decreased after GLP-1 treatment in n0-STZ rats (P < 0.001), but it was not decreased after exendin-4 treatment.

In n0-STZ/GLP-1 and n0-STZ/Ex groups, pancreatic insulin content and total β-cell mass were both significantly increased (P < 0.001) compared with values in untreated n0-STZ rats (Table 1). The β-cell masses in n0-STZ/GLP-1 and in n0-STZ/Ex rats represented 51 and 71%, respectively, of the control Wistar rats’ β-cell mass, whereas the n0-STZ β-cell mass represented only 21% of the control Wistar value.

Because an increase in β-cell mass can result from either larger cells (hypertrophy) or more cells (hyperplasia), we measured the individual cross-sectional area of the β-cells as an indication of β-cell size in the two treated n0-STZ groups. The mean cross-sectional area of β-cells in n0-STZ/GLP-1 or n0-STZ/Ex rats did not differ from that in the untreated n0-STZ group (Wistar: 103.3 ± 0.81 μm²; n0-STZ: 103.2 ± 1.9 μm²; n0-STZ/GLP-1: 107.4 ± 2.6 μm²; n0-STZ/Ex: 108.8 ± 5.5 μm²). Thus, the significant increase in the total β-cell mass found in the two treated groups when compared with the untreated n0-STZ group can be mostly ascribed to β-cell hyperplasia.

There are two pathways producing new β-cells: 1) replication of preexisting β-cells and 2) neogenesis, i.e., differentiation from duct cells. To evaluate β-cell replication, we used the BrdU labeling index of β-cells as determined by double immunocytochemical staining for insulin and BrdU (Fig. 1A and Fig. 2A and B). The BrdU labeling index in the untreated n0-STZ rats was found to be significantly increased (P < 0.001) compared with the Wistar group. In the n0-STZ/GLP-1 and n0-STZ/Ex groups, it was similarly increased (Fig. 1A and Fig. 2A and B). To estimate activation of neogenesis, the number of single β-cells incorporated into the duct epithelium and the number of β-cell clusters budding from ducts were quantified. The number of isolated β-cells in pancreatic tissue in n0-STZ rats represented 185 ± 28.2% of the Wistar value, and the number of β-cell buds in pancreatic tissue in n0-STZ rats represented 106 ± 16.8% of the Wistar value. These two parameters were strongly increased in n0-STZ/GLP-1 and n0-STZ/Ex rats compared with untreated n0-STZ rats (P < 0.001) (Fig. 1B and Fig. 2C and D).

Involvement of β-cell apoptosis during β-cell regeneration in the n0-STZ/GLP-1 and n0-STZ/Ex groups was also evaluated on day 7 to determine whether there is an earlier implication of apoptosis in pancreas remodeling after treatment. The very low frequency of apoptotic β-cells per pancreas detected by the TdT dUTP nick end labeling method (no more than three to six apoptotic β-cells per at least 1,500 β-cells counted) in each studied group suggests that β-cell apoptosis was not influenced during GLP-1 or exendin-4 treatment.

Long-term effects of early treatment with GLP-1 or exendin-4: studies at 2 months. Because of the precedent results, we examined whether the neonatal treatment by GLP-1 or exendin-4 exerted long-lasting beneficial effects on insulin secretion. The characteristics of 2-month-old Wistar, diabetic n0-STZ, n0-STZ/GLP-1, and n0-STZ/Ex rats used in the present study are shown in Table 2. Body weight values were similar in each group. Compared with untreated n0-STZ females, 2-month-old n0-STZ females previously treated with GLP-1 or exendin-4 exhibited a significantly lower plasma glucose concentration (P < 0.001), and these values are similar to the Wistar value. Plasma insulin levels are increased in the n0-STZ/GLP-1 rats but decreased in n0-STZ/Ex rats compared with the untreated n0-STZ rats. Pancreatic insulin content in n0-STZ/GLP-1 and n0-STZ/Ex female rats was increased compared with that in n0-STZ female rats (P < 0.001). In n0-STZ/GLP-1 and n0-STZ/Ex female rats, total β-cell mass was significantly increased (P < 0.001) compared with values in untreated n0-STZ rats (Table 2 and Fig. 3) and represented 53 and 61%, respectively, of the control Wistar β-cell mass,
concentrations were significantly lower in the two treated 15, 20, and 30 min after the glucose load, plasma glucose in the fasted state in each studied group (Fig. 4). At 5, 10, and 30 min after the glucose load, plasma glucose concentrations were significantly lower in the two treated groups compared with n0-STZ groups (except for the 30-min point in n0-STZ/Ex rats compared with untreated n0-STZ rats). All values for glycemia obtained with treated n0-STZ rats were still slightly but significantly higher than those obtained in Wistar rats. The glucose disappearance rate (K) (Fig. 4) was improved in n0-STZ/GLP-1 rats ($P < 0.05$) but not in n0-STZ/Ex rats compared with untreated n0-STZ rats. The early treatment with GLP-1 did not affect the insulin response to glucose, and Wistar values for insulinenia were significantly elevated above those obtained from n0-STZ, n0-STZ/GLP-1, and n0-STZ/Ex rats. Values obtained for plasma insulin from n0-STZ rats were lower than those from untreated n0-STZ rats at 5, 10, 15, 20, and 30 min after the glucose load. The insulinogenic index is the same in n0-STZ, n0-STZ/GLP-1, and n0-STZ/Ex rats ($\Delta I/\Delta G$) (Fig. 4).

We used an isolated perfused pancreas experiment to examine the insulin secretion in vitro in response to glucose or arginine. Basal rates of insulin secretion from the perfused pancreases were similar in the three experimental groups: n0-STZ, n0-STZ/GLP-1, and n0-STZ/Ex (Fig. 5A). Exposure to 16.7 mmol/l glucose for 20 min did not elicit a clear-cut increase of insulin output in the untreated n0-STZ group, which was also the case in the n0-STZ/GLP-1 and n0-STZ/Ex groups (Fig. 5A and B). Arginine (19 mmol/l) caused an important insulin release in untreated n0-STZ pancreases that was greater in n0-STZ/GLP-1 rats compared with untreated n0-STZ rats.

**DISCUSSION**

Neonatal rats treated with STZ at birth exhibit insulin-deficient acute diabetes 3–5 days after birth characterized by a rapid spontaneous remission (3,4). We have previously shown that this remission is accompanied by an incomplete spontaneous β-cell regeneration and a progressive increase in the pancreatic insulin stores starting from 3–5 days after birth (5,20). Moreover, immunocytochemical and ultrastructural studies of pancreatic tissue from such animals indicate that during this favored regeneration, replication from preexisting differentiated β-cells that survived the toxic effect of STZ represents an important but not exclusive mechanism of regeneration, and obviously many of the β-cells found 3–5 days after birth may originate from undifferentiated duct cells (5,21,22).

Adult rats that were treated with STZ at birth (n0-STZ model) exhibited type 2 diabetes with specific failure of insulin release in response to glucose. This pattern of glucose unresponsiveness resembles that shown in some studies of human type 2 diabetes. The n0-STZ model is recognized as an adequate tool for investigations in diabetes pharmacotherapy. Recent works on diabetic models have reported that GLP-1 and exendin-4 could improve the diabetic state (23–27) and exert a trophic effect on the pancreatic β-cell mass (15–17). In this context, we have now investigated the short- and long-term effect of a treatment with GLP-1 and exendin-4 during the regenerating process in the n0-STZ model.

In our study, treatment with either GLP-1 or exendin-4 supplied during a limited period (days 2–6 after birth) after diabetes installation increased body weight, markedly decreased basal plasma glucose, and improved the recovery of pancreatic insulin stores when tested on day 7.

**FIG. 1.** A: BrdU labeling index of the β-cell in 7-day-old Wistar (■), n0-STZ (□), n0-STZ/GLP-1 (●), and n0-STZ/Ex (○) rats. B: β-Cell neogenesis activation in 7-day-old Wistar (■), n0-STZ (□), n0-STZ/GLP-1 (●), and n0-STZ/Ex (○) rats. It is evaluated through quantification of duct-associated β-cells per unit of total tissue area and β-cell buds per unit of total tissue area. Data are expressed as % of corresponding values in the Wistar group. Values are means ± SE for four observations in each group. ***$P < 0.001$.

whereas n0-STZ β-cell mass represented only 37% of the control Wistar value. This increase in the total β-cell mass found in the two treated groups is also due to β-cell hyperplasia and not to β-cell hypertrophy (Wistar: 198.1 ± 2.1 $\mu$m²; n0-STZ: 202.9 ± 4.1 $\mu$m²; n0-STZ/GLP-1: 201.4 ± 1.9 $\mu$m²; n0-STZ/Ex: 197.4 ± 1.5 $\mu$m²).

To examine the glucose-stimulated insulin secretion in vivo, an intravenous glucose tolerance test was performed in the fasted state in each studied group (Fig. 4). At 5, 10, 15, 20, and 30 min after the glucose load, plasma glucose concentrations were significantly lower in the two treated
Furthermore, we found that GLP-1 or exendin-4 therapy improved the \( \beta \)-cell regeneration because on day 7, total \( \beta \)-cell mass was greater in the n0-STZ/GLP-1 and n0-STZ/Ex groups than in the untreated n0-STZ group. Our data suggest that glycemic improvement after treatment with GLP-1 or exendin-4 could be due, at least in part, to enhancement of \( \beta \)-cell regeneration. Because the relative contribution of the replication of existing \( \beta \)-cells to the total \( \beta \)-cell growth is the same in the n0-STZ/GLP-1 and n0-STZ/Ex rats compared with the untreated n0-STZ rats 24 h after the last dose of the two treatments, it could be proposed that under these conditions, an enhancement of \( \beta \)-cell replication due to GLP-1 or exendin-4 injection may not be detected. To test this hypothesis, we measured the BrdU labeling index on day 6, 1 h after the last injection of GLP-1 or exendin-4. The BrdU labeling indexes on day 6 were found to be similar in the three n0-STZ groups (n0-STZ: 6.89 ± 0.46; n0-STZ/GLP-1: 6.78 ± 0.32; n0-STZ/Ex: 6.59 ± 0.38%; \( n = 3 \) in each group) and increased compared with the 6-day-old Wistar group (4.20 ± 0.15%)

### TABLE 2

<table>
<thead>
<tr>
<th>Rats</th>
<th>Wistar</th>
<th>n0-STZ</th>
<th>n0-STZ/GLP-1</th>
<th>n0-STZ/Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>291 ± 4.5 (10)</td>
<td>208 ± 5.9 (11)</td>
<td>206 ± 6.7 (8)</td>
<td>180 ± 3.0* (10)</td>
</tr>
<tr>
<td>Pancreas weight (mg)</td>
<td>838 ± 29.5 (10)</td>
<td>920 ± 39.6 (10)</td>
<td>878 ± 62.6 (8)</td>
<td>902 ± 38.2 (10)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>6.9 ± 0.20 (12)</td>
<td>9.7 ± 0.12 (12)</td>
<td>7 ± 0.17† (10)</td>
<td>6.1 ± 0.21† (12)</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td>705 ± 61 (12)</td>
<td>218 ± 43 (19)</td>
<td>255 ± 18‡ (10)</td>
<td>168 ± 13† (12)</td>
</tr>
<tr>
<td>Pancreatic insulin</td>
<td>83.6 ± 4.7 (10)</td>
<td>44.5 ± 2.5 (11)</td>
<td>57 ± 3.1‡ (8)</td>
<td>59.9 ± 1.6† (10)</td>
</tr>
<tr>
<td>( \mu )g/pancreas</td>
<td>99.5 ± 3.8 (11)</td>
<td>48.7 ± 2.4 (14)</td>
<td>65 ± 3.5‡ (8)</td>
<td>67.6 ± 3.5† (10)</td>
</tr>
<tr>
<td>( \mu )g/g pancreas</td>
<td>58.2 ± 0.1 (4)</td>
<td>2.18 ± 0.16 (4)</td>
<td>3.14 ± 0.15† (4)</td>
<td>3.58 ± 0.3† (4)</td>
</tr>
<tr>
<td>( \mu )g/pancreas</td>
<td>7.90 ± 0.65 (4)</td>
<td>2.85 ± 0.29 (4)</td>
<td>3.66 ± 0.34‡ (4)</td>
<td>4.45 ± 0.28† (4)</td>
</tr>
</tbody>
</table>

Data are means ± SE (n). Animals were in the fasted state. *\( P < 0.05; † P < 0.001; \) and ‡\( P < 0.01 \) compared with the fasted state.
All of these values are similar to those found on day 7 in the four tested groups. We concluded that during the regenerating process in the n0-STZ rats, GLP-1 or exendin-4 did not further increase β-cell replication. Therefore, we propose that GLP-1 or exendin-4 administration preferentially modulates the β-cell neogenesis pathway. Actually, neogenesis is considered the process of β-cell differentiation from progenitor cells located in the duct epithelium, suggesting that the β-cells closely associated with the ducts are those issued from undifferentiated precursor duct cells (28,29). In our study, we found a significantly higher number of single β-cells and β-cell clusters associated with the pancreatic ducts in the two treated n0-STZ groups than in the untreated n0-STZ group, supporting the proposal that neogenesis seems to be the main pathway implicated in the increased β-cell regeneration induced by early treatment with GLP-1 and exendin-4 in the n0-STZ model. A similar observation was made in the 90% pancreatectomized adult rats (16).

During the first 2 weeks after birth, the involvement of apoptosis in the remodeling of the rodent endocrine pancreas was reported to be very low, whereas it was high only during the period of days 13–17 (30). Our findings in the neonatal n0-STZ model indicate that GLP-1 or exendin-4 exerted no influence on β-cell apoptosis on day 7 in the pancreas remodeling under our conditions.

The mechanism by which GLP-1 or exendin-4 treatment enhances the growth of the neonatal diabetic pancreas is not clear. The question that arises is whether the effects of GLP-1 or exendin-4 on the stimulation of β-cell regeneration in the n0-STZ pancreases are direct. The beneficial effect of GLP-1 or exendin-4 on β-cell regeneration can be related to a reduction of hyperglycemia in the treated n0-STZ rats and, more specifically, in the n0-STZ/GLP-1 rats because they exhibit near-normoglycemia after the 5-day treatment. Indeed, in islets of diabetic animals and in long-term culture of immortalized insulin-secreting cells, chronic exposure to high glucose can result in loss of β-cell gene expression (insulin and pancreatic duodenal homeobox gene-1) (31,32). Recently, studies on diabetic models (Goto-Kakizaki rats, pancreatectomized rats, and Psammomys obesus) (33–35) reported that these changes in islet gene expression could reflect a loss of pancreatic β-cell differentiation due to chronic exposure to hyperglycemia. However, we are not inclined to exclude a direct action of the two peptides on β-cell regeneration. The majority of GLP-1 actions are believed to be transduced by a single GLP-1 receptor originally cloned from pancreatic β-cells (36). Moreover, some studies demonstrated that the two peptides isolated from the venom of lizard, exendin-4 and exendin (9-39), can bind the cloned receptor with high affinity and serve as a GLP-1 receptor agonist and antagonist, respectively (26,36,37). Therefore, GLP-1 and exendin-4 might exert their trophic actions on β-cells by a direct effect via the GLP-1 receptor. The reported abnormal glucose tolerance and islets with fewer β-cells in the GLP-1 receptor–null mouse are consistent with a direct effect of GLP-1 or exendin-4 on β-cells (38,39). More evidence supporting β-cell regeneration being a direct effect of GLP-1 or exendin-4 comes from the finding that GLP-1 receptor mRNA and proteins are expressed in the pancreatic ductal cells, a site for β-cell neogenesis (16).

Moreover, our findings show that GLP-1 and exendin-4 treatment during the neonatal period promotes mainly the neogenesis pathway in the n0-STZ model. Because the two peptides are known to strongly increase the circulating insulin levels by potent stimulation of insulin secretion, we could imagine that the effect of GLP-1 and exendin-4 on β-cell neogenesis is partially mediated by the circulating insulin levels that act as growth factor. Nevertheless, our findings show a very low level of circulating insulin in the two treated groups, suggesting that the GLP-1 or exendin-4 effect is independent of insulin.
level. To support this hypothesis, AR42J cells, derived from a ductal carcinoma, differentiate into insulin-producing cells in response to GLP-1, indicating that insulin is not required for GLP-1–induced endocrine differentiation in this in vitro cultured cell model (40). However, we cannot exclude that the transient increase of plasma insulin after GLP-1 or exendin-4 injection could act as the growth factor during the treatment period.

To determine whether the GLP-1 or exendin-4 treatment applied on n0-STZ rats during the neonatal period exerts long-lasting beneficial effects on \( \beta \)-cell mass and \( \beta \)-cell function, we have raised n0-STZ/GLP-1 and n0-STZ/Ex rats to adult age. Two-month-old n0-STZ/GLP-1 and n0-STZ/Ex female rats exhibit basal normoglycemia, an increased insulin pancreatic content, and a beneficial long-term effect on the \( \beta \)-cell mass (Table 2).

The discrete differences existing between the two treated n0-STZ groups concerning body weight and plasma insulin/glucose ratio could be due to an inadequate dose of exendin-4. Indeed, for neonatal treatment with exendin-4, we chose 3 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \), which is in the same range as that reported by Xu et al. (16). However, in this study, exendin-4 was administered to adult rats.

Surprisingly, despite such beneficial effects, basal and glucose-stimulated insulin secretion (in vivo after an intravenous glucose load or in vitro using isolated perfused pancreas) were not improved in the adult n0-STZ/GLP-1 and n0-STZ/Ex females compared with untreated adult n0-STZ females. Such a pattern (improved basal glycemia without restoration of insulin release in response to glucose) suggests that the long-lasting influence of the GLP-1 or exendin-4 treatment on basal glycemia and glucose clearance is probably related to a persistent improvement of glucose disposal by peripheral tissues and/or liver. In the literature, there are some reports of an outside pancreatic action of GLP-1 that may contribute to the regulation of glucose metabolism. It has been proposed that GLP-1 acts directly on glucose metabolism in some extra-pancreatic tissues, such as squalletal muscles (41), adipose tissue (42), and liver (43,44). However, all of these studies addressed acute GLP-1 effects, and to our knowledge, there is no report in the literature illustrating a favorable in vivo long-term effect of GLP-1 on diminution of liver glucose production and/or stimulation of glucose uptake by peripheral tissues.

Finally, we are left without a satisfactory explanation as to why we failed to improve glucose-induced insulin release in our model despite increasing \( \beta \)-cell mass and

![Image](image_url)
One possibility compatible with our data is that a very rapid regenerative process in the neonatal period induced by GLP-1 or exendin-4 has led to the recruitment of β-cells that did not attain a fully functional phenotype. Indeed, a very similar pattern of dissociation between efficient β-cell regeneration and lack of improvement in β-cell function has been reported in mildly diabetic rats after a 48-h glucose infusion (49).

Lastly, the absence of glucose-induced insulin secretion by β-cells regenerated after GLP-1 or exendin-4 treatment could be caused by chronic hyperglycemia, even if it is mild and takes place only during food ingestion. In such a perspective, our data would be compatible with the concept that stringent normalization (and not near-normalization) of plasma glucose levels is a prerequisite to obtain some improvement in the β-cell response to glucose in the n0-STZ rat as well as in other diabetic models (10,50,51).

In summary, our results using the n0-STZ rat model show for the first time that GLP-1 or exendin-4 applied during the neonatal diabetic period exerts both a short- and long-term beneficial effect on β-cell mass recovery and glucose homeostasis. However, the increase in β-cell mass that is maintained in the adult n0-STZ rats previously treated contrasts with the poor β-cell responsiveness to glucose. The reason why β-cells regenerated after early GLP-1 or exendin-4 treatment are lacking in terms of glucose-induced insulin release remains unclear and needs further investigation.

ACKNOWLEDGMENTS
This work was partly supported by grants from MERCK-LIPHA.

Part of this work was presented at the 60th Scientific Sessions of the American Diabetes Association, 9–13 June 2000, in San Antonio, Texas, and has appeared in abstract form in Diabetes 49 (Suppl. 1):A257, 2000.

REFERENCES