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# Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old *db/db* mice

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## Abstract

*Aims/hypothesis.* Glucagon-like peptide-1 ameliorates the symptoms of diabetes through stimulation of insulin secretion and enhancement of beta-cell mass. We have therefore investigated the effects of glucagon-like peptide-1 on the development of diabetes, using *db/db* mice as a model of Type II diabetes.
*Methods.* The potent glucagon-like peptide-1 analogue Exendin-4 or vehicle (control) was administered (i.p.; 1 nmol/kg) to obese 6-week old *db/db* mice daily for 14 days ($n$=10).
*Results.* By 8 weeks of age, control *db/db* mice developed hyperglycaemia (fasting: 10.4±0.5 mmol/l), hyperinsulinaemia and impaired glucose tolerance. However, Exendin-4 treatment prevented hyperglycaemia (fasting: 6.1±1.0 mmol/l, $p$<0.01), with reduced plasma insulin concentrations ($p$<0.001) and improved glucose tolerance ($p$<0.05). Peripheral insulin sensitivity was not affected. However, insulin release in vivo and in vitro from the perfused pancreas was improved by Exendin-4, as were pancreatic insulin concentrations (0.54±0.02 vs 0.32±0.01 µg/mg protein, $p$<0.05). These changes occurred in conjunction with increased beta-cell mass (3.01±0.31 vs 2.22±0.22 mg, $p$<0.05) and proliferation ($BrdU^+$ beta-cells: 1.08±0.20 vs 0.47±0.11%, $p$<0.05), as well as decreased apoptosis ($Tunel^+$ beta-cells: 0.37±0.06 vs 1.20±0.21%). Western blot demonstrated increased expression of Akt1 (by fivefold, $p$<0.01) and p44 MAP kinase (by sixfold, $p$<0.01), and decreased activation of caspase-3 (by 30%, p<0.05).
*Conclusion/interpretation.* Our results suggest that Ex4 treatment delays the onset of diabetes in 6–8 week old *db/db* mice, through a mechanism involving Akt1 and expansion of the functional beta-cell mass. [Diabetologia (2002) 45:1263–1273]

**Keywords** GLP-1, exendin-4, OGTT, insulin, pancreas, perfusion, beta-cell mass, apoptosis, proliferation, Akt1.

The maintenance of beta-cell mass is a dynamic process, undergoing both increases and decreases to maintain glycaemia within a narrow physiological range [1, 2, 3]. The majority of patients with obesity causing insulin resistance are not diabetic, as their capacity for beta-cell compensation is maintained but, 15–20% of these individuals become diabetic, when the beta cells lose their compensatory ability [1]. Although one such approach to preventing and treating diabetes could be through the enhancement of beta-cell mass, the factors that determine beta-cell mass under physiologic conditions or in response to insulin resistance are not clear. Evidence has been provided for roles of a variety of different hormones as beta-cell growth factors, including growth hormone, the insulin-like growth factors, prolactin and, most recently, the intestinal hormone, glucagon-like peptide-1 (GLP-1) [4, 5].

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*Abbreviations:* GLP-1, Glucagon-like peptide-1; Ex4, exendin-4; OGTT, oral glucose tolerance test; PKB, protein kinase B; MAPK, mitogen-activated protein kinase

GLP-1 is a 30 amino acid peptide secreted from the L cells of the gastrointestinal tract in response to nutrient ingestion [6]. Recent studies have established that GLP-1 enhances glucose-dependent insulin secretion, reduces glucagon secretion, delays gastric emptying and induces satiety [7]. GLP-1 could also improve peripheral insulin resistance, although this remains controversial [8, 9]. Consistent with these biological actions, GLP-1 administration has been shown to reduce hyperglycaemia when administered to patients with Type II (non-insulin-dependent) diabetes mellitus [10]. Thus, GLP-1 has been proposed for use as a therapeutic agent for the treatment of hyperglycaemia in patients with Type II diabetes [7].

The GLP-1 receptor is a 7 transmembrane, G protein-coupled protein expressed in a number of tissues, including the beta cells and the brain [11]. Within the beta cell, ligand binding to the GLP-1 receptor results in enhanced activity of the signalling pathways involved in glucose-dependent insulin secretion [11, 12]. Although cAMP is the best characterized of the GLP-1 receptor intracellular signal transducers, recent evidence has suggested that both phosphatidylinositol 3-kinase (PI3-K) and the mitogen-activated protein kinases (MAPKs) could also be activated by GLP-1 binding to beta cells [13, 14]. Consistent with a possible role for these kinases in the actions of GLP-1, it has been recently reported that GLP-1 can enhance beta-cell mass through stimulation of beta-cell proliferation and enhancement of islet neogenesis in normal mice, as well as in a variety of rodent models of Type II diabetes [15, 16, 17, 18, 19, 20]. However, all of these studies to date have been conducted in animals with pre-established diabetes. This study, therefore, aimed to establish the effects of GLP-1 on the development of diabetes, using pre-diabetic *db/db* mice as our model [21]. These mice lack a functional leptin receptor, and they spontaneously develop obesity, hyperinsulinaemia, and glucose intolerance at 4–6 week of age, progressing to frank diabetes by 8 weeks of age. They have thus been used extensively as a model for Type II diabetes.

## Subjects and methods

*Animals.* Five-week-old female *db/db* mice (BKS.Cg-m+/+Lepr$^{db}$, stock number 000642) were purchased from Jackson Laboratories (Bar Harbor, Me., USA). Background control mice (C57BLKS/J) were obtained from Charles River Canada (Montreal, Quebec, Canada) at the same age. Mice were housed under controlled light (12 h light/12 h dark) and temperature conditions, and had free access to food (normal rodent chow) and water. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee.

*Evaluation of mice and drug administration.* All experiments were initiated in mice at 6 weeks of age after glucose concentrations were measured in fasting blood samples to ensure that the mice were not diabetic. Vehicle (control) or the potent, degradation-resistant GLP-1 analogue, Exendin-4 (Ex4; [22]) was injected (1 nmol/kg i.p.; Bachem, Torrance, Calif., USA) daily at 09 00 hs. The injection on day 0 and day 7 was delayed until after completion of an oral glucose tolerance test (OGTT) or insulin tolerance test, in order to avoid any short-term drug effect. Injection of Ex4 was omitted on day 14.

*OGTT and insulin tolerance test.* Groups of mice were subjected to an OGTT or insulin tolerance test at day 0, 7, and 14. For OGTT, mice were fasted for 15 h, whilst for insulin tolerance test, mice remained fed. For both groups of mice, a basal blood sample (20 µl) was collected from a tail vein ($t$=0) for measurement of glucose using the One Touch Basic glucose meter (Lifescan Canada, Burnaby, British Columbia, Canada). For OGTT, the mice were gavaged with glucose (1.59/kg) and additional blood samples were collected at $t$=10, 20, 30, 60, 90 and 120 min for glucose measurement. For the insulin tolerance test, the mice were injected (i.p.) with human biosynthetic insulin (Novo Nordisk Pharmaceutical Industries, Toronto, Ontario, Canada) at a dose of 2.5 U/kg, and blood samples were collected at t=20, 30, 40, 60, 90, 120, 150, 180 and 210 min for glucose measurement. On day 14, a separate group of mice was subjected to OGTT, and blood samples (50 µl) were collected in a heparinised microhematocrit tube at $t$=0, 15, 30, 60 and 90 min. Blood samples were centrifuged and the plasma was stored at –20°C for determination of insulin concentrations using a highly sensitive Insulin ELISA kit designed for small sample volumes (Crystal Chem, Chicago, Ill., USA).

*Insulin radioimmunoassay.* Blood samples (~1 ml) were obtained by cardiac puncture in fasted mice on day 14, and plasma was collected and stored at –20°C. Plasma insulin concentrations were measured using an insulin radioimmunoassay kit (Linco Research, St. Louis, Miss., USA).

*Pancreas perfusion.* Mice were fasted for 15 h (to ensure consistent starting conditions and to determine fasting glycaemia), and anaesthetized with 35 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The abdominal aorta and portal vein were cannulated with PE-10 and PE-50 tubing, respectively for perfusion and sample collection. The procedure for surgical isolation has been described previously [23]. The perfusate was a modified Krebs-Ringer buffer containing 2% dextran (Pharmacia Biotech, Sweden) and 1% bovine serum albumin (RIA grade, Sigma-Aldrich, Oakville, Ontario, Canada) and was gassed with 95% $O_2$/5%$CO_2$ to achieve a pH of 7.4. The pancreas was sequentially perfused at 1 ml/min with buffer containing 2 mmol/l glucose (5 min), 20 mmol/l glucose (10 min), 20 mmol/l glucose with or without $1\times10^{-8}$ mol/l GLP-1 (10 min) and 2 mmol/l glucose (5 min), for a total perfusion period of 30 min. At the end of the experiment, the pancreas was homogenized in 5 ml of 1 N HCl containing 5% HCOOH, 1% trifluoroacetic acid and 1% NaCl and the peptides were purified by reversed-phase extraction using a C18 SepPak cartridge (Water Associates, Milford, Mass., USA). Extracts were dried in a vacuum dryer prior to RIA for insulin as previously described [23].

*Pancreas harvesting and tissue processing.* Some mice were injected (i.p.) with 100 mg/kg 5-bromo-2′deoxyuridine (BrdU; Amersham, Oakville, Ontario, Canada), 6 h before removal of the pancreas. After cardiac puncture under sodium pentobarbital anesthesia, a midlaparotomy was performed and the pancreas was immediately dissected from surrounding tissues, cleared of fat and lymph nodes, blotted, weighed, and placed in Bouin's fixative. The procedure from completion of cardiac

puncture to placement of tissue in fixative was completed within 5 min. After overnight fixation, the pancreas was washed in cold running water, cut into 8–10 segments and placed in 10% buffered formalin until processing for paraffin embedding [3]. All of the segments from a single pancreas were embedded into one block, thus permitting analysis of the entire pancreas in a single section.

*Beta-cell mass analysis.* Pancreatic sections (4 µm) were processed as previously described [3]. Briefly, following dewaxing, dehydration and antigen retrieval (by boiling in citrate buffer), sections were incubated overnight at 4°C with guinea pig anti-insulin antibody (Dako Diagnostics, Mississauga, Ontario, Canada). The samples were then incubated for 1 h with biotinylated anti-guinea pig antibody (Vector Laboratories, Burlington, Ontario, Canada), and subsequently treated for 1 h with avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, Calif., USA). Sections were then stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) for 10 min. After DAB staining, the sections were washed with tap water and counterstained with hematoxylin.

Beta-cell mass from the insulin antibody-stained sections was measured using a Nikon (ECLIPSE-E1000) microscope connected to a video camera equipped with a colour monitor and ImagePlus software, and the cross-sectional area occupied by all of the beta-cells and the cross-sectional area of all pancreatic tissue was quantified. Total beta-cell area and total pancreas mass for each animal was calculated as the sum of the determinations from each of the 8–10 segments of pancreas. A total of 1000–1500 beta cells was counted for each pancreas. Total beta-cell mass for each pancreas was determined as the product of the total cross-sectional beta-cell area over total tissue area and the weight of the pancreas before fixation.

*Measurement of beta-cell replication.* Pancreatic sections were double stained for insulin, as described above, and for BrdU using anti-BrdU mouse IgG (Sigma-Aldrich) and DAB staining following incubation with biotinylated anti-mouse IgG, as described previously [3]. The beta cells, identified as a red field for insulin staining (chromagen: New Fuchsin Substrate, DAKO), and the BrdU positive beta cells, identified by dark brown staining of nuclei (chromagen: 3,3′-Diaminobenzidine, Sigma), were counted using a Nikon microscope under high magnification (×1000). Results are expressed as the percentage of $BrdU^+$ beta cells.

*Apoptosis detection.* Pancreas sections were double immunostained for insulin, as described above, and for fragmented DNA by Tunel assay, which detects fragmented DNA characteristic of apoptotic cells. Tunel staining was carried out using an ApopTag Kit (Intergen, Purchas, New York, N.Y., USA) according to the manufacturer's instructions. The islet tissue was identified as a red field for insulin staining (chromagen: New Fuchsin Substrate, DAKO), and apoptotic cells were identified by dark brown staining of nuclei (chromagen: 3,3′-Diaminobenzidine, Sigma). The results are expressed as the percentage of $Tunel^+$ beta cells.

*Immunoblotting.* The isolated pancreas was homogenised in lysis buffer [24] containing 1% Triton X-100 and a mixture of protease and phosphatase inhibitors. The protein content was measured by Bradford assay (Bio-Rad Laboratories, Hercules, Calif., USA) and equal amounts of protein were separated by 7–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred on to polyvinylidene difluoride filters (Bio-Rad Laboratories). After probing with specific primary antibodies [Akt1: rabbit IgG (1:1000), New England Bio-Labs (Mississauga, Ontario, Canada); MAPK, mouse IgG (1:1000), Oncogene Research Products (San Diego, Calif., USA); caspase-3 rabbit IgG (1:2000), Biomol Research Laboratories (Plymouth Meeting Penn., USA); and IRS-1: rabbit IgG (1:500), Santa Cruz Biotechnology (Santa Cruz, Calif., USA), the immunoreactive bands were visualised either with horseradish peroxidase-conjugated (HRP) sheep anti-mouse IgG for monoclonal antibodies or with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Penn., USA) for polycolonal antibodies using an ECL detection technique (Amersham, Oakville, Ontario, Canada), as described previously [24]. Developed films were scanned and quantitated using NIH Image software (National Institute of Health, Bethesda, Md., USA).

*Statistical analysis.* All data are presented as mean ± SEM. Statistic analysis was performed using Student's *t* test or analysis of variance (ANOVA) with '*n*-1' *post hoc* custom hypotheses tests, as appropriate, on SAS software (Statistical Analysis Systems, Cary, N.C., USA). A *p* value of less than 0.05 was considered to be statistically significant.

## Results

*Ex4 normalizes fasting blood glucose in db/db mice.* Ex4 treatment was initiated in *db/db* mice at 6 weeks of age. All experiments were carried out at least 24 h after the last injection to ensure clearance of the Ex4 from the circulation [25]. As shown in Figure 1, fasting blood glucose was in the normal range in both PBS (vehicle) and Ex4 treated groups at the initiation of treatment (day 0, PBS vs Ex4: 5.2±0.5 vs 5.1±0.5; $n$=10). On day 7 of the treatment, though still in the normal range, the vehicle-treated group showed a noticeable increase in fasting blood glucose when compared to mice treated with Ex4 (PBS vs Ex4: 6.5±0.8 vs 4.9±0.5; $n$=10). By the age of 8 weeks (day 14), PBS-injected mice had developed hyperglycaemia (10.4±0.5, $n$=10, $p$<0.001 vs day 0), whereas Ex4-treated mice maintained fasting blood glucose concentrations in the normal range

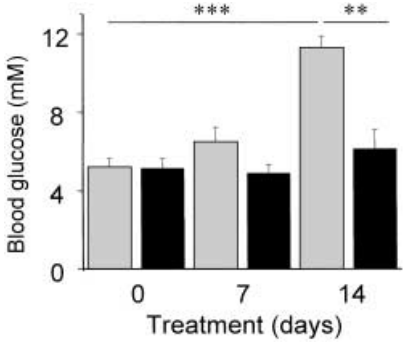



**Fig. 1.** Effect of Ex4 on fasting blood glucose in db/db mice. Fasting blood glucose concentrations were measured at day 0, day 7 and day14 in Ex4 (i.p., 1 nmol/per kg, *black bars*) or PBS (vehicle, *grey bars*) injected *db/db* mice starting at the age of 6 weeks ($n$=10 each). **$p$<0.01; ***$p$<0.001

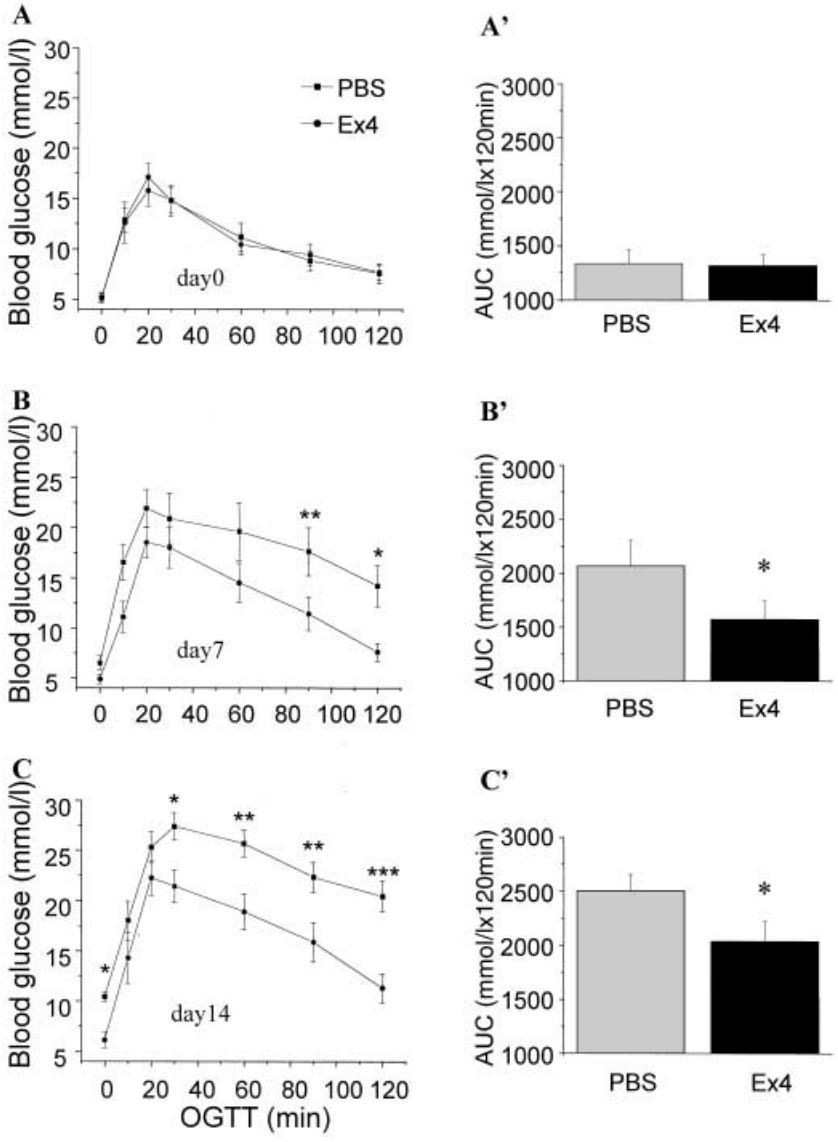



**Fig. 2A–C.** Effect of Ex4 on OGTT in *db/db* mice. Glucose concentrations in *db/db* mice undergoing an OGTT (1.5 g/kg) after Ex4 (i.p., 1 nmol/per kg, *circles* and *black bars*) or vehicle (PBS, *squares* and *grey bars*) treatment ($n$=10 each), at day 0 (**A**), day 7 (**B**) and day 14 (**C**). *$p$<0.05; **$p$<0.01; ***$p$<0.001. The areas under the glycaemic curves (AUC) are shown in the right panels (**A'**, **B'** and **C'**)

(6.1±1.0, $n$=10, $p$<0.01 vs vehicle-treated mice). The fasting glycaemia of the Ex4-treated mice was in the normal range indicating that these treated mice were not diabetic. There were no significant changes in weight gain between the two groups of mice (data not shown).

*Ex4 improves OGTT, insulin tolerance and insulin release.* Before initiation of Ex4 treatment (day 0), the OGTT patterns for the two groups of mice were similar (Fig. 2A) and there was no noticeable difference between the groups when the glycaemic response was expressed as the area under the curve (AUC) (Fig. 2A', PBS vs Ex4: 1333±130 vs 1316±104 mmol/l*120 min, $p$>0.05, $n$=10). However, as compared to background (control) C57BLKS/J mice, both groups of *db/db* mice had impaired oral glucose tolerance (AUC for C57BLKS/J mice at 6 weeks: 1014±68 mmol/l*120 min, $n$=5, $p$<0.05 vs 6-week-old *db/db* mice). A further impairment of glucose tolerance was observed in the vehicle-treated mice at both day 7 (Fig. 2B, 2B') and day 14 (Fig. 2C, 2C') when compared to that at 6 weeks of age (day 0, Fig. 2A), as determined by AUC ($p$<0.05, $n$=10). However, an improvement in glucose tolerance was observed in the mice treated with Ex4 when compared to that of PBS-treated mice at both experimental day 7 (Fig. 2B), and day 14 (Fig. 2C). The AUC of the OGTT results showed that the differences between the two groups were statistically significant on both day 7 (Fig. 2B', PBS vs Ex4: 2056±251 vs 1572±175 mmol/l*120 min, $p$<0.05, $n$=10) and day 14 (Fig. 2C', PBS vs Ex4: 2501±157 vs 2036±194 mmol/l*120 min, $p$<0.05,

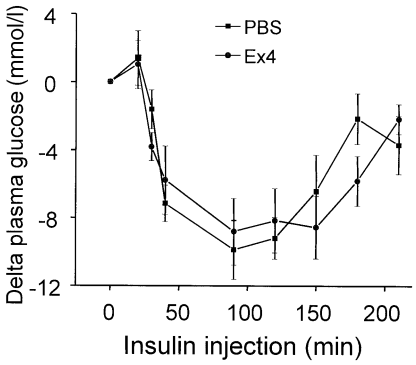



**Fig. 3.** Effect of Ex4 on insulin tolerance in *db/db* mice. Glucose concentrations of *db/db* mice undergoing an insulin tolerance test (i.p. insulin injection: 2.0 U g/kg) on day 14 of Ex4 (i.p., 1 nmol/per kg, ●) or vehicle (PBS, ■) treatment in *db/db* mice ($n$=5 each). No significant differences were seen between the two groups of animal ($p$>0.05)

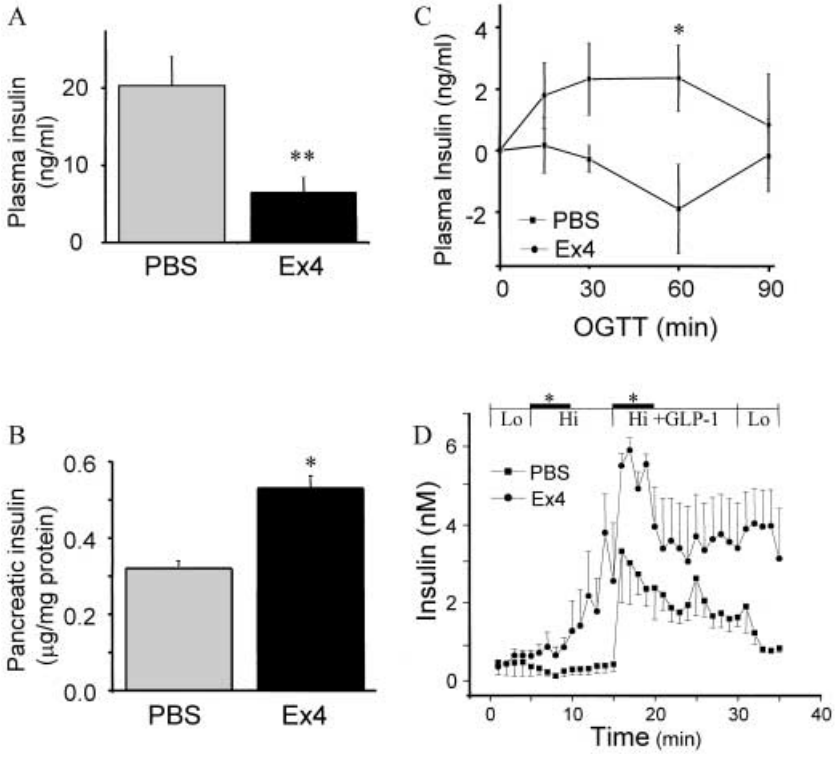
**Fig. 4A–D.** Effect of Ex4 on plasma insulin, pancreatic insulin, insulin release in vivo, and insulin release in vitro. **A** Plasma insulin concentrations were compared after two-week treatment with Ex4 (i.p. injection, 1 nmol/per kg, *black bar*) or PBS (vehicle, *grey bar*) in fasted *db/db* mice. **B** On day 14 of treatment, total pancreatic insulin content was measured and expressed as µg/mg protein. **C** On day 14 of treatment, the plasma insulin concentration was measured during an OGTT using an Insulin-ELISA kit. Insulin concentrations at $t$=60 min and the AUC from two groups of animals were statistically different ($p$<0.05, $n$=5). **D** Insulin secretion by the perfused pancreas from *db/db* mice treated for 14 days with Ex4 (*circles*), or vehicle, (*squares*), in response to 2 mmol/l (Lo) and 20 mmol/l (Hi) glucose, and 20 mmol/l glucose plus GLP-1 ($1\times10^{-8}$ mol/l). *Significantly different from PBS mice ($p$<0.05)

$n$=10), and were higher for the *db/db* mice than in control, 8-week C57BLKS/J mice (974±61 mmol/l*120 min, $p$<0.01, $n$=5).

The enhancement of glucose tolerance in the *db/db* mice treated with Ex4 might be due to an improvement in insulin action on peripheral tissues and/or elevated pancreatic beta-cell function. To evaluate the effect of Ex4 on peripheral insulin action, we carried out an insulin tolerance test in these obese mice by determining blood glucose concentrations after an i.p. injection of insulin (Fig. 3). Insulin sensitivity was reduced in both groups of *db/db* mice compared to background (control) C57BLKS/J mice (data not shown). However, no significant difference in insulin sensitivity (based upon the slopes of the downward curves) was observed between the vehicle-treated and Ex4-treated mice, suggesting that peripheral insulin target sites were not the major contributors to the normoglycaemia and improved glucose tolerance observed in the mice treated with Ex4.

On day 14, consistent with their hyperglycaemia, the fasting plasma insulin concentrations in PBS-mice were extremely high (Fig. 4A, 20.4±3.8 ng/ml) compared to the background C57BLKS/J (control) mice (1.4±0.4, $p$<0.01, $n$=5). Ex4 treatment significantly reduced plasma insulin concentrations (6.5±2.0 ng/ml, $p$<0.01, $n$=5), which was consistent with the normal fasting glycaemia in these mice (Fig. 1).

When the pancreas of these *db/db* mice was excised (Fig. 4B), the vehicle-treated mice had relatively lower pancreatic insulin concentrations (0.32±0.01 µg/mg protein, $n$=5), when compared to that of Ex4-treated mice (0.54±0.02 µg/mg protein, $p$<0.05, $n$=5), or the



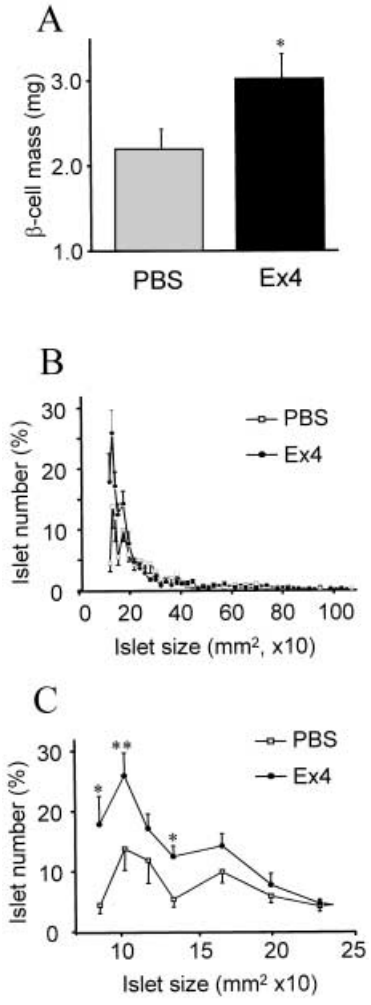



**Fig. 5A–C.** Effect of Ex4 on beta-cell mass in db/db mice. **A** Effect of Ex4 treatment on beta-cell mass ($p<0.05$, $n=5$; Ex4 treatment: *black bar*, control: *grey bar*). **B** Islet distribution analysis plotting the number of islets of different sizes vs. percentage of total islet number. The percentage of small islets to the total islets significantly increased in Ex4-treated mice (–○–) compared with that of vehicle-treated mice (–□–), as shown in (**C**), with an enlarged x-axial scale. *$p<0.05$, **$p<0.01$

background C57BLKS/J (control) mice (0.56± 0.03 μg/mg protein, $p<0.05$, $n=5$). The reduced pancreatic insulin storage in PBS-treated mice suggested that these mice might have a reduced capacity for insulin secretion in response to a glucose challenge. Indeed, this prediction was supported by an indirect study for pancreatic insulin release in which plasma insulin concentrations were measured during an OGTT (Fig. 4C). As seen, during the time course of the OGTT, the PBS-mice showed an abnormal plasma insulin response to the oral glucose challenge. In contrast, Ex4 treatment significantly improved the insulin response, consistent with the relatively normal pancreatic insulin concentrations in these mice (Fig. 4B).

To test the hypothesis that Ex4 treatment normalizes glycaemia and improves glucose tolerance in *db/db* mice through improved pancreatic islet function, in vitro pancreas perfusion experiments were utilised. As shown in Figure 4D, 20 mmol/l glucose alone induced a two to fivefold increase in insulin secretion from the perfused pancreas of Ex4-treated mice. However, the pancreas of vehicle-treated mice was unresponsive to the same concentration of glucose ($p<0.05$ vs Ex4, $n=5$). Addition of 10 nmol/l GLP-1 to the 20 mmol/l glucose perfusate induced insulin secretion from the pancreas of both groups of mice. However, the pancreas of Ex4-treated mice had a much greater response than vehicle-treated mice ($p<0.05$, $n=5$).

*Ex4 increases beta-cell mass and replication and decreases beta-cell apoptosis.* To determine the mechanisms by which Ex4 increased pancreatic insulin content and insulin secretion in response to a glucose challenge, beta-cell mass was determined in pancreas sections immunostained for insulin. This analysis demonstrated a 1.4-fold increase in beta-cell mass in Ex4-treated mice, as compared to PBS-treated mice (Fig. 5A, PBS vs Ex4: 2.2±0.2 vs 3.0±0.3 mg, $p<0.05$, $n=5$). Beta-cell mass in both groups of *db/d*b mice was greater than seen in control, 8-week-old C57BLKS/J mice (1.13±0.06 mg, $p<0.01$, $n=3$). Furthermore, examination of pancreatic sections from both groups of *db/db* mice revealed that the number of small islets including single beta cells was increased in Ex4-treated mice; islet distribution analysis (Fig. 5B) revealed a statistically significant increase in the percentage of small islets in Ex4-treated mice (Fig. 5C), suggesting an increase in islet neogenesis in response to Ex4 treatment. It is known that the absolute beta-cell mass is dynamic and is determined by two major factors: the rate of beta-cell proliferation and the rate of apoptosis [26]. On day 14, beta-cell proliferation was evaluated in both groups, based upon incorporation of BrdU. As shown in Figure 6, the Ex4-treated mice had significantly higher numbers of BrdU positive beta cells ($p<0.05$, vs PBS, $n=5$), reflecting a higher beta-cell proliferation rate. Furthermore, analysis of Tunel staining demonstrated a decrease in beta-cell apoptosis in Ex4-treated mice as compared to the vehicle-treated mice (Fig. 6, $p<0.05$, $n=5$).

*Ex4 up-regulates pancreatic Akt1 and MAPK.* Results of one dimensional (SDS PAGE) and two dimensional (first: pH, second: SDS PAGE) gel analysis showed that at least 15 to 20 proteins were up-regulated after 14 days of Ex4 treatment, while only a few proteins were down-regulated (data not shown). Western blot analysis using specific antibodies showed that these up-regulated proteins included Akt1 and MAPK (Fig. 7). Akt is a serine/threonine kinase that plays an important role in cell survival and in the protection of cells from apoptosis [27]. Furthermore, within the pancreas, the isoform Akt1 is expressed selectively in beta cells [28, 29]. A fivefold increase in expression

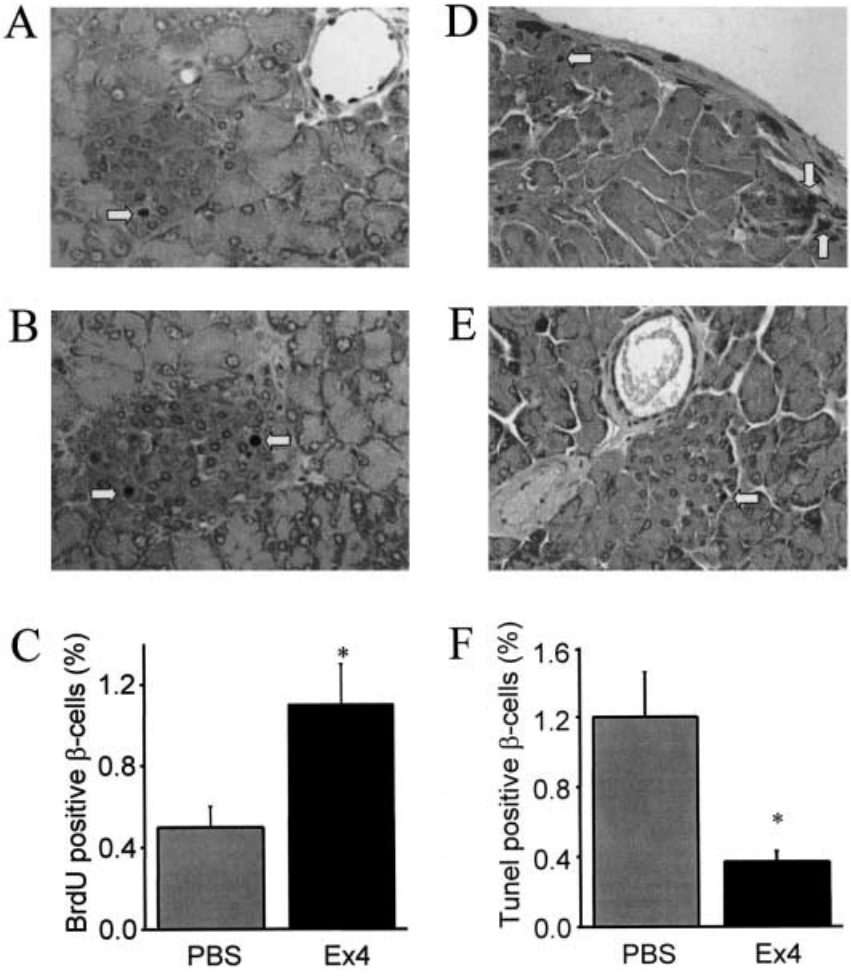



**Fig. 6A–F.** Effect of Ex4 on beta-cell replication and apoptosis. **A–B** Pancreatic sections were stained for insulin and BrdU. Representative examples of $BrdU^+$ beta cell(s) are shown in pancreatic sections from (**A**) vehicle- and (**B**) Ex4-treated *db/db* mice [$BrdU^+$ cells are indicated by the *arrow(s)*]. **C** 14 days Ex4 treatment (■) increased the number of $BrdU^+$ B-cells compared to that of vehicle-treated *db/db* mice ($p<0.05$, $n=5$). **D–E** Pancreatic sections were stained for insulin and by Tunel assay. Representative examples of $Tunel^+$ beta-cell(s) are shown in pancreatic secretions from (**D**) vehicle- and (**E**) Ex4-treated *db/db* mice [$Tunel^+$ cells are indicated by the *arrow*(*s*)]. **F** 14 days Ex4 treatment (■) decreased the number of $Tunel^+$ beta cells compared to that of vehicle treated *db/db* mice ($p<0.05$, $n=5$)

of total Akt1 protein was found in the pancreas of Ex4-treated mice as compared to that of vehicle-treated mice (Fig. 7B, $p<0.01$, $n=5$). suggesting an important role for Akt1 in regulating beta-cell function. MAPKs are ubiquitous signaling molecules [30, 31], activated by growth factors, including insulin and IGF-1, which are known to be important in mitogenesis. In the pancreatic beta cell, it had been demonstrated that MAPK signalling pathways can be activated by glucose or other secretagogues [14, 32], probably mediating some of the pleiotropic actions of these agents on the beta cell [14]. In this study, elevated expression (by sixfold, $p<0.01$) of a specific 44 kDa isoform of MAPK (ERK1) was associated with increased beta-cell mass in Ex4-treated mice (Fig. 7), suggesting a possible role for the MAPK pathway in regulating Ex4-induced beta-cell proliferation. In addition, Western blot analysis using a specific antibody against caspase-3, a member of the caspase family of cysteine proteases that are implicated in the apoptosis cascade [33], demonstrated a decrease in the content of the bioactive form of this apoptotic enzyme (~20 kDa; by 30%, $p<0.05$) in mice treated with Ex4, while the inactive proenzyme (~32 kDa) was increased (by 2.5-fold, $p<0.01$; Fig. 7D), consistent with the results of pancreatic section analysis for apoptosis (Fig. 6). Finally, no changes were observed in the levels of IRS-1, a protein involved in the insulin and IGF-1 signalling, in Ex4- vs PBS-treated mice.

## Discussion

This study aimed to determine the effects of GLP-1 administered to pre-diabetic *db/db* mice on the development of diabetes. The *db/db* mouse genetically lacking the leptin receptor, is a rodent model for Type

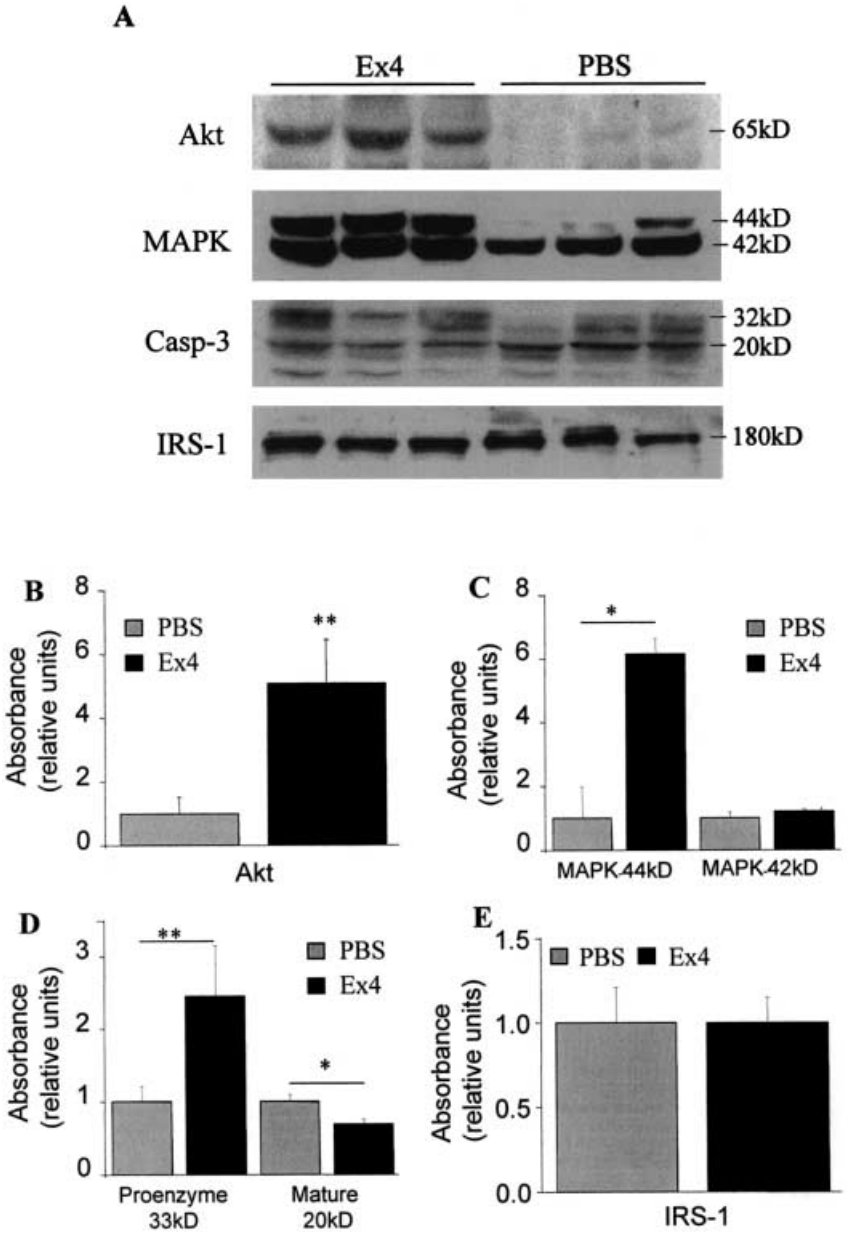



**Fig. 7A–E.** Effect of Ex4 on pancreatic protein expression. **A** Western blot analysis of pancreatic proteins using specific antibodies against Akt1, MAPK, Caspase-3 and IRS-1 (lane 1–3: pancreas from individual mice treated with Ex4; lane 4–6: pancreas from individual mice treated with PBS). **B** Akt1 protein expression was significantly increased in Ex4-treated mice (■). **C** 44 kDa-MAPK (ERK1), but not 42 kDa-MAPK (ERK2), protein expression was significantly increased in mice treated with Ex4 (■). **D** The inactive form of caspase-3 (proenzyme) was significant increased in mice treated with Ex4 (■); however, the bioactive form of the enzyme (mature) was significantly decreased in the Ex4-treated mice. **E** IRS-1 protein expression was not changed between the two groups of mice ($p>0.05$). *$p<0.05$; **$p<0.01$ ($n=4–5$)

II diabetes [21]. Our study has confirmed that obese mice developed diabetes by 8 weeks of age, in association with hyperinsulinaemia and insulin resistance. However, age-matched *db/db* mice treated with a potent GLP-1 analogue, Ex4, for 14 days had normoglycaemia, indicating that Ex4 treatment delayed the onset of diabetes in these mice. Whether Ex4 can completely prevent the onset of diabetes in these mice was not evaluated in the present study, as the *db/db* mice based upon the C57BLKS/J background have a severe form of progressive diabetes with loss of beta cells that leads to premature mortality.

Administration of GLP-1 or Ex4 stimulates short-term increases in insulin release and suppresses glucagon release thereby lowering blood glucose [7, 9, 34]. However, this effect of Ex4 was avoided in our study, as the half-life of Ex4 in rats is 67 min [25], and all experiments were carried out at least 24 h after Ex4

injection. Furthermore, body weight gain in these obese mice was not affected by once-daily Ex4 treatment (1 nmol/kg) for 14 days, consistent with the observation by others that once daily injection of Ex4 does not significantly affect body weight gain in *db/db* mice [35]. These results further suggest that the maintenance of fasting normoglycaemia in Ex4-treated *db/db* mice was not due to changes in food intake or body weight or both, which have been observed in Zucker rats after twice daily i.p. injection of Ex4 [36].

*Db/db* mice had impaired oral glucose tolerance as early as 6 weeks of age, and this impairment progressed with increasing age. Our results show that this loss of glucose tolerance in *db/db* mice was prevented, at least in part, by treatment of the mice with Ex4. Although controversial, GLP-1 has been reported to increase basal and insulin-stimulated glucose uptake [37], thus improving peripheral insulin sensitivity [8, 9]. However, the possibility that Ex4 maintained normoglycaemia and improved glucose tolerance in these mice through improved peripheral insulin action was ruled out because Ex4 treatment did not change insulin sensitivity in these mice. These findings indicate that peripheral insulin target sites are not likely to be the major contributor to the normoglycaemia and improved glucose tolerance observed in Ex4- as compared to saline-treated *db/db* mice. Indeed, once-daily Ex4 treatment reduced but was not able to prevent the progressive loss of glucose tolerance that occurs in these mice, probably due to their increasing obesity consequent to the absence of leptin action.

As GLP-1 is known to increase beta-cell mass in diabetic and ageing animals [15, 16, 17, 18, 19, 20], we hypothesized that enhanced islet beta-cell function might be prominent in the effects of Ex4 in *db/db* mice. Indeed, both our in vivo and in vitro results showed increased insulin release in response to glucose challenge in the obese mice treated with Ex4, further supporting this prediction. Findings in rats [38] and in humans with Type II diabetes [39] also demonstrated that GLP-1 improved islet function mainly by increasing glucose-dependent insulin secretion, rather than by improving peripheral insulin sensitivity. However, the results of our study also suggest that Ex4 induced chronic changes in islet function, as we observed the effects of Ex4 24 h after the last injection. This was confirmed by the demonstration that Ex4 treatment significantly increased beta-cell mass, in association with an increased ability of the pancreas to release insulin. These findings suggest that a functional beta-cell mass is important in controlling glycaemia in the obese *db/db* mice and is essential in preventing the onset of diabetes in these mice. Supporting this concept is the landmark United Kingdom Prospective Diabetes Study, which demonstrated that Type II diabetes is a progressive disease in which this progression is mainly due to declining beta-cell function [19, 40].

Functional beta-cell mass is dynamic and is controlled by the balance between beta-cell survival and beta-cell death [26]. The increased beta-cell mass shown in Ex4-treated mice occurred through both increased beta-cell proliferation and decreased beta-cell apoptosis, and these changes were associated with higher expression of the protein kinases Akt1 and MAPK (Fig. 7). Akt (also known as PKB) is a key enzyme mediating the insulin signal to GLUT4 in peripheral tissues, and thus plays an important role in glucose homeostasis [41]. Furthermore, a role for the PI3-K/Akt pathway in mediating cell survival has been found recently in isolated islets of Langerhans [42]. Recent data has also suggested that the activation of Akt by growth factors such as insulin and IGF-1 [28, 42, 43] could converge at the level of PI3-K to convey differential signals from stimuli to subcellular effectors [43], further supporting the essential role of Akt/PKB in regulating beta-cell function. A recent study has shown that ablating Akt2 in mice results in insulin resistance and a diabetes mellitus-like syndrome [44]. However, as the Akt2 null mouse does not exhibit any reduction in beta-cell mass [44], other isoforms of this kinase could be involved in the regulation of beta-cell proliferation and apoptosis. Consistent with this hypothesis, Akt1 is specifically expressed in the islet of Langerhans [42] where it is predominantly localized in the beta cells [28, 29]. The results of our study demonstrated that Ex4 treatment of *db/db* mice significantly increased Akt1 protein expression in association with increased beta-cell proliferation and decreased beta-cell apoptosis, suggesting a possible role of Akt1 in mediating GLP-1-induced proliferation and anti-apoptotic effects (Fig. 8). The notion is supported, at least in part, by recent findings showing that GLP-1 activates pancreatic duodenal homeobox gene 1 (PDX-1) DNA binding activity, as well as increases beta-cell proliferation, via a PI3-K dependent mechanism in INS-1 cells [13, 14]. Consistent with these findings, we have recently shown in INS-1 cells that GLP-1 induces Akt phosphorylation in parallel with the PI3-K-dependent incorporation of $^{3}$H-thymidine (QW & PLB unpublished observations). Thus, when taken together, these data strongly suggest a role for Akt1 in the GLP-1 induced stimulation of beta-cell mass.

MAPKs are ubiquitous components of signalling cascades [30, 31] that are initiated by various extracellular stimuli including growth factors such as insulin and IGF-1, and that lead to cell proliferation, protection of cells from apoptosis, and cell differentiation [45]. Recently, a signalling pathway involving specific isoforms of MAPK (ERK1 and ERK2) was identified in beta-cells [46, 47]. Ex4-treated mice showed a significant increase in the ERK1 (44 kDa) protein content but not in ERK2 (42 kDa) (Fig. 7), suggesting that ERK1 plays a role in regulating islet function by GLP-1. A possible role for ERK1 in regulating islet function has also been suggested by studies showing that secre-

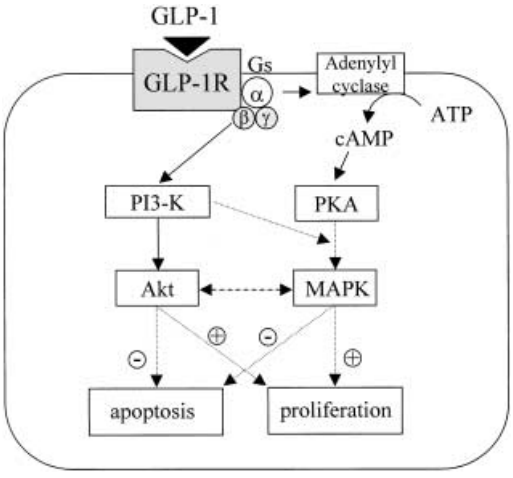



**Fig. 8.** A model showing the proposed roles of Akt1 and MAPK (ERK1) in transducing the stimulatory GLP-1 signal to beta-cell proliferation and the inhibitory GLP-1 signal to apoptosis. Activation of the GLP-1 receptor results in activation of PI3K/Akt1 initiating a signalling cascade involving cell proliferation and protection of cells from death. MAPK (ERK1) can be activated by the cAMP dependent pathway and, also through activation of PI3-K. Both the PI3K/Akt1 and MAPK signaling pathways are required for glucose-dependent and growth factor-induced cell mitogenesis, and possibly in the protection of beta-cells from apoptosis. The downstream effectors of the protein kinase A (PKA) pathway that lead to insulin secretion are not shown. *Solid lines* established pathways; *dotted lines* putative pathways

tagogue-stimulated insulin release is associated with the activation of ERK1, by a $Ca^{++}$ and glucose-dependent pathway in INS-1 cells [14]. Because of findings that glucose-stimulated insulin secretion is not inhibited by MAPK inhibitors, and that activation of MAPK causes rapid translocation of MAPK to the nucleus [48], it has been suggested that MAPK plays a role in a transcriptional regulation/proliferative action in beta-cells, rather than in the acute stimulus-secretion coupling process. Thus, the up-regulation of MAPK by Ex4 in the pancreas of the *db/db* mice may be linked to the observed increases in beta-cell proliferation. However, it must be noted that expression of ERK1 (p44 MAPK) is not limited to the beta-cells within the pancreas. Nonetheless, as the GLP-1 receptor is only found in the pancreatic islets and duct cells, and is not expressed by acinar cells [15, 49], these findings suggest that the marked effects of Ex4 on total pancreatic MAPK content could have been exerted indirectly.

Little is known about the mechanisms by which Akt and MAPK mediate the GLP-1 signal to intracellular effectors, and how the two molecules are themselves regulated. Recent data [29] showed that when Akt1 activity is suppressed by transfection of kinase-dead Akt1 into INS-1 cells, the MAPK signalling pathway appeared to be enhanced, suggesting that there is a requirement for both the PI3-K and MAPK signalling pathways for glucose-dependent, growth factor-induced cell mitogenesis, and possibly cell death.

In conclusion, our results suggest a potential therapeutic role for GLP-1 in the prevention of diabetes. Further study of the mechanisms by which Akt and MAPK mediate the GLP-1 signal in beta-cell growth and survival will contribute to a better understanding of the fundamental mechanisms underlying Type II diabetes, and could lead to new pharmacological approaches to prevent the development of Type II diabetes.

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