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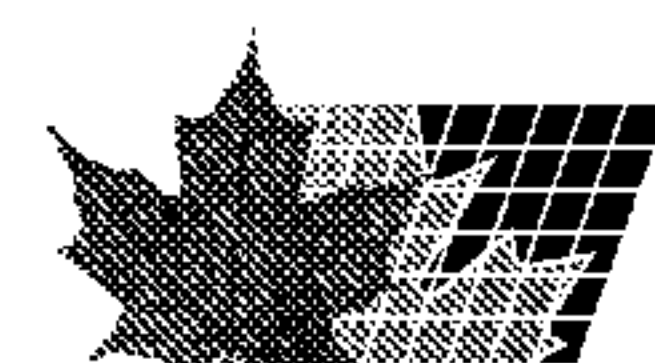
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(54) Titre : **METHODES ET COMPOSITIONS POUR LA PREVENTION DU DIABETE**

(54) Title: **METHODS AND COMPOSITIONS FOR PREVENTING DIABETES**

(57) **Abrégé/Abstract:**

The invention relates to a method of preventing and or/delaying diabetes in a subject, comprised administration to the subject an effective amount of a compound that increases beta-cell proliferation and/or reduces beta cell apoptosis in the subject. The compound preferably comprises of a GLP-1 polypeptide or an exendin-4 polypeptide.



**Abstract**

The invention relates to a method of preventing and or/delaying diabetes in a subject, comprised administration to the subject an effective amount of a compound that increases beta-cell proliferation and/or reduces beta cell apoptosis in the subject. The  
5 compound preferably comprises of a GLP-1 polypeptide or an exendin-4 polypeptide.

## **METHODS AND COMPOSITIONS FOR PREVENTING DIABETES**

### **Field of the Invention**

The invention relates to methods of preventing and/or delaying the onset of diabetes in a subject.

### **5 Background of the Invention**

There are two types of diabetes. Type 1 diabetes occurs when the pancreas produces very little or no insulin. Type 2 diabetes occurs when the pancreas does not produce enough insulin or when the body does not effectively use the insulin that is produced. The majority of patients have type 2 diabetes (over 90-95% of patients).

- 10 Diabetes is the leading cause of death of all diseases in North America. Currently, in Canada, over 2 million Canadians have diabetes. The number of diabetes patients is much higher in the United States. Numerous epidemiologic studies indicate that there will be an increase in the prevalence of prediabetic and diabetic populations worldwide. According to the World Health Organization (WHO), by 2025, the number of people with  
15 diabetes is estimated to rise to 300 million. The prevalence of diabetes increases with age. As the general population ages, the number of people with diabetes will rise substantially.

- Diabetes care represents a large proportion of all health care costs in North America. The WHO estimates that four to five percent of health budgets are spent on diabetes-  
20 related illnesses. In Canada, it is estimated that at least \$9 billion (USD) is spent annually on treating people with diabetes and its complications. It is known that a person with diabetes incurs medical costs that are two to five times higher than those of a person without diabetes. Current therapies, such as insulin injection, do not successfully prevent either the progression of diabetes or the long-term complications associated with  
25 this disease. Many researchers are trying to find treatments of diabetes.

- A product proposed for use as a therapeutic agent for the treatment of hyperglycemia in patients with type 2 diabetes is GLP-1 [7]. GLP-1 is a 30 amino acid peptide secreted from the L cells of the gastrointestinal tract in response to nutrient ingestion [6, 7, 8, 9]. GLP-1 administration has been shown to reduce hyperglycemia when administered to  
30 patients with type 2 diabetes [10].

The GLP-1 receptor is a 7 transmembrane, G protein-coupled receptor 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 signaling pathways involved in glucose-dependent insulin secretion [11,12]. All GLP-1 studies to date have  
 5 been conducted in animals with pre-established diabetes[15-19]. 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 2 diabetes. No published study prior to this invention established the effects of GLP-1 on the prevention of onset of diabetes, using pre-diabetic *db/db* mice as our model.

10 One could also target enzymes, such as Akt (also known as PKB), that mediate the insulin signal to GLUT4 in peripheral tissues, and thus play an important role in glucose homeostasis [41]. Furthermore, a role for the PI3-K/Akt pathway in mediating cell survival had been demonstrated recently in isolated islets of Langerhans [42]. Recent data has also suggested that the activation of Akt by growth factors such as insulin and  
 15 IGF-1 [28,42,43] may 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.

Other enzymes which could be targeted are MAPKs, which are ubiquitous components of signaling cascades [30,31] that are initiated by various extracellular stimuli including  
 20 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 signaling pathway involving specific isoforms of MAPK (ERK1 and ERK2) was identified in beta cells [46,47]. A possible role for ERK1 in regulating islet function has also been suggested by studies showing that secretagogue-stimulated insulin release is associated with the activation of  
 25 ERK1, via 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.

30 With diabetes accounting for such high mortality rates in Canada, the impact of this disease category on Canadian economy is staggering. Actually, the professional organizations such as CDA (Canadian Diabetes Association) and ADA (American

Diabetes Association) have been making great efforts of educating people to gain knowledge of prevention of diabetes that include implementation of lifestyle changes ie. dietary, exercise and so on. However, education itself has not been enough to create lifestyle changes that will prevent or delay the onset of diabetes, so there remains a  
 5 need for compounds that will achieve this result.

Diabetes could be prevented or delayed if insulin release and glucose tolerance could be increased in a subject as soon as signs of diabetes became apparent to a physician. One therapeutic target in the pancreas is the beta-cell. A landmark United Kingdom Prospective Diabetes Study demonstrated that type 2 diabetes is a progressive disease  
 10 and that this progression is due to declining beta cell function [19,40]. The maintenance of beta cell mass is a dynamic process, undergoing both increases and decreases to maintain glucose levels within a narrow physiological range [1-3]. The majority of patients with obesity causing insulin resistance are not diabetic, as their capacity for beta cell compensation is maintained; however, 15-20% of these individuals become diabetic  
 15 when the beta cells lose their compensatory ability [1]. The development of effective means to prevent and treat diabetes via modulating the beta cell is thus becoming of great importance.

### Summary of the Invention

The invention shows a therapeutic role for GLP-1 and exendin-4 and similar compounds  
 20 in the prevention or delay of the onset of diabetes. Akt and MAPK mediate the GLP-1 and exendin-4 signal in beta cell growth and survival.

### Brief Description of the Drawings

Preferred embodiments of the invention will be described in relation to the drawings in which:

25 **Figure 1. Effect of exendin-4 on fasting blood glucose in db/db mice.** Fasting blood glucose levels were measured at day 0, day 7 and day 14 in exendin-4 (i.p. injection, 1nmol/per kg, black bars) or PBS (vehicle, gray bars) injected *db/db* mice starting at the age of 6 weeks (n=10 each). \*\*: p < 0.01; \*\*\*: p < 0.001.

**Figure 2. Effect of exendin-4 on OGTT in db/db mice.** Glucose concentrations in  
 30 *db/db* mice undergoing an OGTT (1.5 g/kg) after exendin-4 (i.p., 1nmol/per kg, circles

and black bars) or vehicle (PBS, squares and gray 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-glycemic-curves (AUC) are shown in the right panels (A', B' and C').

**Figure 3. Effect of exendin-4 on insulin tolerance test 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 exendin-4 (i.p., 1nmol/per kg, circles) or vehicle (PBS, squares) treatment in *db/db* mice (n=5 each). No significant differences were seen between the two groups of animal ( $p > 0.05$ )

**Figure 4. Effect of exendin-4 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 exendin-4 (i.p. injection, 1nmol/per kg, black bar) or PBS (vehicle, gray bar) in fasted *db/db* mice. (B) On day 14 of treatment (black bar, total pancreatic insulin content was measured and expressed as  $\mu\text{g}/\text{mg}$  protein. (C) On day 14 of treatment, the plasma insulin concentration was measured during an OGTT using an Insulin-ELISA kit. Insulin levels 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 exendin-4 (circles, or vehicle, squares, in response to 2 mM and 20 mM glucose, and 20 mM glucose plus GLP-1 ( $1 \times 10^{-8}$  M)). \*Significantly different from PBS mice ( $P < 0.05$ ).

**Figure 5. Effect of exendin-4 on B cell mass in db/db mice.** (A) Effect of exendin-4 treatment on B cell mass ( $p < 0.05$ ,  $n = 5$ ; treatment: black bar, control:gray 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 exendin-4-treated mice (circles) compared with that of vehicle-treated mice (squares), as shown in (C), with an enlarged x-axial scale. \*:  $p < 0.05$ , \*\*:  $p < 0.01$

**Figure 6. Effect of exendin-4 on B cell replication and apoptosis.** (A-B) Pancreatic sections were stained for insulin and BrdU. Representative examples of BrdU<sup>+</sup> beta cell(s) are shown in pancreatic sections from (A) vehicle- and (B) exendin-4-treated *db/db* mice (BrdU<sup>+</sup> cells are indicated by the arrow(s)). (C) 14 days exendin-4 treatment (black bar) increased the number of BrdU<sup>+</sup> beta 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<sup>+</sup> beta cell(s) are shown in pancreatic secretions from (D) vehicle- and (E) exendin-4-treated *db/db* mice (Tunel<sup>+</sup> cells are indicated by the arrow(s)). (F) 14 days exendin-4 treatment (black bar) decreased the number of Tunel<sup>+</sup> beta cells compared to that of vehicle treated *db/db* mice ( $p < 0.05$ ,  $n =$   
 5 5).

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

**Figure 8. A model showing the 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  
 20 initiating a signaling cascade involving cell proliferation and protection of cells from death. MAPK (ERK1) can be activated via a 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.

**Figure 9. Akt kinase activation by GLP-1 in INS-1 cells.** INS-1 cells at 70-80 confluence were serum deprived for 2 hours and treated with or without GLP-1 (100 nM and 10 nM) for 4 hours. Cells lysates were collected and the proteins were separated by SDS-PAGE, and Western blot analysis was performed using anti-Akt1 rabbit IgG (1:500, Cell Signaling Technology, Inc.), and anti-Phospho-Akt (Ser473) (1:500, Cell Signaling  
 30 Technology, Inc.). The lower panel of images was obtained from the same blot as the image shown on the top panel, after stripping and re-probing procedures.

**Figure 10. Effect of GLP-1 on INS-1 cell proliferation in INS-1 cells.** INS-1 cells at 70-80 confluence cultured in 24-well plate were serum deprived for 2 hours and pre-treated with or without wortmannin (20 nM) for 20 min, and incubated with different concentrations of GLP-1 for 16 hours. The proliferation assay was performed during the last 4-hours incubation period using [3H-methyl]-thymidine (0.5µCi/0.5ml/well). TCA (5%) was used to precipitate the DNA and the radioactivity was counted by scintillation counter. The results indicate that GLP-1-stimulated  $\beta$  cell proliferation is PI3-kinase/Akt dependent.

**Figure 11. Drug induced INS-1 cell apoptosis is prevented by GLP-1 treatment**  
The INS-1 cells apoptosis was evaluated using APOPercentage technique (Biocolor Ltd. Ireland). Cells at 70 % confluence cultured in 96-well plate were serum deprived for 30 min, pre-treated with or without wortmannin (20 nM, 20 min), and subsequently with GLP-1 (10 nM) for 16 hours. The staurosporine-induced apoptotic cells were determined by APOPercentage Dye labelling.

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#### **Detailed Description of the Invention**

The invention provides a method for preventing and or delaying the onset of diabetes in a subject. A subject in need of such treatment is one that is at risk for developing diabetes, for example an obese person. The World Health Organization (WHO) defines obesity by reference to body mass index (BMI). This is a measure derived from dividing body weight in kg by the square of height in metres. A BMI between 18.5 and 25 is normal weight. An individual is overweight with a BMI between 25 and 30. An obese subject is defined as a subject with a BMI equal to or greater than 30. The subject may also be a person whose blood glucose is higher than average for that person's age and weight (normal blood glucose may be routinely determined from medical reference sources), although not high enough that the person is diagnosed diabetic. The subject may also be a person with a genetic history of diabetes who has not yet developed diabetes. Diabetes is diagnosed when the blood sugar levels are higher than an accepted normal range. According to CDA (Canadian Diabetes Association) standards, diabetes onset occurs when a subject has a fasting blood glucose level over 7.0 mmol/L, or a random (anytime of day) sugar that is greater than 11.1 mmol/L. Once diagnosed, any effort/means made to the patient, in order to combat the hyperglycemia, is treatment, rather than prevention. Some people, although not diabetic, (eg obese people, whose excess weight is usually associated with insulin resistance) have poor

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health and a higher risk of development of type 2 diabetes. To reduce or minimize their risk of developing type 2 diabetes, the compounds of the invention are administered to prevent or delay the onset of type 2 diabetes. The compounds of the invention may also be administered to prevent or delay the onset of type 1 diabetes.

5 The method of the invention comprises administering to the subject in need of prevention and/or delay of onset of diabetes, an effective amount of a compound that increases beta-cell proliferation or neogenesis. Proliferation refers to one beta-cell dividing into two beta cells. Neogenesis refers to the production of a totally new beta-cell from a progenitor cell or a stem cell. The compound also reduces beta cell  
 10 apoptosis in the subject. Increased proliferation and neogenesis and reduced apoptosis provide increased beta-cell mass. Protein kinase (Akt1 and/or p44MAPK) expression in the beta cell is increased by the compound. The compound also decreases caspase-3 activation. The effective amount of the compound increases insulin release and glucose tolerance in the subject. The compound preferably comprises a GLP-1 polypeptide or  
 15 an exendin-4 polypeptide. It may also be a compound having at least 60% sequence identity to a GLP-1 polypeptide or at least 60% sequence identity to an exendin-4 polypeptide. Other forms of GLP-1, analogues, derivatives thereof and fragments are disclosed in US Patent No. 6,268,343 and are incorporated herein by reference. The invention includes the use of all the aforementioned compounds in preventing and/or  
 20 delaying the onset of diabetes, such as type 2 diabetes. The invention also includes use of all the aforementioned compounds for preparation of a medicament for preventing and/or delaying the onset of diabetes, such as type 2 diabetes. The invention also includes a pharmaceutical composition, such as a prophylactic composition, for all the aforementioned uses. The sequences of GLP-1 and exendin-4 are as follows:

25 GLP-1 HAEGTFTSDV SSYLEGQAAK EFIAWLVKGR-NH<sub>2</sub>

(SEQ ID NO:1)

Exendin-4 HEGTFTSDL SKQMEEEAVR LFIEWLKNNG PSSGAPPPS-NH<sub>2</sub>

(SEQ ID NO:2)

Exendin-4 is a 39-residue peptide from the salivary secretions of *Heloderma suspectum*  
 30 having some pharmacological properties similar to GLP-1. In contrast to GLP-1,

exendin-4 forms monomeric helices in aqueous media. This helical state displays unusual thermal stability due to a hydrophobic C-terminal capping interaction. The absence of this C-capping interaction and the presence of a flexible, helix-destabilizing glycine at residue 16 in GLP-1 are proposed to be the basis for the diminished stability of the monomeric helical state of GLP-1. The greater intrinsic stability of the exendin-4 helix likely reduces the entropic cost of binding at a receptor that requires the C-terminal helix state.

In one embodiment, this invention showed the effects of GLP-1 on the prevention of onset of diabetes, using, in one example, pre-diabetic *db/db* mice as a model [21]. These mice lack a functional leptin receptor, and they spontaneously develop obesity, hyperinsulinemia, and glucose intolerance at 46 week of age, progressing to frank diabetes by 8 weeks of age. They have thus been used extensively and are recognized as a model for type 2 diabetes.

This invention showed 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 2 diabetes [21]. These obese mice develop diabetes by the age of 8 weeks, in association with hyperinsulinemia and insulin resistance. However, age-matched *db/db* mice treated with a potent GLP-1 analog, exendin-4, for 14 days exhibited normoglycemia, showing that exendin-4 treatment delayed the onset of diabetes in these mice.

Administration of GLP-1 and/or exendin-4 stimulates acute increases in insulin release and suppresses glucagon release thereby lowering blood glucose [7,9,34]. However, this effect of exendin-4 was avoided in the present study, as the half-life of exendin-4 in rats is 67 min [25], and all experiments were carried out at least 24 hr after exendin-4 injection. Furthermore, body weight gain in these obese mice was not affected by once-daily exendin-4 treatment (1 nmol/kg) for 14 days. These results further show that the maintenance of fasting normoglycemia in exendin-4-treated *db/db* mice was not due to changes in food intake and/or body weight.

*Db/db* mice exhibited impaired oral glucose tolerance as early as 6 weeks of age, and this impairment progressed significantly with increasing age. This loss of glucose tolerance in *db/db* mice was prevented, at least in part, by treatment of the mice with

exendin-4. 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 exendin-4 maintained normoglycemia and improved glucose tolerance in these mice through improved peripheral insulin action was ruled out  
 5 by the demonstration that exendin-4 treatment did not change insulin sensitivity in these mice. These findings show that peripheral insulin target sites are not likely to be the major contributor to the normoglycemia and improved glucose tolerance observed in exendin-4 as compared to saline-treated *db/db* mice. Indeed, once-daily exendin-4 treatment reduced but was unable to prevent the progressive loss of glucose tolerance  
 10 in these animals, likely 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 aging animals [15-19], we then showed that enhanced islet beta cell function might be prominent in the effects of exendin-4 in *db/db* mice. Indeed, both *in vivo* and *in vitro* results showed increased  
 15 insulin release in response to glucose challenge in the obese mice treated with exendin-4. This perception is also supported by findings in rats [38] and in humans with type 2 diabetes [39], which demonstrated that GLP-1 improved islet function mainly by increasing glucose-dependent insulin secretion, rather than improving in peripheral insulin sensitivity. Exendin-4 also induced chronic changes in islet function, as shown by  
 20 the observed effects of exendin-4 24 hr after the last injection. This was confirmed by the demonstration that exendin-4 treatment significantly increased beta cell mass, in association with an increased ability of the pancreas to release insulin. These findings showed that a functional beta cell mass is important in controlling glycemia in the obese  
*db/db* mice and is essential in preventing the onset of diabetes in these mice.

25 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 demonstrated in exendin-4 treated mice occurred through both increased beta cell proliferation and decreased beta cell apoptosis, and these changes were associated with elevated expression of the protein kinases Akt1 and MAPK (Fig 7). Exendin-4 treatment of *db/db* mice significantly  
 30 elevated Akt1 protein expression level in association with increased beta cell proliferation and decreased beta cell apoptosis, showing a role of Akt1 in mediating GLP-1-induced proliferation and anti-apoptotic effects (Fig 8). In INS-1 cells that GLP-1 induces Akt phosphorylation in parallel with the PI3-K-dependent incorporation of <sup>3</sup>H-

thymidine. The invention shows the role for Akt1 in the GLP-1 induced stimulation of beta cell mass.

Exendin-4-treated mice showed a significant increase in the ERK1 (44kDa) protein levels but not ERK2 (42kDa) (Fig 7), showing that ERK1 plays a role in regulating islet function by GLP-1. The up-regulation of MAPK by exendin-4 in the pancreas of the *db/db* mice is linked to the observed increases in beta cell proliferation. 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 B and D cells of the pancreatic islets, and is not expressed by acinar cells [49], these findings show that the marked effects of exendin-4 on total pancreatic MAPK levels have been exerted indirectly.

#### **Modifications to polypeptide sequence**

Changes which result in production of a chemically equivalent or chemically similar amino acid sequence are included within the scope of the invention. Polypeptides having sequence identity to exendin-4 or GLP-1 receptor are tested to ensure that they are suitable for use in the methods of the invention. US Patent No. 6,268,343, which is incorporated by reference in its entirety, describes a number of GLP-1 derivatives. Variants of the polypeptides of the invention may occur naturally, for example, by mutation, or may be made, for example, with polypeptide engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine.

Therefore, the invention includes polypeptides having conservative changes or substitutions in amino acid sequences. Conservative substitutions insert one or more amino acids, which have similar chemical properties as the replaced amino acids. The invention includes sequences where conservative substitutions are made that do not destroy compound activity.

Polypeptides comprising one or more d-amino acids are contemplated within the invention. Also contemplated are polypeptides where one or more amino acids are acetylated at the N-terminus. Those with skill in the art recognize that a variety of techniques are available for constructing polypeptide mimetics with the same or similar  
5 desired compound activity as the corresponding polypeptide compound of the invention but with more favorable activity than the polypeptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See, for example, Morgan and Gainor, Ann. Rep. Med. Chem., 24:243-252 (1989). Examples of polypeptide mimetics are described in U.S. Patent Nos. 5,643,873. Other patents describing how to make and  
10 use mimetics include, for example in, 5,786,322, 5,767,075, 5,763,571, 5,753,226, 5,683,983, 5,677,280, 5,672,584, 5,668,110, 5,654,276, 5,643,873. Mimetics of the polypeptides of the invention may also be made according to other techniques known in the art. For example, by treating a polypeptide of the invention with an agent that chemically alters a side group by converting a hydrogen group to another group such as  
15 a hydroxy or amino group. Mimetics preferably include sequences that are either entirely made of amino acids or sequences that are hybrids including amino acids and modified amino acids or other organic molecules.

The invention also includes hybrid and polypeptides, for example where a nucleotide sequence is combined with a second sequence.

20 The invention also includes polypeptide fragments of the polypeptides of the invention which may be used to confer compound activity if the fragments retain activity. The invention also includes polypeptides fragments of the polypeptides of the invention which may be used as a research tool to characterize the polypeptide or its activity. Such polypeptides preferably consist of at least 5 amino acids. In preferred embodiments,  
25 they may consist of 6 to 10, 11 to 15, 16 to 25, 26 to 50, 51 to 75, 76 to 100 or 101 to 250 amino acids. Fragments may include sequences with one or more amino acids removed, for example, C-terminus amino acids in a compound sequence.

#### **Enhancement of compound polypeptide activity**

30 The activity of the compound polypeptide is increased or decreased by carrying out selective site-directed mutagenesis. A DNA plasmid or expression vector containing the nucleic acid molecule or a nucleic acid molecule having sequence identity is preferably

used for these studies using the U.S.E. (Unique site elimination) mutagenesis kit from Pharmacia Biotech or other mutagenesis kits that are commercially available, or using PCR. Once the mutation is created and confirmed by DNA sequence analysis, the mutant polypeptide is expressed using an expression system and its activity is  
5 monitored.

The invention also includes polypeptides which have sequence identity at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to a sequence of the invention (or a partial sequence thereof). Modified polypeptide molecules are discussed below.  
10 Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above). BLAST is a series of programs that are available online at  
15 <http://www.ncbi.nlm.nih.gov/BLAST>. The advanced blast search (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1>) is set to default parameters. (ie Matrix BLOSUM62; Gap existence cost 11; Per residue gap cost 1; Lambda ratio 0.85 default).

References to BLAST searches are:

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Zhang, J. & Madden, T.L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." *Genome Res.* 7:649\_656. Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

- 5 The invention includes polypeptides with mutations that cause an amino acid change in a portion of the polypeptide not involved in providing activity or an amino acid change in a portion of the polypeptide involved in providing activity so that the mutation increases or decreases the activity of the polypeptide.

#### **Pharmaceutical compositions**

- 10 The compound and functional equivalent polypeptides are useful when used alone, but are also useful when combined with other components such as a carrier in a pharmaceutical composition. All or part of a compound may be administered to a subject, such as a human, in soluble form to prevent or delay the onset of diabetes.

- 15 The pharmaceutical compositions can be administered to humans or animals by a variety of methods including, but not restricted to topical administration, oral administration, aerosol administration, intratracheal instillation, intraperitoneal injection, and intravenous injection. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration. An example of a dosage for humans would be 0.2 nmol/kg of body weight or between 0.05 to 0.8 nmol/kg of body weight. Polypeptides may be
- 20 introduced into cells using *in vivo* delivery vehicles such as liposomes.

- 25 The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the nucleic acid molecule or polypeptide is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA).

- 30 On this basis, the pharmaceutical compositions could include an active compound or substance, such as a compound nucleic acid molecule or polypeptide, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of

combining the active molecules with the vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within tissue.

#### **Screening for activators and inhibitors**

- 5 Polypeptides having sequence identity to exendin-4 or GLP-1 receptor are tested to ensure that they are suitable for use in the methods of the invention. Small organic molecules are also tested. The invention includes compounds which are identified with the screening methods of the invention and which are suitable for methods and uses of the invention and in pharmaceutical compositions of the invention. In a preferred  
10 embodiment, the invention includes an assay for evaluating whether a candidate compound is capable of increasing Akt-1 or MAPK expression or activity or decreasing caspase-3 expression or activity, by culturing cells (preferably beta-cells) in the presence of at least one compound whose ability to modulate (inhibit or activate) expression activity is sought to be determined and thereafter monitoring the cells for either an  
15 increase or decrease in the level of Akt-1 or MAPK expression or activity or decreasing caspase-3 expression or activity.

A receptor binding assay is the preferred method to evaluate the specificity of a compound for the cell membrane receptor, as all signaling transducing events are initiated from this ligand-receptor binding. If a candidate compound binds to the receptor  
20 (for example, as identified with a gel-shift mobility assay), this binding indicates that the compound is suitable for use in the subsequent steps of the invention. Receptor activation assays are used to further determine the suitability of a candidate compound for the methods of the invention. For example, cAMP determination can be used to evaluate the receptor activation (GLP-1 receptor is GPCR). In addition, an Akt kinase  
25 assay can further show the activation of Akt. In the initial screens, when there are large numbers of compound candidates, a receptor binding assay can be used. Compounds that bind to the receptor are preferably subjected to cAMP determination, and finally an Akt kinase assay. Small organic molecules may also be tested as candidate compounds for their suitability for use in the methods of the invention. To this end, cAMP  
30 determination is optionally used to screen for GPCR binding and activation. As per the rationale described above, Akt kinase assay, or MAPK assay is optionally used to evaluate the cellular effectivity of the compounds.

To validate both screened peptide and organic molecule compounds, beta cell mass analysis can be performed in the pre-diabetic animal model after treatment of the animal with the compounds for a longer period (i.e. 28 weeks). To this end, an additional Insulin-release assay can also performed using an insulin radioimmunoassay kit (Linco Research, St. Louis, MO). These experimental approaches confirm the growth effects of the screened compounds on the beta cells.

**Descriptions of sample protocols used to test candidate compounds are provided below.**

10 *Beta cell mass analysis:*

Pancreatic sections (4 mm) are processed as previously described [DT Finegood Diabetes 50: 1021-1029, 2001]. Briefly, following dewaxing, dehydration and antigen retrieval (by boiling in citrate buffer), sections are incubated overnight at 4°C with guinea pig anti-insulin antibody (Dako Diagnostics, Mississauga, ON, Canada). The samples are then incubated for 1 h with biotinylated anti-guinea pig antibody (Vector Laboratories, Burlington, ON, Canada), and subsequently treated for 1 h with avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA.). Sections are then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) for 10 min. After DAB staining, the sections are washed with tap water and counterstained with hematoxylin. Beta cell mass from the insulin antibody-stained sections is measured using a Nikon (ECLIPSE-E1000) microscope connected to a video camera equipped with a color 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 are calculated as the sum of the determinations from each of the 8-10 segments of pancreas. A total of 1000-1500 beta cells are counted per pancreas. Total beta cell mass per pancreas is determined as the product of the total cross-sectional beta cell area /total tissue area and the weight of the pancreas before fixation.

*Receptor binding assay:*

30 Compound is iodinated by a classical Chloramines-T method (WM Hunter et al, Nature 194, 495-6 1962). The receptor binding assay is performed as described previously (Q

Wang et al, Cell Physiol. Biochem. 8 304-13, 1998): isolated islet cells and insulin secreting cells are suspended in PBS and centrifuged at 600 G for 10 min, and the cell pellets are resuspended in aliquots of PBS. The binding of the iodinated compound to the intact cells is carried out in 7 x 35 mm polystyrene tubes at 4°C in 300 µl assay  
 5 buffer (PBS containing 0.2% BSA) with the labeled compound (20,000 cpm) in the presence or absence of unlabelled compounds. After 4.5 hours of incubation, when the assay system reaches an equilibrium state, cold PBS is added, and the samples are centrifuged for 10 min at 600 g at 4°C. The supernatant is discarded. After washing the cell pellets with cold PBS, the radioactivity is measured in a gamma counter.

#### 10 *cAMP determination:*

cAMP determination is a method that can evaluate the G-protein coupled receptor (GPCR) activation (D Lee et al, Biochim Biophys Acta 1490 311-23 2000). Intracellular cAMP levels were determined in isolated islet cells or cultured insulin-secreting cells that  
 15 cultured in 35 mm<sup>2</sup> dishes. They are preincubated in the buffer containing 130 mM NaCl, 5 mM KCl, 1 mM sodium phosphate, 1 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 20 mM HEPES buffer (pH 7.4), 6 mM glucose, and 0.1% BSA (RIA grade, Sigma) for 1 h. The PKA inhibitors were added for 20 min, and isobutyl methylxanthine (100 µM) for 10 min before addition of the compound for 20 min. Cells were washed three times in ice-cold PBS, cAMP extracted with hydrochloric acid (0.1M, 300 µl) and measured as per the enzyme  
 20 immunoassay kit (Assay Designs Inc., Ann Arbor, MI).

#### *PI 3-kinase activity assay:*

PI3-kinase is upstream of Akt (Wang Q et al, Mol. Cell. Biol. 19(6):4008-18, 1999). Whole cell lysates are obtained from isolated islet cells and insulin-secreting cell line (eg  
 25 INS-1 cells and beta TC cells) pre-treated with the compound for 20 min, and PI 3-kinase is immunoprecipitated using an antibody against the p85-regulatory subunit of PI 3-kinase (Santa Cruz Biotechnology). Activity is detected and quantitated by measuring the formation of [32P]PI 3-phosphate (Q Wang et al, Biochem. J. 331(3):917-928, 1998). Briefly, after overnight incubation with the antibody-coated beads, the bound protein is washed three times with buffer I (PBS containing 1% Nonidet P-40 and 100 µM  
 30 Na<sub>3</sub>VO<sub>4</sub>), three times with buffer II (100 mM Tris-HCl (pH 7.5), 500 mM LiCl, and 100 µM Na<sub>3</sub>VO<sub>4</sub>), and finally three times with buffer III (Tris-HCl (pH 7.5), 100 mM NaCl, 1

mM EDTA and 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>). After washing, immunoprecipitates are resuspended in 50  $\mu$ l buffer III with the addition of 10  $\mu$ l 100 mM MgCl<sub>2</sub> and 10  $\mu$ l PI (2  $\mu$ g/ml). The samples sit at room temperature for 5 min before the addition of 10  $\mu$ l ATP (ATP 440  $\mu$ M with 30  $\mu$ Ci/10  $\mu$ l [<sup>32</sup>P]ATP). The samples are then shaken at room temperature for 10 min. The reaction is stopped by the addition of 20  $\mu$ l 8 N HCl and 160  $\mu$ l chloroform-methanol (1:1). The lipids are extracted by standard methods, dried down, resuspended in 20  $\mu$ l chloroform-methanol (1:1), and separated on thin layer silica gel plates (pretreated with 10% w/v potassium oxalate) in a solvent system of chloroform-methanol-water-NH<sub>4</sub>OH (60:47:11:2.2, vol/vol/vol/vol). Incorporation of <sup>32</sup>P into PI 3 phosphate is detected by autoradiography, and activity is quantified using a Molecular Dynamics PhosphorImager System (Sunnyvale, CA).

#### *Apoptosis assay:*

After compound treatment (0.5 to 16 hours), the apoptotic rate of isolated islet cells and/or insulin-secreting cell line (eg INS-1 cells and beta TC cells) is determined using APOPercentage Assay Kit (Biocolor Ltd. Ireland) according to manufacture's instruction. In animal model, pancreas sections obtained from subjects treated with or without compound were double immunostained for insulin, as described above, and for fragmented DNA by Tunel assay, which detects fragmented nuclei characteristic of apoptotic cells. Tunel staining was performed using ApopTag Kit (Intergen, Purchas, NY) according to manufacture's instruction. 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<sup>+</sup> beta cells.

#### *Akt kinase assays:*

After compound treatment for 20 min, whole cell lysates are obtained from isolated islet cells and insulin-secreting cell line (eg INS-1 cells and beta TC cells), using lysis buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 30 mM sodium pyrophosphate, 10 mM NaF, 1mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM dithiothreitol [DTT], and 100 nM okadaic acid. (Wang Q et al, Mol. Cell. Biol. 19(6):4008-18, 1999). Akt antibody is precoupled (16 hours) to a mixture of protein A- and protein G-Sepharose beads. These antibody-bead complexes are washed twice with phosphate-

buffered saline (PBS) and once with lysis buffer (4°C). Akt is immunoprecipitated by incubating 200 mg of total cellular protein with the anti-Akt-bead complexes for 2 to 3 h with constant rotation (4°C). Akt immunocomplexes are washed four times with 1 ml of wash buffer (25 mM HEPES [pH 7.8], 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 5 0.1% [wt/vol] bovine serum albumin, 1 M NaCl, 1mM DTT, 1mM phenylmethylsulfonyl fluoride, 1 mM microcystin, and 100nM okadaic acid) and twice with 1ml of kinase buffer (50 mM Tris-HCl [pH 7.5], 10mM MgCl<sub>2</sub>, and 1mM DTT). The immunocomplexes are incubated with constant agitation for 30 minutes at 30°C with 30 mL of reaction mixture (kinase buffer containing 5 mM ATP, 2 mCi of [<sup>32</sup>P]ATP, and 100 mM Crosstide). 10 Following the reaction, 30 microlitre of the supernatant is transferred onto Whatman p81 filter paper and washed four times for 10 minutes each time with 3 ml of 175 mM phosphoric acid and once with distilled water for 5 min. The filters are air dried and then subjected to liquid scintillation counting.

*MAP kinase assay:*

15 After 20 min treatment of the compound, beta cells are labeled with 1.25 microcurie <sup>32</sup>Pi/group (NEN Life Science Products, Boston, MA) in phosphate-free RPMI medium without serum for 3 h at 37°C. The cells are harvested and placed in RPMI with 100 ng/ml LBP (PS-binding protein) and treated with compounds for 30 min. After the compound incubation, the cells are stimulated with LPS for 15 min at 37°C. The cells 20 are harvested, resuspended in lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M Na<sub>3</sub>PO<sub>4</sub> (pH 7.2), 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 μM okadaic acid, 100 μg/ml PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg/ml pepstatin, all from Boehringer Mannheim), and sonicated. MEK is immunoprecipitated from the lysate, and the sample separated on a 10% SDS-PAGE discontinuous gel, and immuno-blotting is 25 performed using anti-phosphor-MEK antibody (Oncogene Research Products, San Diego, CA).

Other assays (as well as variations of the above assay) will be apparent from the description of this invention and techniques such as those disclosed in U.S. Patent No. 5,851,788, 5,736,337 and 5,767,075 which are incorporated by reference in their entirety. 30 For example, a candidate compound may be incubated with beta cells to determine if the candidate compound inhibits caspase-3 by Western blot analysis using specific antibody against phosphor- caspase-3 (Oncogene Research Products, San Diego, CA).

Sequences of some compounds optionally used in the assays described in this application are as follows:

Human Akt/Protein kinase B:

```

1      mkterprpnt fiirclqwtv viertfhvet peereewtta iqtvadgikk qeeeemdfsr
5  61    gspsdnsgae emevslakpk hrvtmnefey lkllgkgtfg kvilvkekat ayyamkilkk
      121    evivakdeva htltenvvqq nsrhpfitrl kysfqthdrl cfvmeyangg elffhlsrer
      181    vfaedrarfy gaeivsaldy lhseknvvyr dlklenlml dkgihikidf glckegikdg
      241    atmktfcgtp eylapevled ndygravdww glgvvmyemm cgrlpfynqd heklfelilm
      301    eeirfptlg peaksllsll lkkdpkqrlg ggsedakeim qhrfftgiwv qhvyekklsp
10  361    pfkpqvtset dtryfdeeft aqmititppd qddsmecvds errphfpqfs yspata

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(SEQ ID NO:3)

Human MAP kinase:

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1      msdskcdsqf ysvqvadstf tvlkryqqk pigsgaaggiv caafdtvlgv nvavkklsrp
      61    fqngthakra yrelvllkv nhkniislln vftpqklee fqdvyvlmel mdanlcqvih
15  121    meldhermsy llyqmlcgik hlhsagiihr dlkpsnivvk sdctlkildf glartactnf
      181    mmtpyvvtry yrapevilgm gykenvdiws vgcimgelvk gcvifqgtdh idqwnkvieq
      241    lgtpsaefmk klqptvmyv enrpkypgik feelfpdwif pseserdkik tsqardllsk
      301    mlvidpdkri svdealrhy itwydpaea eapppqiya qleerehaie ewkeliykev
      361    mdweerskng vvkdpssdaa vssnatpsqs ssindissms teqtlasdtd ssldastgpl
20  421    egcr (SEQ ID NO:4)

```

Human caspase-3:

1       mentensvds ksiknlepki ihgsesmdsg mswdtgykmd ypemglciii nnknfhkstg  
61       mtsrsgtdvd aanlretfrn lkyevrnknd ltreeivelm rdvskedhsk rssfvcvlls  
121      hgeegiifgt ngpvdllkit nffrgdrors ltgkpklfii qacrgteldc gietdsgvdd  
181      dmachkipvd adflyaysta pgyysswrnsk dgswfiqslc amlkqyadki efmhiltvvn  
5       241     rkvatefesf sfdatfhakk qipcivsmll kelyfyh (SEQ ID NO:5)

### Examples

#### GLP-1 treatment prevents the onset of diabetes in young db/db mice

Glucagon-like peptide-1 (GLP-1) stimulates glucose-dependent insulin secretion, and is thus under investigation for the management of type 2 diabetes. Recent studies have  
10 implicated GLP-1 as a B cell growth factor. Therefore, to determine whether GLP-1 can prevent the onset of diabetes, 6 wk db/db mice were treated for 2 wk with daily ip injections of vehicle or exendin-4 (1 nmol/kg), a GLP-1 receptor agonist. Fasting blood glucose (FBG) was normal in mice at the initiation of treatment (PBS vs exendin-4: 5.2±0.5 vs 5.1±0.5 mM; n=10 each). At 8 wk, PBS-treated mice were diabetic (FBG:  
15 10.4±0.5 mM), whereas exendin-4-treated mice demonstrated a significantly reduced FBG (6.1±1.0 mM; p<0.001), and were thus, not diabetic. Oral glucose tolerance tests demonstrated that exendin-4 treatment significantly improved glucose tolerance (area-under-the-curve/AUC PBS vs exendin-4: 3467±237 vs 2888±285, p<0.01; n=10). However, an insulin sensitivity test showed no significant difference between the 2  
20 groups (p>0.05; n=5), suggesting that peripheral insulin target sites are not the major contributors to the normal blood glucose levels in mice treated with exendin-4. Fasting plasma insulin levels (by RIA) in PBS-mice were higher than those of exendin-4-treated mice (PBS vs exendin-4: 17.4±2.4 vs 9.6±1.8 ng/ml; p<0.02; n=10), consistent with hyperinsulinemia due to hyperglycemia. However, the pancreatic insulin content was  
25 significantly lower in PBS- as compared with exendin-4-treated mice (5.6±0.07 and 9.1±0.03 [μg]/pancreas, respectively; p<0.03; n=10). Furthermore, insulin levels (by Elisa) during oral glucose tolerance test indicated increased release of insulin in exendin-4-treated mice as compared to PBS-treated mice (Delta-AUC for PBS vs exendin-4: 31.1.3±41.0 vs 103.3.1±31.3 P<0.05; n=5). To determine the mechanism by  
30 which exendin-4 increased pancreatic insulin content and secretion, beta cell mass was

determined from the total area immunostained for insulin as compared to the total pancreatic area, using ImagePlus software. This analysis demonstrated a 1.37-fold increase in B cell mass in exendin-4-treated mice as compared with PBS-mice ( $p < 0.05$ ). The results of this study demonstrate that exendin-4-treatment prevents the onset of diabetes in 8 wk db/db mice, through a mechanism involving expansion of the B cell mass, resulting in increased insulin production. The precise mechanism by which exendin-4 improve pancreatic beta cell function in the control of glucose homeostasis is currently under investigation.

***Glucagon-like peptide-1 (GLP-1) stimulates insulin secretion***

Akt1 mediates GLP-1-stimulated beta cell proliferation and protection from apoptosis in INS-1 cells. Glucagon-like peptide-1 (GLP-1) stimulates glucose-dependent insulin secretion in vitro and in vivo. GLP-1 treatment prevents the onset of diabetic symptoms through stimulation of insulin secretion and enhancement of beta cell mass in 8-wk old db/db mice. The GLP-1 stimulated increase in beta cell mass is associated with an increased beta cell proliferation and a decreased rate of beta cell apoptosis. These events are coupled with increased pancreatic expression of the beta cell signaling molecule Akt1, showing that Akt1 is important in mediating the mitogenic and/or anti-apoptotic effects of GLP-1 on beta cells. Akt is a serine/threonine kinase and is a central mediator of cellular proliferation and protection against apoptosis. In order to further confirm that the GLP-1 stimulated increase in beta cell mass is dependent on Akt, an in vitro cell culture system INS-1 pancreatic beta cell line, showed that GLP-1 promotes beta cell proliferation, and/or inhibits beta cell apoptosis. A 16-hr incubation of INS-1 cells with GLP-1 or its potent analog exendin-4 ( $1 \times 10^{-11}$  to  $10^{-7}$  M) increased the rate of  $^3\text{H}$ -thymidine incorporation into DNA by up to 2fold ( $0.9 \pm 0.15$  vs  $1.9 \pm 0.16$ ;  $p < 0.01$ ;  $n=4$ ), showing that GLP-1 promotes cellular proliferation. Western blot analysis demonstrated that GLP-1 or exendin-4 treatment of INS-1 cells (10 nM, 4hr) resulted in marked phosphorylation of Akt (by 2.5-fold,  $p < 0.01$ ,  $n=3$ ), while the total protein levels of Akt1 remained unchanged. Using the APOPercentage Apoptosis Assay technique, we showed that staurosporine-induced apoptosis of INS-1 cells was significantly prevented by 16-hr pretreatment of the cells with 10 nM GLP-1 or exendin-4 ( $p < 0.01$ ). As Akt1 is immediate downstream molecule from PI-3 kinase, the role of Akt in mediating the effects of GLP-1 on cellular proliferation and protection of the cells against death was further shown using the PI3-kinase inhibitor wortmannin. The results demonstrated that

GLP-1-induced cellular proliferation and protection from drug-induced cellular death were fully blocked by co-treatment with wortmannin (20 nM). Therefore, Akt1 mediates the effects of GLP-1 on beta cell proliferation and apoptosis, resulting in enhanced beta cell mass.

## 5 ***Exendin-4 normalizes fasting blood glucose in db/db mice***

Exendin-4 treatment was initiated in *db/db* mice at 6 weeks of age. All experiments were performed at least 24 hrs after the last injection to ensure clearance of the exendin-4 from the circulation [25]. As shown in figure 1, fasting blood glucose was in the normal range in both PBS (vehicle) and exendin-4 treated groups at the initiation of treatment (day 0, PBS vs. exendin-4:  $5.2 \pm 0.5$  vs.  $5.1 \pm 0.5$ ;  $n = 10$ ). On day 7 of the treatment, though still in the normal range, vehicle-treated group showed a noticeable increase in fasting blood glucose when compared to mice treated with exendin-4 (PBS vs. exendin-4:  $6.5 \pm 0.8$  vs.  $4.9 \pm 0.5$ ;  $n = 10$ ). By the age of 8 weeks (day 14), PBS-injected mice had developed hyperglycemia ( $10.4 \pm 0.5$ ,  $n = 10$ ,  $p < 0.001$  vs. day 0), whereas exendin-4-treated mice maintained fasting blood glucose levels in the normal range ( $6.1 \pm 1.0$ ,  $n = 10$ ,  $p < 0.01$  vs. vehicle-treated mice). The fasting glycemia of the exendin-4-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).

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## ***Exendin-4 improves OGTT, insulin tolerance and insulin release***

Before initiation of exendin-4 treatment (day 0), the OGTT patterns for the two groups of mice were similar (Fig 2 A) and there was no noticeable difference between the groups when the glycemic response was expressed as the area under curve (AUC) (Fig 2 A', PBS vs. exendin-4:  $1333 \pm 130$  vs.  $1316 \pm 104$  mM\*120 min,  $p > 0.05$ ,  $n = 10$ ). However, as compared to background (control) C57BLKS/J mice, both groups of *db/db* mice exhibited impaired oral glucose tolerance (AUC for C57BLKS/J mice 6 wk:  $1014 \pm 68$  mM\*120 min,  $n = 5$ ,  $p < 0.05$  vs. 6 wk-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

30

was observed in the mice treated with exendin-4 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. exendin-4:  $2056 \pm 251$  vs.  $1572 \pm 175$  mM\*120 min,  $p < 0.05$ ,  $n=10$ ) and day 14 (Fig 2C', PBS vs. exendin-4:  $2501 \pm 157$  vs.  $2036 \pm 194$  mM\*120 min,  $p < 0.05$ ,  $n=10$ ), and were higher for the *db/db* mice than in control, 8 wk C57BLKS/J mice ( $974 \pm 61$  mM\*120 min,  $P < 0.01$ ,  $n=5$ ).

The enhancement of glucose tolerance in the *db/db* mice treated with exendin-4 might be due to an improvement in insulin action on peripheral tissues, and/or elevated pancreatic beta cell function. To evaluate the effect of exendin-4 on peripheral insulin action, we performed an insulin tolerance test in these obese mice by determining blood glucose levels after an i.p. injection of insulin (Fig 3). Insulin sensitivity was reduced in both groups of *db/db* mice as 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- and exendin-4-treated mice, suggesting that peripheral insulin target sites were not the major contributors to the normoglycemia and improved glucose tolerance observed in the mice treated with exendin-4.

On day 14, consistent with their hyperglycemia, the fasting plasma insulin levels in PBS-mice were extremely high (Fig 4A,  $20.4 \pm 3.8$  ng/ml) compared to the background C57BLKS/J (control) mice ( $1.4 \pm 0.4$ ,  $p < 0.01$ ,  $n = 5$ ). Exendin-4 treatment significantly reduced plasma insulin levels ( $6.5 \pm 2.0$  ng/ml,  $p < 0.01$ ,  $n = 5$ ), which was consistent with the normal fasting glycemia 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 ( $0.32 \pm 0.01$   $\mu$ g/mg protein,  $n = 5$ ), when compared to that of exendin-4-treated mice ( $0.54 \pm 0.02$   $\mu$ g/mg protein,  $p < 0.05$ ,  $n = 5$ ), or the background C57BLKS/J (control) mice ( $0.56 \pm 0.03$   $\mu$ g/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 levels 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, exendin-4 treatment significantly improved the insulin response, consistent with the relatively normal pancreatic insulin concentrations in these mice (Fig 4B).

- To test whether exendin-4 treatment normalizes glycemia and improves glucose tolerance in *db/db* mice through improved pancreatic islet function, *in vitro* pancreas perfusion experiments were utilized. As shown in Figure 4D, 20 mM glucose alone induced a 2- to 5-fold increase in insulin secretion from the perfused pancreas of exendin-4-treated mice. However, the pancreas of vehicle-treated mice was unresponsive to the same concentration of glucose ( $p < 0.05$  vs. exendin-4,  $n = 5$ ).
- 10 Addition of 10 nM GLP-1 to the 20 mM glucose perfusate induced insulin secretion from the pancreas of both groups of mice. However, the pancreas of exendin-4-treated mice had a much greater response than vehicle-treated mice ( $p < 0.05$ ,  $n = 5$ ).

***Exendin-4 increases beta cell mass and replication, and decreases beta cell apoptosis.***

- 15 To determine the mechanisms by which exendin-4 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 exendin-4-treated mice, as compared to PBS-treated mice (Fig 5A, PBS vs. exendin-4:  $2.2 \pm 0.2$  vs.  $3.0 \pm 0.3$  mg,  $p < 0.05$ ,  $n = 5$ ). beta cell mass in
- 20 both groups of *db/db* mice was greater than seen in control, 8 week-old C57BLKS/J mice ( $1.13 \pm 0.06$  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 exendin-4-treated mice; islet distribution analysis (Fig 5B) revealed a statistically significant increase in the percentage of small islets in exendin-4-
- 25 treated mice (Fig 5C), suggesting an increase in islet neogenesis in response to exendin-4 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 exendin-4-treated mice had
- 30 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 exendin-4-treated mice as compared to the vehicle-treated mice (Fig 6,  $p < 0.05$ ,  $n = 5$ ).

***Exendin-4 up-regulates pancreatic Akt1 and MAPK.***

Results of one dimensional (SDS PAGE) and two dimensional (first: pH, second: SDS PAGE) gel analysis demonstrated that at least 15 to 20 proteins were up-regulated after 14 days of exendin-4 treatment, while only a few proteins were down-regulated (data not shown). Western blot analysis using specific antibodies demonstrated that these upregulated proteins included Akt1 and MAPK (Figure 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 B cells [28]. A 5-fold increase in expression of total Akt1 protein was found in the pancreas of exendin-4-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 B 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 signaling 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 the present study, elevated expression (by 6-fold,  $p < 0.01$ ) of a specific 44 kDa isoform of MAPK (ERK1) was associated with increased beta cell mass in exendin-4-treated mice (Fig 7), suggesting a possible role for the MAPK pathway in regulating exendin-4-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 levels of the bioactive form of this apoptotic enzyme (~20 kDa; by 30%,  $p < 0.05$ ) in mice treated with exendin-4, while the inactive proenzyme levels (~32 kDa) were 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 signaling, in exendin-4-treated vs. PBS-treated mice.

## Materials and Methods

**Animals** Five-week old female *db/db* mice (BKS.Cg-m<sup>+</sup>/+Lepr<sup>db</sup>, stock number 000642) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Background control mice (C57BLKS/J) were obtained from Charles River Canada (Montreal, QC, Canada) at the same age. Mice were housed under controlled light (12 hours light/12 hours 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.

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**Evaluation of mice & drug administration** All experiments were initiated in mice at 6-weeks of age after glucose levels were measured in fasting blood samples to ensure that the mice were not diabetic. Vehicle (control) or the potent, degradation-resistant GLP1 analog, exendin-4 (Ex4; [22]) was injected (1nmol/kg i.p.; Bachem, Torrance, CA) daily at 0900 hours. 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 acute drug effect. Injection of exendin-4 was omitted on day 14.

**OGTT & insulin tolerance test** Groups of mice were subjected to an OGTT or insulin tolerance test at day 0, 7, and 14. For OGTT, animals were fasted for 15 hours, whilst for insulin tolerance test, mice remained fed. For both groups of animals, a basal blood sample (20 microliter) was collected from a tail vein ( $t = 0$ ) for measurement of glucose using the One Touch Basic glucose meter (Lifescan Canada, Burnaby, BC, Canada). For OGTT, the mice were gavaged with glucose 1.5 g/kg, and additional blood samples were collected at  $t = 10, 20, 30, 40, 60, 90$  and 120 minutes for glucose measurement. For the insulin tolerance test, the mice were injected (i.p.) with human biosynthetic insulin (Novo Nordisk Pharmaceutical Industries Inc. Toronto, ON, Canada) at a dose of 2.5 U/kg, and blood samples were collected at  $t = 0, 20, 30, 40, 60, 90, 120, 150, 180$  and 210 minutes for glucose measurement. On day 14, a separate group of mice was subjected to OGTT, and blood samples (50 microliter) were collected in a heparinized microhematocrit tube at  $t = 0, 15, 30, 60$  and 90 minutes. Blood samples were

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centrifuged and the plasma was stored at  $-20^{\circ}\text{C}$  for determination of insulin levels using a highly sensitive Insulin ELISA kit designed for small sample volumes (Crystal Chem Inc. Chicago, IL.).

- 5     **Insulin radioimmunoassay** Blood samples (~1 ml) were obtained by cardiac puncture in fasted animals on day 14, and plasma was collected and stored at  $-20^{\circ}\text{C}$ . Plasma insulin levels were measured using an insulin radioimmunoassay kit (Linco Research, St. Louis, MO).
  
- 10    **Pancreas perfusion** Mice were fasted for 15 hours (to ensure consistent starting conditions and to determine fasting levels of glycemia), 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  
15    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, ON, Canada) and was gassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  to achieve a pH of 7.4. The pancreas of the mice were sequentially perfused at 1 ml/min  
20    with or without  $1 \times 10^{-8}$  M GLP-1 (10 min) and 2 mM 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 1N HCl containing 5%  $\text{HCOOH}$ , 1% trifluoroacetic acid and 1% NaCl and the peptides were purified by reversed-phase extraction using a C18 SepPak cartridge (Water Associates, Milford, MA). Extracts were dried in a vacuum dryer prior to RIA for insulin  
25    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, ON, Canada), 6 h before removal of the pancreas. After cardiac puncture under sodium pentobarbital anesthesia,  
30    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 micrometer) 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, ON, Canada). The samples were then incubated for 1 h with biotinylated anti-guinea pig antibody (Vector Laboratories, Burlington, ON, Canada), and subsequently treated for 1 h with avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA.). 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 color 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 sum of the determinations from each of the 8-10 segments of pancreas. A total of 1000 – 1500 B cells were counted per pancreas. Total beta cell mass per pancreas was determined as the product of the total cross-sectional beta cell area /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 (x1000). Results are expressed as the percentage of BrdU<sup>+</sup> beta cells.

**Apoptosis detection** Pancreas sections were double immunostained for insulin, as described above, and for fragmented DNA by Tunel assay, which detects fragmented nuclei characteristic of apoptotic cells. Tunel staining was performed using ApopTag Kit (Intergen, Purchas, NY) according to manufacture's instruction. 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<sup>+</sup> beta cells.

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**Immunoblotting** The isolated pancreas were homogenized in lysis buffer [24] containing 1% Triton X-100 and a mixture of protease and phosphatase inhibitors. The protein content was measured by Biorad assay (Bio-Rad Laboratories, Hercules, CA) and equal amount 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 BioLabs Ltd., Mississauga, ON, Canada; MAPK, mouse IgG (1:1000), Oncogene Research Products, San Diego, CA; caspase-3 rabbit IgG (1:2000), Biomol Research Laboratories Inc., Plymouth Meeting PA; and IRS-1: rabbit IgG (1:500), Santa Cruz Biotechnology Inc., Santa Cruz, CA), the immunoreactive bands were visualized either with horseradish peroxidase-conjugated (HRP) sheep anti-mouse IgG for monoclonal antibodies or with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grov. PA) for polyclonal antibodies using an ECL detection technique (Amersham, Oakville, ON, Canada), as described previously [24]. Developed films were scanned and quantitated using NIH Image software (National Institute of Health, Bethesda, MD).

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**Statistical analysis.** All data are presented as mean  $\pm$  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, NC). Significance was assumed at  $p < 0.05$ .

It will be appreciated that the description above relates to the preferred embodiments by way of example only. Many variations on the computer system and methods for delivering the invention will be obvious to those knowledgeable in the field, and such obvious variations are within the scope of the invention as described and claimed, whether or not expressly described.

All references, including journal articles, patents and patent applications, in this application are incorporated by reference herein in their entirety.

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We claim:

1. A method of preventing and or/delaying diabetes in a subject in need thereof, comprising administering to the subject an effective amount of a compound that increases beta-cell proliferation and/or reduces beta cell apoptosis in the subject.
- 5 2. The method of claim 1, wherein the increased proliferation and/or reduced apoptosis provide increased beta-cell mass.
3. The method of claim 1, wherein the compound increases protein kinase expression in the beta cell.
4. The method of claim 1, wherein the protein kinase comprises Akt1 and/or p44MAPK.
- 10 5. The method of claim 1, wherein the compound decreases caspase-3 activation.
6. The method of claim 1, wherein the effective amount of the compound increases insulin release and glucose tolerance in the subject.
7. The method of claim 1, wherein the effective amount comprises a dose of 0.05nmol/kg of body weight to 0.5nmol/kg of body weight.
- 15 8. The method of claim 1, wherein the compound comprises a GLP-1 polypeptide or an exendin-4 polypeptide.
9. The method of claim 1, wherein the compound has at least 60% sequence identity to a GLP-1 polypeptide or at least 60% sequence identity to an exendin-4 polypeptide.

Application number / numéro de demande: 2389462

Figures: \_\_\_\_\_

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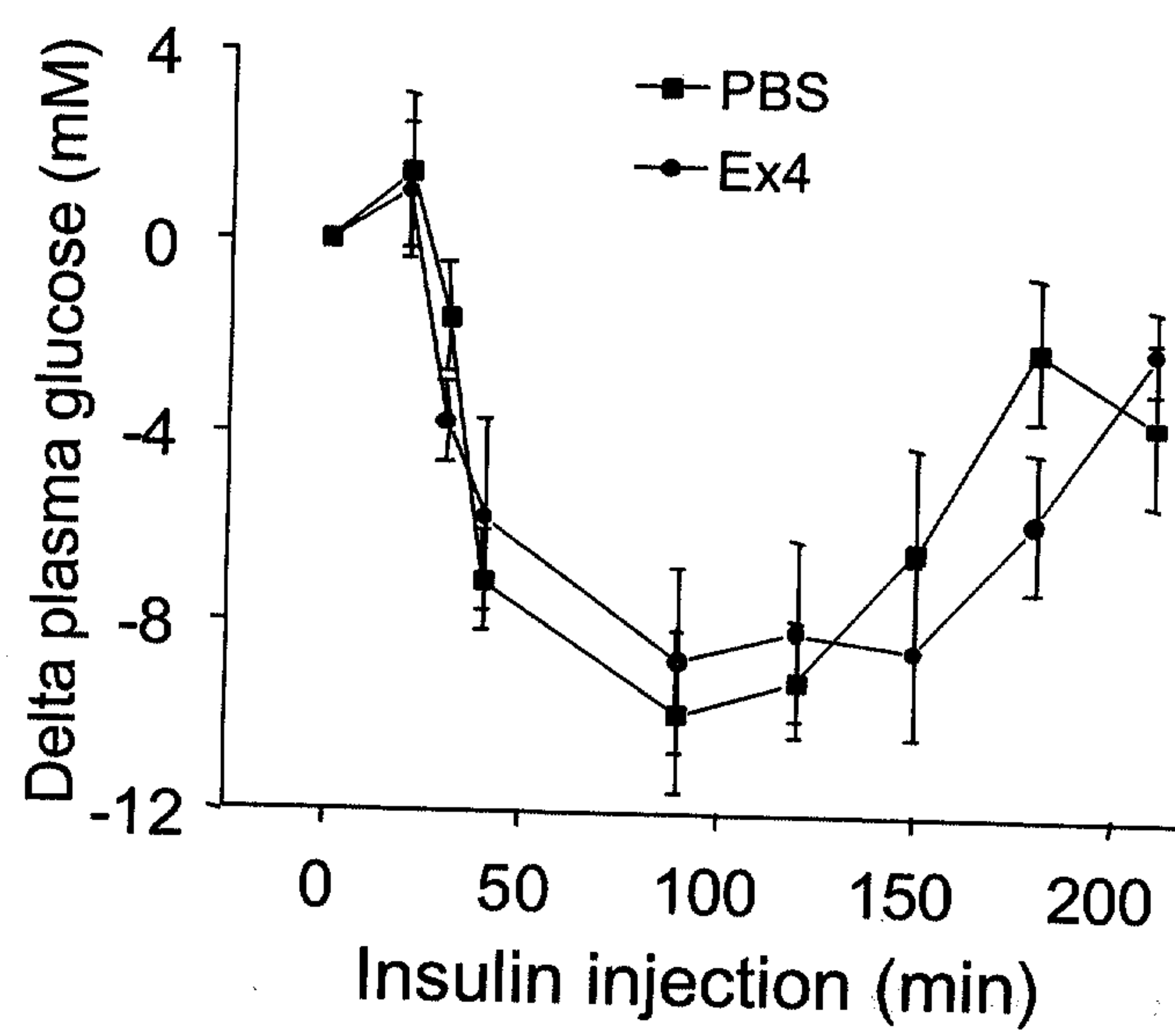


Figure 3. Wang &amp; Brubaker

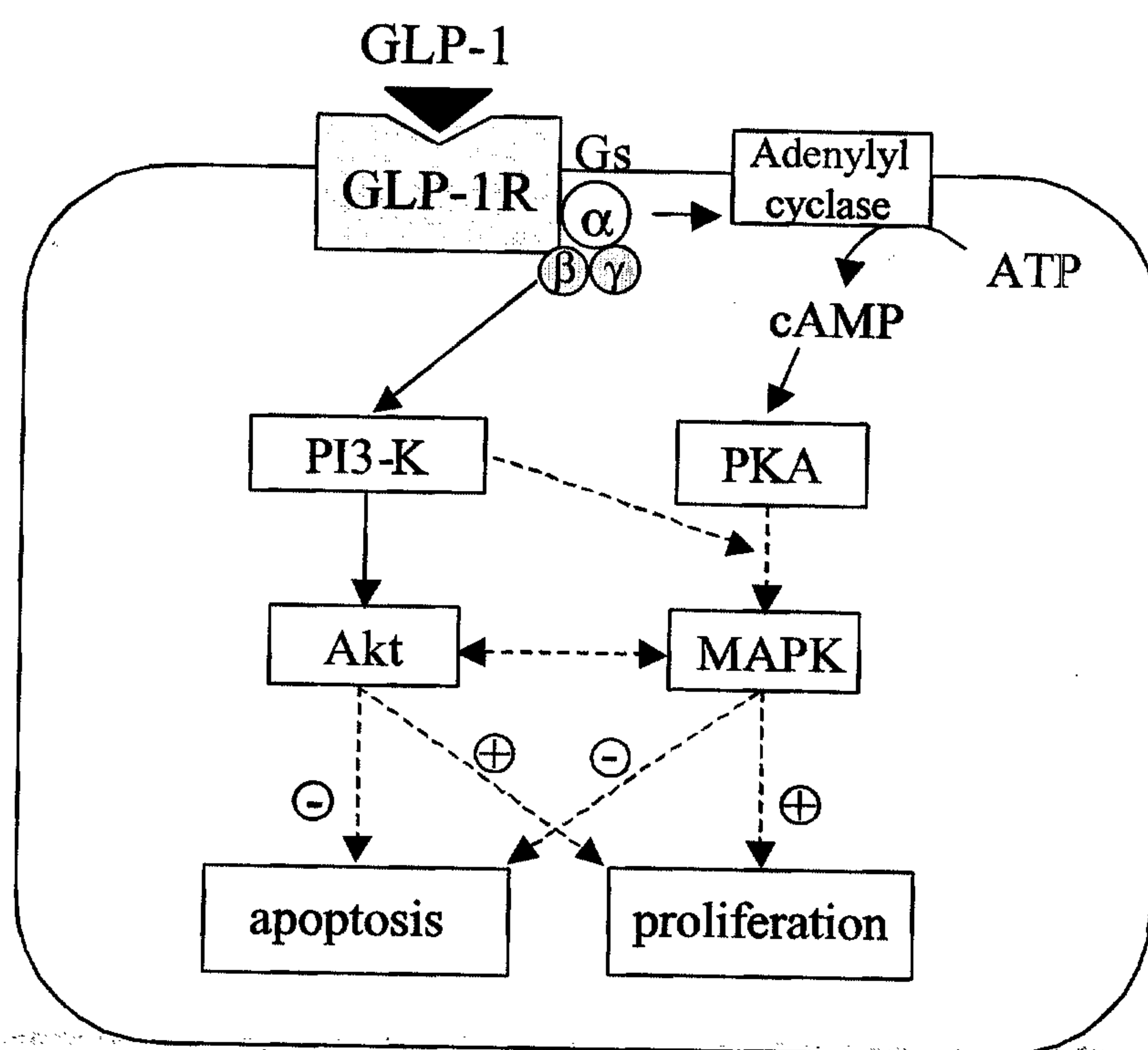


Figure 8. Wang &amp; Brubaker

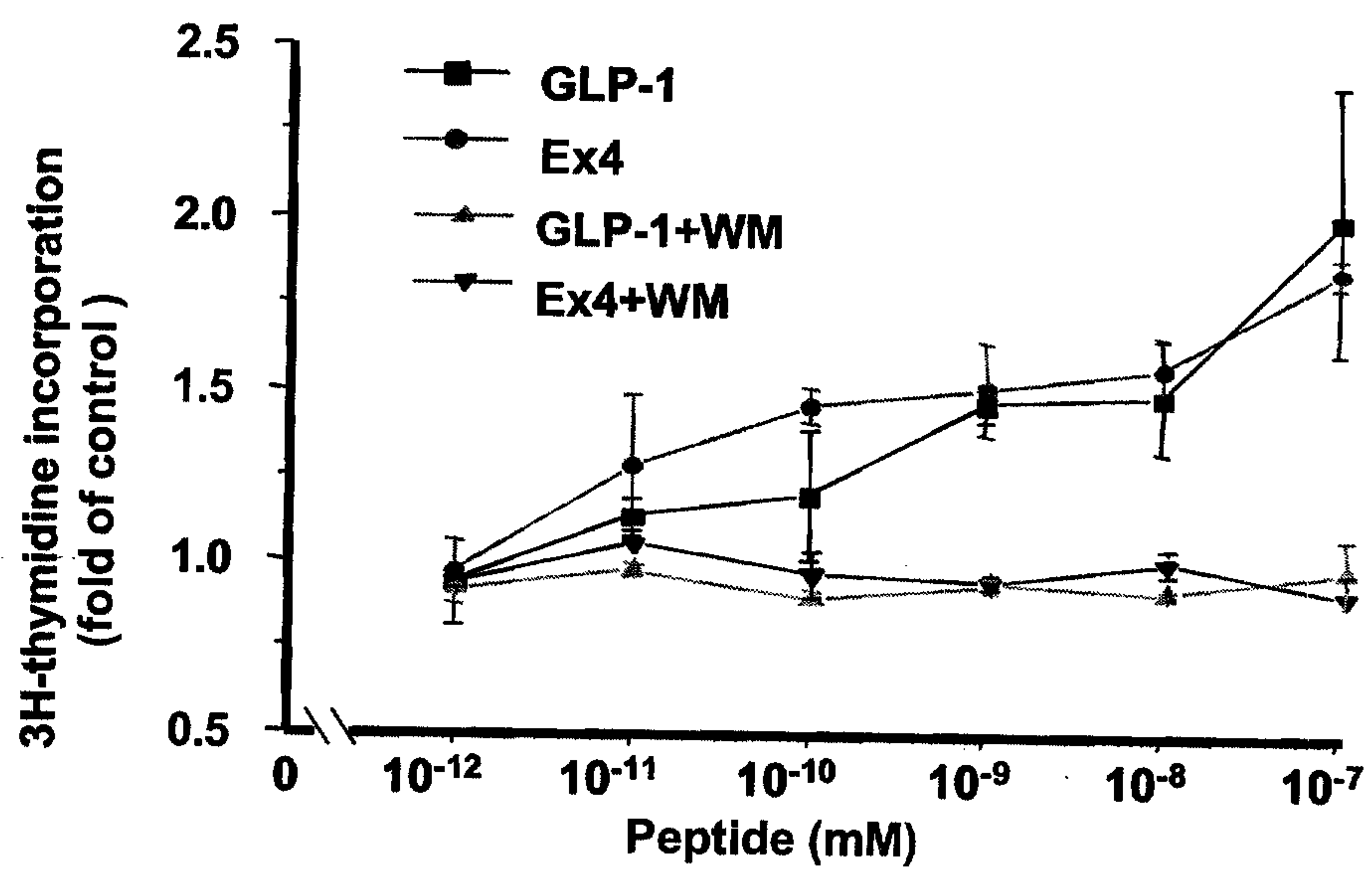


Figure 10. Wang &amp; Brubaker