(54) Title: BLOCKADE OF GLUCOSE TOXICITY TO THE β CELLS IN THE ISLETS OF LANGERHANS

(57) Abstract

The present invention demonstrates that streptozotocin (STZ) is a specific toxin for the pancreatic β cells and that STZ specifically inhibits the activity of O-GlcNac-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase), therefore, blocks O-linked N-acetylglucosamine (O-GlcNAc) removal from intracellular proteins. The present invention is also directed to a transgenic animal with impaired glucosamine synthesis and various methods of treating diabetes and of screening for drugs for treating diabetes.
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BLOCKADE OF GLUCOSE TOXICITY TO THE β CELLS IN THE ISLETS OF LANGERHANS

BACKGROUND OF THE INVENTION

Federal Funding Legend
10 This invention was created in part using funds from the federal government under NIH grant DK55262. Consequently, the U.S. government has certain rights in this invention.

Cross-reference to Related Application
15 This patent application claims benefit of provisional patent application U.S. Serial number 60/097,021, filed August 18, 1998, now abandoned.

Field of the Invention
20 The present invention relates generally to the fields of protein biochemistry and medical therapy for diabetes. More specifically, the present invention relates to drugs that block glucose toxicity to the β cells in the islets of Langerhans and thereby prevent the accumulation of damage to the β cells and methods for screening for such drugs. The present invention further relates to transgenic mice with impaired glucosamine synthesis and the usage of such transgenic mice for screening for diabetes-treating drugs.
Description of the Related Art

In early diabetes, β cell function is sufficient such that oral hypoglycemic agents are adequate for the treatment of the condition. In type 2 diabetes (adult onset type), high blood glucose concentrations appear to lead to the loss of capacity of the β cells in the islets of Langerhans to produce sufficient amounts of insulin to control the blood sugar. Thus, a vicious cycle arises where high blood sugar destroys β cell function leading to even higher blood sugar and worsening of the condition. Complications would therefore occur, such as accelerated atherosclerosis, kidney, eye and peripheral nerve disease.

Presently, the drugs used to prevent the progression of type 2 diabetes and the complications are directed at lowering the blood sugar and dependent on insulin injections. Although the conventional approaches do contribute greatly to the control of the disease, they do not completely succeed in controlling the blood sugar at all times.

For the past 30 years, streptozotocin (STZ) has been used to create animal models of type 1 diabetes. A single dose of 50-100 mg/kg of streptozotocin to a rat results, within 4 to 8 hours, in the death of most of the β cells and the development of a picture similar to type 1 diabetes. No other tissues sustain significant damage in this animal model by the administration of this dose of streptozotocin, other than secondarily through the development of diabetes. Streptozotocin is an antibiotic with a structure that is remarkably similar to N-acetylglucosamine (GlcNAc) (Figure 1) (1). A nitrosourea group is present in a position that corresponds to the acetate in GlcNAc. This structure has suggested that streptozotocin toxicity derives from its ability
to behave as an alkylating agent (2, 3) or as an NO donor (2, 4). Exposure of cells to NO donors has been associated with apoptotic death (5, 6) and streptozotocin has been shown to cause DNA fragmentation in β cells (7), a possible indication that these cells undergo apoptosis in response to this drug.

The alkylating property of streptozotocin has also made this drug useful as a chemotherapeutic agent in humans for the treatment of islet cell tumors, particularly insulinomas. At the high doses used for chemotherapy, streptozotocin is nephrotoxic and hepatotoxic in addition to its toxicity to the islets. Thus, the these properties of streptozotocin may explain its non-specific toxicity to tissues other than the β cells. The specificity of streptozotocin for the β cell has been suggested to result from the GLUT2 glucose transporter that is expressed in the β cell. This transporter appears to transport streptozotocin (8) and GlcNAc (9), perhaps more efficiently than other glucose transporters such as GLUT1 (8). While this preferential transport might explain partially the β cell specificity of streptozotocin, it does not explain how other tissues that express GLUT2, such as liver, kidney and small intestine (10) are less susceptible than β cells to streptozotocin toxicity.

That streptozotocin is a structural analog of GlcNAc suggested that the specificity of this drug for the β cell might result from alterations in glucosamine metabolism. Glucosamine is a product of glucose metabolism and is synthesized from fructose-6-phosphate by the apparently unique and rate limiting enzyme, glutamine:fructose-6-phosphate aminotransferase (GFAT) (11-13). This metabolic step provides the hexosamine substrates that
are necessary for glycoprotein synthesis. Quantitatively, most
glycosylation occurs on those proteins destined for export or the
cell surface; however, in eukaryotic cells, there is also a
cytoplasmic form of glycosylation that involves the O-linkage of
the monosaccharide, GlcNAc, to cytoskeletal and nuclear proteins
at serine or threonine residues (14).

An enzyme responsible for this intracellular form of
protein modification, O-GlcNAc transferase (OGT), has been
characterized (15) and its cDNA cloned (15, 16). Studies on the
tissue distribution of O-GlcNAc transferase have indicated that the
O-GlcNAc transferase mRNA, although ubiquitous, is particularly
abundant in the pancreas (16). The high abundance of O-GlcNAc
transferase in the pancreas and the structural similarity of
streptozotocin to GlcNAc suggested that the specificity of toxicity
of streptozotocin to the pancreatic β cells might be related to this
high abundance of O-GlcNAc transferase. This postulate depends
on whether O-GlcNAc transferase is localized to the β cells or some
other cell type in the pancreas.

The prior art is deficient in the lack of effective means
of blocking glucose toxicity to the β cells in the Islets of
Langerhans, thereby preventing the accumulation of damage to
the β cells in diabetes. The present invention fulfills this long-
standing need and desire in the art.

**SUMMARY OF THE INVENTION**

The present invention demonstrates that the O-GlcNAc
transferase mRNA is highly abundant in the rat pancreatic islet in
a distribution that corresponds to the distribution of β cells. Also
disclosed is that streptozotocin markedly alters the metabolism of O-GlcNAc by inhibiting an enzyme that removes this sugar from proteins. It is proposed that this correlation between the high abundance of O-GlcNAc transferase in the β cells and the ability of streptozotocin to block O-GlcNAc removal may explain the β cell specificity of streptozotocin toxicity.

The present invention also demonstrates that using a transgenic mouse with impaired β-cell glucosamine synthesis, these mice are also resistant to the diabetogenic effect of streptozotocin plus glucose yet succumb to streptozotocin plus glucosamine. This study links the apoptotic effect of streptozotocin on β-cells with the O-GlcNAc modification potentially implicating this pathway of glucose metabolism with β-cell apoptosis glucose toxicity.

In one embodiment of the present invention, there is provided a method of treating an individual with type 2 diabetes using an effective dose of a drug that blocks glucose metabolic pathway.

In another embodiment of the present invention, there is provided a method of treating an individual with type 2 diabetes using an effective dose of a drug which activates O-linked N-acetylglucosamine removal from intracellular proteins.

The above disclosed methods may be synergized with conventional approaches, such as insulin injection.

In still yet another embodiment of the present invention, there is provided a method of screening for a drug for blocking production and/or accumulation of O-GlcNAc-modified proteins and therefore, treating type 2 diabetes, comprising the steps of contacting an animal with a drug candidate; staining
pancreatic islets from the treated animal with an antibody directed against O-GlcNAc-modified proteins; obtaining a distribution of the antibody in pancreatic β cells of the treated animals; comparing the distribution in the treated animal and a distribution in an untreated animal. If the distribution in the treated animal is less than the distribution in the untreated animal, the tested drug blocks the production and/or accumulation of O-GlcNAc-modified proteins, therefore, is suitable for treating type 2 diabetes.

In yet another embodiment of the present invention, there is provided a method of screening for a compound that blocks production of O-GlcNAc, comprising the steps of contacting a cell in a glucose-containing medium with a compound suspected to block O-GlcNAc production; measuring the level of glucosamine in the cell; and comparing the level with level of glucosamine in an untreated cell. If the level of glucosamine in the treated cell is less than the level of glucosamine in the untreated cell, the tested compound blocks O-GlcNAc production.

In still yet another embodiment of the present invention, there is provided a transgenic animal such as a mouse containing an antisense glutamine: fructose-6-phosphate aminotransferase (GFAT) transgene. Such a transgenic animal or mouse may be used for screening for diabetes-treating drugs.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.
BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the chemical structures of N-acetylglucosamine and streptozotocin.

Figure 2A shows immunohistochemical localization of insulin in a normal rat pancreas. A 5 μm section of pancreas was incubated with a rabbit polyclonal anti-insulin antiserum. The bound antibody was detected by the immunoperoxidase method using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Insulin was localized to the central part of each islet.

Figure 2B shows localization of the O-GlcNAc transferase mRNA in the pancreas by in situ hybridization. A 10 μm thick frozen section of pancreas was probed with an 35S-UTP-labeled antisense riboprobe corresponding to nucleotides 828 to 1801 of the rat O-GlcNAc transferase cDNA. The photomicrograph of the section under dark field illumination that resulted from a 9 day exposure of the section to photographic emulsion and hematoxylin-eosin counterstain, is shown. Figures 2C and 2D show effect of streptozotocin on monoclonal antibody RL2 detectable
glycoproteins in the pancreas. Rats, approximately 150 g each, were deprived of food overnight, then injected intraperitoneally with a 100 mg/kg dose of streptozotocin (Figure 2D) or with vehicle (Figure 2C) (citrate buffer, pH 4). At various times after the injection, the pancreas was removed for examination by immunohistochemistry. A 5 μm section of pancreas, after appropriate permeablation, was incubated with the monoclonal antibody, RL2. Bound RL2 was detected by the immunoperoxidase method using DAB as a chromogen. Shown is the distribution of RL2 detectable glycoproteins in the control (Figure 2C) pancreas and in the pancreas 2 hour after the streptozotocin injection (Figure 2D). Both the control and streptozotocin animals had been fed after the injection. The blood sugar of the control animal was 155 mg/dl and of the streptozotocin animal, 162 mg/dl.

Figure 3 shows effect of streptozotocin on the glycosylation of an Sp1-derived peptide. BSC40 cells were infected with a recombinant vaccinia virus that directed the expression of a fusion protein composed of GST and a 97 amino acid peptide containing the sequence of the B domain of the transcription factor, Sp1. After infection, cells were placed in glucose-free DMEM with 10% serum and treated with (Figure 3A) or without (Figure 3B) 5 mM streptozotocin for 24 hours. The GST fusion proteins were affinity and HPLC-purified, and then subjected to MALDI mass spectrometry analysis.

Figure 4 shows effect of streptozotocin on O-GlcNAc turnover on the Sp1 peptide. BSC40 cells were infected with vaccinia virus to express the GST fusion protein and were treated with (dashed line) or without (solid line) 5 mM STZ. 16 hours after infection, [35S]methionine and [3H]glucosamine were added to
the cells for 4 hours in glucose-free DMEM. After washing the cells 3 times, they were placed in glucose-free DMEM containing 10% serum and either 2.5 mM glucosamine (solid square) or 2.5 mM streptozotocin (hollow circle). The GST-fusion proteins were purified by affinity chromatography at the indicated times following the removal of the label and the radioactivity attributable to $^{35}$S and $^{3}$H was determined by dual-counting scintillation spectroscopy.

**Figure 5** shows effect of streptozotocin on O-GlcNAcase and jack bean hexosaminidase activity. Purified O-GlcNAcase and hexosaminidase were incubated with the indicated concentrations of streptozotocin for 15 min at 4°C in hexosaminidase assay buffer prior to the addition of the substrate, $p$-nitrophenyl-$\beta$-D-$N$-acetylglucosaminide. The reaction was incubated at 37°C for 1 hour and the OD at 400 nM was determined. Plotted are the % of the activities, as a function of streptozotocin concentration, of the O-GlcNAcase (hollow square) and hexosaminidase (solid circle) relative to the activity measured in the absence of STZ.

**Figure 6** shows effect of streptozotocin on RL2 detectable glycoproteins in BSC40 cells. Confluent BSC40 cells were incubated overnight in glucose-free DMEM with 10% serum. The cells were treated with or without 5 mM streptozotocin in glucose- and serum-free medium as indicated on the figure. In addition, the cells were also treated as indicated with 100 $\mu$M 6-diazo-5-oxonorleucine (DON) 1 hour prior to the initiation of streptozotocin treatment and either 5 mM Glc or 5 mM GlcN during the streptozotocin treatment. After 6 hours, the proteins, in high-salt extracts from the cells, were separated by SDS gel
electrophoresis and analyzed by a Western blot that was probed with the RL2 monoclonal antibody.

**Figure 7** shows a summary of the metabolic pathway leading from glucose metabolism to O-linked protein glycosylation. The structure of protein-O-GlcNAc is shown. Glc: glucose; Gln: glutamine; Glu: glutamate; other abbreviations are defined below.

**Figure 8** shows the effect of hyperglycemia and streptozotocin on O-GlcNAc accumulation in pancreatic islets *in vivo*. The rats were infused with glucose with or without STZ-pretreatment and the pancreases were examined for the distribution of RL2 detectable glycoprotein. **Figures 8A and 8D**, overnight fasted; **Figures 8B and 8E**, 45 min after glucose infusion; **Figures 8C and 8F**, 170 min after insulin treatment; **Figures 8D, 8E and 8F**, STZ-pretreatment. (magnification 400 X).

**Figure 9** shows the effect of glucose and glucosamine on the cytotoxicity of streptozotocin to β cells. **Column A**: Single dose streptozotocin (50 mg/kg). **Column B**: Single dose streptozotocin (50 mg/kg) followed by glucose. **Column C**: Single dose streptozotocin (50 mg/kg) followed by glucosamine. **Row 1**: The pancreas was stained by immunohistochemistry with a polyclonal insulin antibody (magnification 400 X). **Row 2**: Bright field view of the in situ hybridization with a $^{35}$S-UTP-labeled insulin antisense riboprobe (magnification 400 X). **Row 3**: Dark field view of the same sections shown in row 2. The hybridization signal appears as dark grains in the bright field and white grains in the dark field views. **Row 4**: *In situ* TUNEL assay for DNA nicking (magnification 600 X). The insets in **B4** and **C4**: apoptotic cells are indicated by arrows (magnification 1000 X). STZ: streptozotocin; Glu: glucose; GlcN: glucosamine.
Figure 10 shows the effect of an 8 hour intravenous glucosamine infusion on the pancreas of the Sprague-Dawley rats. Figures 10A and 10C, the pancreas from control normoglycemic rats. Figures B and D, the pancreas from the rats receiving sustained infusion of glucosamine for 8 hours (magnification 400 X). Figures 10A and 10B: Hematoxylin-eosin staining; Figures 10C and 10D: Insulin immunohistochemical staining.

Figure 11 shows the characterization of the antisense-GFAT transgenic mice. Figure 11A: Upper left panel: The schematic diagram of the transgene. Upper right panel shows the PCR analysis of DNA derived from the tails of F1 mice (lanes 2 and 4 are gene positive). M1: 1 kb markers; M2: 100 bp markers; P: 0.5 pg plasmid DNA. The expected amplified target sequence is about 1.28 kb. Lower panel: antisense GFAT mRNA was determined to be expressed specifically in the β-cells of transgenic mice by in situ hybridization with 35S-UTP-labeled GFAT sense riboprobe (left: bright field; right: dark field, magnification 400 X). Figure 11B shows the effect of the antisense GFAT transgene on the β-cell O-GlcNAc accumulation in hyperglycemic mice. The mice were injected with streptozotocin followed by glucose and the pancreas was examined by immunohistochemical staining with RL2 antibodies. Left panel: the pancreas from a wild type littermate mouse after receiving treatment with streptozotocin and glucose (blood glucose = 14 mM). (magnification 400 X). Right panel: the pancreas from a RIP-mGFAT(AS) transgenic mouse after receiving treatment with streptozotocin and glucose (blood glucose = 19 mM).

Figure 12 shows the blood glucose response following treatment with multiple low doses of STZ. The arrows indicate the
streptozotocin injections (40 mg/kg, 5 consecutive days). Blood glucose was determined in samples from a tail bleed at the indicated times. Results are given as the mean ± S.E. for 14 transgenic and 16 wild type littermate mice. *: p<0.05; **: p<0.01 (unpaired t-test).

DETAILED DESCRIPTION OF THE INVENTION

Streptozotocin, an analog of N-acetylglucosamine (GlcNAc), is a specific toxin for the pancreatic β cell. The present invention demonstrates that treatment of rats with streptozotocin results in an early β cell-specific increase in the level of intracellular protein modification by O-linked GlcNAc (O-GlcNAc). Using a model O-GlcNAc-peptide based on the transcription factor Sp1, it was shown that treatment of cultured cells with streptozotocin during peptide biosynthesis results in hyperglycosylation of the peptide as a result of the ability of streptozotocin to specifically inhibit the activity of O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase). While this inhibitory activity of streptozotocin probably can occur in all cells, using in situ hybridization, β cells were found to express very high levels of the mRNA encoding the enzyme responsible for cytoplasmic protein O-glycosylation, O-GlcNAc transferase. These findings suggest that the pancreatic β cell is particularly sensitive to the toxicity of streptozotocin because it expresses such high levels of O-GlcNAc transferase.

When streptozotocin blocks O-GlcNAc removal from intracellular proteins, the cell with the most rapid on-rate for O-
GlcNAc, the β cell, will experience the most rapid accumulation of this protein modification. The present invention also shows that the on-rate of O-GlcNAc is substrate driven in several cell types, it is speculated that the β cell, with its high level of O-GlcNAc transferase, may also respond to elevations of blood sugar with increased protein modification by O-GlcNAc. Thus, this proposed mechanism of streptozotocin toxicity on the β cell may result from an exaggeration of a heretofore unrecognized physiological response to glucose mediated through the high level of O-GlcNAc transferase in these cells.

In one embodiment of the present invention, there is provided a method of treating an individual with type 2 diabetes using an effective dose of a drug that blocks glucose metabolic pathway. Preferably, the drug inhibits the activity of one or more than one enzyme involved in the pathway. The enzyme can be glutamine:fructose-6-phosphate amidotransferase, which is responsible for conversion of glucose to glucosamine, or O-linked N-acetylglucosamine transferase, which modifies the proteins in the nucleus.

In another embodiment of the present invention, there is provided a method of treating an individual with type 2 diabetes using an effective dose of a drug which activates O-linked N-acetylglucosamine removal from intracellular proteins. Specifically, the drug enhances the activity of O-GlcNAc-selective N-acetyl-β-D-glucosaminidase.

The above disclosed methods may be synergized with conventional approaches, such as insulin injection.

In still yet another embodiment of the present invention, there is provided a method of screening for a drug for
blocking production and/or accumulation of O-GlcNAc-modified proteins and therefore, treating type 2 diabetes, comprising the steps of injecting an animal with a drug candidate; staining pancreatic islets from the treated animal with an antibody directed against O-GlcNAc-modified proteins; obtaining a distribution of the antibody in pancreatic β cells of the treated animals; comparing the distribution in the treated animal and a distribution in an untreated animal. If the distribution in the treated animal is less than the distribution in the untreated animal, the tested drug blocks the production and/or accumulation of O-GlcNAc-modified proteins, therefore, is suitable for treating type 2 diabetes.

In yet another embodiment of the present invention, there is provided a method of screening for a compound that blocks production of O-GlcNAc, comprising the steps of contacting a cell in a glucose-containing medium with a test compound suspected of blocking O-GlcNAc production; measuring the level of glucosamine in the cell; and comparing the level with level of glucosamine in an untreated cell. If the level in treated cell is less than the level in the untreated cell, the test compound blocks O-GlcNAc production.

In still yet another embodiment of the present invention, there is provided a transgenic mouse containing antisense glutamine:fructose-6-phosphate aminotransferase (GFAT) transgene. Specifically, 150 bp 5' UTR and complete coding sequence of mouse GFAT cDNA is inserted in the antisense direction into an XbaI-XbaI site between the rat insulin II promoter (RIP) and the SV40 small T-antigen intron and polyadenylation sequences to form RIP-mGFAT (antisense)-SV40
construct in the transgene. The transgene is expressed in islet β-cells that blocks the glucose-stimulated increase in O-GlcNAc modification, resulting impaired β-cell glucosamine synthesis.

In still yet another embodiment of the present invention, there is provided a method of screening for a drug useful for treating diabetes, comprising the steps of: administering the transgenic mouse disclosed herein with streptozotocin (STZ) in combination with glucosamine to obtain diabetic transgenic mouse; measuring blood glucose level in the diabetic transgenic mouse; administering a drug candidate to the diabetic transgenic mouse; and measuring blood glucose level in the drug-treated diabetic transgenic mouse, wherein if the blood glucose level in the drug-treated diabetic transgenic mouse is lower than that in the diabetic transgenic mouse without the drug treatment, the test drug is useful for treating diabetes.

In yet still another embodiment of the present invention, there is provided a method of screening for a drug useful for treating diabetes, comprising the steps of: administering the transgenic mouse disclosed herein with streptozotocin (STZ) in combination with glucosamine to obtain diabetic transgenic mouse; measuring β-cell apoptosis in the diabetic transgenic mouse; administering the diabetic transgenic mouse with a drug candidate; and measuring β-cell apoptosis in the drug-treated diabetic transgenic mouse, wherein if the β-cell apoptosis in the drug-treated diabetic transgenic mouse is reduced compared to that in the diabetic transgenic mouse without the drug treatment, the test drug is useful for treating diabetes.
The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Cell Culture

BSC40 cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% newborn calf serum (Gibco/BRL, Grand Island, NY), 100 µg of penicillin/ml, and 50 µg of gentamicin/ml at 37°C in a humidified incubator with 7.5% CO₂.

EXAMPLE 2

Expression of Glutathione-S-Transferase (GST) Fusion Protein

A recombinant vaccinia virus was generated as described (17). After BSC40 cells were infected with the vaccinia virus that directed the expression of the fusion protein (17) composed of GST and a 97 amino acid peptide containing the sequence of the B domain of the transcription factor Sp1, cells were placed in glucose-free DMEM with 10% serum. Cells were then treated with or without 5 mM streptozotocin for 20 hours. A whole cell extract was made by freezing and thawing cells twice in a buffer containing 20 mM Tris (pH 7.5), 0.5 M NaCl, 0.5 M GlcNAc, 0.5 mM EDTA, 0.5 M MgCl₂, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 20% glycerol. The supernatant was collected after centrifugation. Glutathione-Sepharose 4B (Pharmacia) was added to the extract for 30 min. The beads were collected and washed three times with the extraction buffer minus the glycerol and GlcNAc. This procedure extracted at least 90% of the fusion protein from the extract. For mass spectroscopic
analysis, the beads were further washed with a buffer containing 20 mM Tris, 100 mM NaCl, and 2.5 mM CaCl₂. The peptide was then cleaved from GST with 4 U of thrombin (Sigma) per mg of fusion protein.

EXAMPLE 3

Mass Spectroscopy of Glycopeptides

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectroscopy of the Sp1 peptides was carried out on a Perspective Biosystems (Framingham, MA) Voyager Elite MALDI-TOF mass spectrometer (17). Samples were mixed with a saturated solution of α-cyano-4-hydroxy-cinnamic acid in a water-acetonitrile (50:50) mixture acidified with 0.1% trifluoroacetic acid. A 1 µl aliquot of the sample was spotted onto the gold plate target. Ionization of the sample was accomplished with a nitrogen-laser operated at 337 nm. A delayed extraction method was used in the determination of molecular mass. Measurement of ion flight times through the drift region of the mass spectrometer were carried out with a Tektronix (Beaverton, OR) TDS784A oscilloscope. The instrument was calibrated with external molecular weight standards.

EXAMPLE 4

Effect of Streptozotocin on O-GlcNAcase and Jack Bean Hexosaminidase Activity

Purified O- GlcNAcase (from Dr. Gerald W. Hart and Glendon Parker) and β-D-N- acetylhexosaminidase (V-LABS) were incubated with the indicated concentrations of freshly dissolved streptozotocin for 15 min at 4°C in hexosaminidase assay buffer
prior to the addition of the substrate, p-nitrophenyl-β-D-N-acetylglucosaminide (Sigma) (18). The reaction was incubated at 37°C for 1 hour and the OD at 400 nM was determined.

**EXAMPLE 5**

Western Blotting with the RL2 Monoclonal Antibody

Confluent BSC40 cells were incubated overnight in glucose-free DMEM and 10% serum. The cells were then washed with glucose-free DMEM and treated with or without 5 mM streptozotocin in this glucose-free medium. In addition, the cells were also treated as indicated with 100 μM 6-diazo-5-oxonorleucine (DON) (Sigma) 1 hour prior to the initiation of streptozotocin treatment and either 5 mM glucose or 5 mM glucosamine during the streptozotocin treatment. After 6 hours, the proteins, in high-salt extracts from the cells, were separated by SDS gel electrophoresis and analyzed by a Western blot that was probed with the RL2 monoclonal antibody as previously described (13). The RL2 antibody was from Dr. L. Gerace, (19).

**EXAMPLE 6**

Pulse Chase Labeling of the O-GlcNAc on the Sp1-Derived Peptide

Confluent BSC40 cells in 15 cm plates were infected with vaccinia virus to express GST fusion protein and were treated with or without 5 mM streptozotocin. 16 hours after infection, 2 μCi of [35S]methionine (1000 Ci/mmol) and 5 μCi of [6-3H]glucosamine (40 Ci/mmol) (Amersham) were added to the cells for 4 hours in glucose-free DMEM. After washing the cells 3 times, they were placed in glucose-free DMEM containing 10% serum and either 2.5 mM glucosamine or 2.5 mM streptozotocin. The GST-
fusion proteins were purified by glutathione affinity chromatography (see above) at the indicated times following the removal of the label and the radioactivity attributable to $^{35}$S and $^3$H was determined by dual-counting scintillation spectroscopy on an appropriately calibrated Beckman LS 5000TD counter.

**EXAMPLE 7**

**Streptozotocin Treatment of Rats**

Sprague Dawley rats weighing between 150 and 170 g, were fasted overnight and then injected intraperitoneally with vehicle (50 mM citrate buffer, pH 4) alone or streptozotocin (100 mg/ml) made fresh in citrate buffer at a dose of 100 mg/kg. Blood glucose concentrations were determined before the injection and at various times after the injection on blood derived from a tail bleed using an "Advantage" blood glucose monitor (Boehringer Mannheim). After the injection, the animals were allowed to feed on standard rat chow and were sacrificed by CO$_2$ inhalation at various times after the injection. The treatment of these animals was approved by the institutional animal care committee.

**EXAMPLE 8**

**Immunostaining**

Formaldehyde fixed and paraffin embedded pancreas sections (5 μm) were prepared and immunostained with RL2 monoclonal antibody (ascites diluted 1:20), or anti-insulin polyclonal antibody (1:50, NCL-INS, Novocastra, UK) using the Vectastain Elite ABC Kit (Vector, Burlingame, CA). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, and sections were counterstained with Gill's
Hematoxylin (Vector). To facilitate immunostaining, sections were pretreated with 2 N HCl for 20 min at 37 °C, and exposed to 0.01% trypsin at 37 °C for 3 min.

EXAMPLE 9

In Situ Hybridization

The 978-bp rat OGT cDNA corresponding to nucleotides 828 to 1801 of the rat OGT cDNA (accession number U76557) (15) was cloned by RT-PCR from rat liver RNA using two oligonucleotides 5'- CATGGATCCGCAATTGAGACGCAACC -3' (SEQ ID NO: 1), and 5'- CATGGTACCTGAGGAGGCAAGAGC -3' (SEQ ID NO: 2). The identity of the PCR product was confirmed by restriction enzyme digestion and partial automated sequencing. The PCR fragment was subcloned into a plasmid. Frozen normal rat pancreas sections (10 μm) were utilized for in situ hybridization. Preparation of the 35S-UTP labeled antisense riboprobe and hybridization were performed as described (20). After hybridization, photographic emulsion dip, exposure and developing, the sections were counterstained with hematoxylin and eosin, and subjected to microscopic examination under bright field and dark field illumination.

EXAMPLE 10

High Abundance of OGT in Pancreatic β Cells

To determine which cells in the pancreas are responsible for the high overall level of O-GlcNAc transferase expression in this organ (16), in situ hybridization on the rat pancreas with a probe derived from the rat O-GlcNAc transferase cDNA was performed (Figure 2B). At the level of exposure used,
the O-GlcNAc transferase mRNA was seen almost exclusively in the central islets, a distribution resembling that of insulin in the rat (Figure 2A). Little O-GlcNAc transferase was seen at the periphery of the islets nor was there an appreciable signal over the exocrine pancreas. Probing with a sense O-GlcNAc transferase riboprobe gave only a background level of signal and probing with an actin antisense probe revealed a general distribution of actin mRNA on the same pancreatic tissue slices (data not shown). That the abundance of the O-GlcNAc transferase mRNA is higher in the intact pancreas than in any other tissue (16) and that the O-GlcNAc transferase mRNA is concentrated in the β cells together suggest that the β cells may contain considerably more O-GlcNAc transferase mRNA than any of the other tissue examined thus far (16).

**EXAMPLE 11**

Streptozotocin Increased O-GlcNAc in β Cells

It is presumed that the very high level of O-GlcNAc transferase mRNA in the β cells results in an abundance of O-GlcNAc transferase protein. This high concentration of O-GlcNAc transferase in the β cells suggests that the β cell-specificity of streptozotocin might be related to the ability of this drug to behave as a GlcNAc analogue capable of modulating protein modification by O-GlcNAc.

To determine the effect of streptozotocin on O-GlcNAc in the pancreatic islets, fasting laboratory rats were injected intraperitoneally with a single dose of streptozotocin and then allowed to feed. The animals were sacrificed at various times following injection, and the pancreatic islets were examined for
the presence of O-GlcNAc modified proteins using the monoclonal antibody, RL2. This antibody recognizes the O-GlcNAc protein modification on a wide variety of proteins (13, 19, 21, 22).

The pancreas from untreated animals, that had been allowed to feed for two hours following their overnight fast, showed the typical (13) stochastic distribution of RL2 positivity predominantly in the nuclei of both the islet and exocrine pancreatic cells (Figure 2C). The peripheral non-β cells of the islets exhibited more staining than the central β cells. However, two hours following streptozotocin treatment, when the blood sugar of the treated and untreated animals were both about 150 mg/100ml and before histological evidence of β cell damage had developed, a marked change in the distribution of RL2 positivity was noted in the STZ-treated animal. The central region of all islets examined became markedly positive for RL2 immunohistochemical staining (Figure 2D). The distribution of RL2 staining was similar to the distribution of O-GlcNAc transferase mRNA and insulin, that is, within the central part of each islet. The exocrine pancreas showed no change in RL2 staining (Figure 2), nor did the liver, kidneys or skin (data not shown). At later times, between 4 to 24 hours following the streptozotocin injection, marked islet cell disruption was observed in conjunction with marked hyperglycemia.

This observation demonstrates that streptozotocin modulates a protein modification in β cells that is detectable by the RL2 antibody. Previous studies had shown that the binding of the RL2 antibody to glycoproteins on western blots was highly dependent on O-GlcNAc in the epitope. Antibody recognition could be blocked by preadsorption of RL2 with GlcNAc but could not be
blocked by glucosamine nor N-acetylgalactosamine (21). It is shown that preadsorption of RL2 with streptozotocin also can block binding to O-GlcNAc proteins on western blots. Thus, the modification induced by streptozotocin treatment of the rats could either result from a change in O-GlcNAc or from the direct modification of the β cell proteins by STZ.

**EXAMPLE 12**

*Increase in O-GlcNAc Modification of a Model Peptide*

To determine the biochemical mechanism by which streptozotocin might cause the observed change in RL2 staining of β cells, a highly defined peptide substrate of O-GlcNAc transferase was generated. The structural similarity of streptozotocin to GlcNAc could allow streptozotocin to be used as a substrate for protein glycosylation by O-GlcNAc transferase or streptozotocin could modulate the activity of other enzymes involved in protein O-glycosylation.

To distinguish these possibilities, the previously characterized glycopeptide model was used, which is based on a segment of the transcription factor, Sp1 (17). When expressed as a GST-fusion protein using a vaccinia virus expression system in BSC40 cells, this Sp1 peptide is glycosylated predominantly at a unique site corresponding to serine 484 in Sp1 (17). Mass spectroscopy of this peptide (Figure 3A) indicates the presence of the non-glycosylated form of molecular mass 11,917.5 and the singly glycosylated form of mass 12,118.1. When this peptide was expressed in this vaccinia system in the presence of 5 mM streptozotocin and then analyzed by mass spectroscopy, a striking change in the spectrum was observed (Figure 3B). The non-
glycosylated form of the peptide was not detected and in its place were peptides with masses increasing in increments of about 203 mass units. These peptides corresponded in molecular mass to the mono-, bi- and perhaps tri-\(O\)-GlcNAc forms.

**EXAMPLE 13**

**Streptozotocin Blocks the Removal of \(O\)-GlcNAc from the Sp1 Model Peptide**

The accumulation of \(O\)-GlcNAc on the Sp1 peptide could have resulted as either a consequence of an increased rate of modification or a decreased rate of \(O\)-GlcNAc removal. Furthermore the modification could have resulted from the direct addition of streptozotocin itself to the peptide. While the addition of unmodified streptozotocin would have increased the mass of the peptide by increments of 240, it remains possible that the streptozotocin became chemically modified, thereby approximating the 203 mass of \(O\)-GlcNAc. To distinguish these possibilities, a dual-label pulse-chase experiment was performed in which the peptide backbone of the Sp1 peptide was labeled with \(^{35}\)S-methionine and the \(O\)-GlcNAc residues were labeled with \(^3\)H-glucosamine. The chase was performed with either glucosamine or streptozotocin. These pulse-chase experiments were performed on cells pretreated with or without streptozotocin for 18 hours prior to the addition of the radioactive labels.

In the cells that had not been pretreated with streptozotocin, chase with glucosamine but not streptozotocin resulted in a loss of the \(^3\)H-\(O\)-GlcNAc label from the peptide (Figure 4). Indeed, the streptozotocin chase resulted in continued accumulation of \(^3\)H-\(O\)-GlcNAc on the peptide. The ability of the
unlabeled glucosamine to "chase" the counts implies that the labeled $O$-GlcNAc on the peptide can be replaced with unlabeled $O$-GlcNAc during the chase. The failure of streptozotocin to chase the GlcNAc counts implies that streptozotocin or a metabolite of streptozotocin does not act as a substrate for $O$-GlcNAc transferase and is thus incapable of replacing the labeled $O$-GlcNAc on the modified peptide.

In contrast to these results, the cells pretreated with streptozotocin and subjected to the same pulse-chase experiment displayed two features (Figure 4). First, the peptide became less labeled with $^3$H-$O$-GlcNAc. Second, neither glucosamine nor streptozotocin could chase the $^3$H-$O$-GlcNAc counts. The failure of glucosamine to chase the GlcNAc counts in cells pretreated with streptozotocin suggests that the STZ-treated cells are unable to remove the labeled GlcNAc from the peptide to allow replacement with unlabeled GlcNAc. The lesser degree of $^3$H-$O$-GlcNAc labeling during the pulse in the streptozotocin pretreated cells suggests that the peptide synthesized during streptozotocin treatment and prior to the pulse label was saturated (see Figure 3B) with unlabeled $O$-GlcNAc and this peptide could not undergo exchange labeling during the pulse. Only that peptide newly synthesized during the pulse could be labeled. Thus, it is likely that streptozotocin blocks the removal of GlcNAc from the peptide, thereby leading to the accumulation of the $O$-GlcNAc modification. This experiment also strongly suggests that streptozotocin itself does not become covalently linked to the peptide.
EXAMPLE 14

Streptozotocin Inhibits O-GlcNAc Selective N-acetyl-β-D-glucosaminidase

Direct measurements on the effect of streptozotocin on O-GlcNAc selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase) (18) in vitro indicated that streptozotocin could inhibit the activity of this enzyme with half-maximal inhibition occurring at a streptozotocin concentration of approximately 1 mM (Figure 5). This concentration dependence is similar to that observed for Sp1-peptide hyperglycosylation (Figure 3) (dose-dependence not shown). Streptozotocin had no significant effect on the activity of jack bean hexosaminidase suggesting that the inhibition of the O-GlcNAcase was not simply a result of competition for substrate based on the hexosamine-like structure of STZ. Streptozotocin treatment of cultured cells for 6 hours also resulted in the loss of nuclear O-GlcNAcase activity but not lysosomal hexosaminidase activity as measured on cell extracts (data not shown).

Inhibitors of O-GlcNAcase activity, whose structure is based on GlcNAc, have been described (18). In addition, the activity of the O-GlcNAcase has been shown to be blocked by N-ethylmaleimide, indicating the presence of a free sulfhydryl group in the active site. Interestingly, streptozotocin is thought to be either an NO donor (2, 4) or alkylating agent (3) raising the possibility that the free sulfhydryl group in the active site of the O-GlcNAcase might be covalently modified either by NO (5) or alkylation, resulting in an irreversible inactivation of the enzyme. Therefore, streptozotocin could be targeted specifically to the O-GlcNAcase as a result of its structural similarity to GlcNAc and could inactivate this enzyme through the covalent modification of
its active site. While this mechanism by itself would not explain the specificity of streptozotocin for the β cell, the abundance of O-GlcNAc transferase in these cells would make them particularly sensitive to a process that delays removal of O-GlcNAc residues.

EXAMPLE 15

Streptozotocin Treatment of Cultured Cells Results in Hyperglycosylation of Several Intracellular Proteins.

The O-GlcNAc modification is present in all eukaryotic cells (14) and in mammalian cells, the enzymes involved in the metabolic steps leading to this modification appear to be ubiquitous. In order to obtain sufficient cellular proteins to study the effect of streptozotocin on the O-GlcNAc modification, the same simian BSC40 cell line used for vaccinia virus expression of the Sp1 peptide was generated. After an overnight incubation, the cells were placed in glucose-free medium to enhance the uptake of STZ. Intracellular proteins extracted from cells incubated in glucose-free medium for 6 hours displayed a low O-GlcNAc content as detected by RL2 immunoblotting. In contrast, proteins from cells treated with streptozotocin in glucose-free medium displayed markedly more glycosylation. (Figure 6). This effect was present but slightly blunted if streptozotocin was added to the cells simultaneously with an inhibitor of glucosamine synthesis, 6-diazo-5-oxonorleucine (13, 21, 22).

These results show that the effects of streptozotocin on protein glycosylation can occur in cells other than β cells, indicating that the process of hyperglycosylation stimulated by streptozotocin is fundamentally the same in these cells. The ability of streptozotocin to stimulate hyperglycosylation when the
enzyme GFAT is briefly and simultaneously inhibited or when cells are deprived of glucose is compatible with the notion that streptozotocin treatment does not result in increased glucosamine synthesis from the activation of GFAT, or increased de novo O-glycosylation through the activation of O-GlcNAc transferase, but rather that streptozotocin preserves the state of glycosylation of the proteins through the inactivation of the O-GlcNAcase. This interpretation is compatible with the pulse-chase experiments and measured effect of streptozotocin on O-GlcNAcase. Cells treated with glucose and glucosamine exhibited more protein O-glycosylation than cells that were glucose-starved over the same time period. In the presence of these sugars, streptozotocin had no further effect. Since streptozotocin is a glucosamine analog, and glucosamine is transported into the cell less efficiently than glucose, glucose may have blocked a further effect of streptozotocin because glucose was taken up by the cell in preference to the streptozotocin.

It has been shown previously in a variety of cell lines (13, 21, 22), and it is shown (Figure 6), that alterations in the concentration of extracellular glucose results in corresponding alterations in protein modification by O-GlcNAc. Glucosamine exposure has an even greater effect on this protein modification (13, 21, 22) (Figure 6) while prolonged blockade of GFAT activity results in less O-GlcNAc (13, 21, 22). The effect of glucose may be less dramatic than the effect of glucosamine because the activity of the enzyme required for metabolism of glucose to glucosamine, GFAT, is regulated by negative feedback by its downstream product UDP-GlcNAc (11, 12). Nevertheless, in a variety of cell types, alterations of extracellular glucose or glucosamine
concentrations have been reflected by changes in intracellular UDP-GlcNAc content (23, 24). That glucose and glucosamine exposure results in increased protein modification by O-GlcNAc suggests that the overall activity of O-GlcNAc transferase is largely regulated by the availability of its substrate, UDP-GlcNAc. While it has not yet been demonstrated this effect of glucose in the normal islet cell, the behavior of the enzymes involved in protein O-glycosylation are likely to be qualitatively similar to the behavior observed in other cell types.

These studies show that the specific β cell toxin, streptozotocin, interferes with the metabolism of proteins modified by O-GlcNAc. It was observed that this drug results in the accumulation of the O-GlcNAc modification on proteins in the β cells of normal islets of intact animals and that this effect precedes β cell death by several hours. This effect of streptozotocin was observed in cultured non-β-cells and that the accumulation of O-GlcNAc on proteins results from the ability of streptozotocin to specifically block the activity of the enzyme, O-GlcNAcase, that removes the monosaccharide from proteins. That streptozotocin results specifically in the accumulation of O-GlcNAc in the β cells in vivo suggests that this accumulation results from the high level of expression of O-GlcNAc transferase in the β cells. The potentially high on-rate of O-GlcNAc in these cells would make them the most sensitive to any inhibition of O-GlcNAc removal.

These results provide a correlation between the β cell toxicity of streptozotocin and its effect on the metabolism of O-GlcNAc-modified proteins. This correlation alone does not prove that the STZ-induced accumulation of O-GlcNAc in the β cells is
itself cytotoxic. This proof would require that the primary and perhaps only biochemical effect of streptozotocin is upon the O-GlcNAcase. Since its discovery, streptozotocin has been proposed to have a variety of biochemical actions. It has been shown to be an alkylating agent capable of modifying DNA (3) and an NO donor (2, 4). The accumulation of excessive NO in β cells has been proposed to be associated with apoptosis (6, 25, 26). However, all of these properties of streptozotocin have failed to adequately explain how this antibiotic is so specifically toxic to the β cell.

It is observed that the β cell is so richly endowed with O-GlcNAc transferase relative to other tissues and that streptozotocin specifically inhibits O-GlcNAcase, coupled with the structural resemblance of streptozotocin to GlcNAc itself are, together, compelling evidence that the β cell toxicity of streptozotocin results from its action on the metabolism of O-GlcNAc-modified proteins. If this is indeed the case, it remains to be demonstrated how the accumulation of O-GlcNAc in the β cell or any other cell type is cytotoxic.

That excessive O-GlcNAc is cytotoxic is supported by the observation that when the O-GlcNAc transferase enzyme was overexpressed as a transgene in C. elegans a lethal phenotype resulted (16). While it is presumed that this rather abundant modification of proteins of the nucleus and cytoskeleton plays some role in the normal function of these proteins in the cell, the role of this modification has not been definitively determined. Hart and coworkers (14) have suggested a variety of roles for this protein modification in cellular function. The dynamic nature of this modification in response to extracellular signals and through the cell cycle and the fact that this modification has been mapped
in certain proteins to the same serine residues that can be phosphorylated have suggested that the O-GlcNAc modification plays a role as important to protein function as does phosphorylation.

The studies (17) on the role of O-GlcNAc in the function of the transcription factor, Sp1, suggest that one function of O-GlcNAc is to occlude hydrophobic protein interaction domains by rendering these domains hydrophilic. From this Sp1 model, it was proposed that this modification would prevent newly synthesized nuclear proteins from prematurely forming, in the cytoplasm, the sort of protein complexes that later occur on the DNA template. If so, then the removal of O-GlcNAc from nuclear proteins would be a vital step in the protein complex assembly necessary for nuclear transcription and organization. Blockade of the enzyme required for the removal of O-GlcNAc could then have dire consequences, particularly in the β cell with its high content of the transferase. While the generality of this proposed mechanism has yet to be demonstrated, streptozotocin should become a useful reagent in the investigation of the role of the O-GlcNAc modification in protein function.

Clinical (26-32) and in vitro (33-35) studies have suggested that the chronic exposure of β cells to supraphysiological concentrations of glucose may result in impaired β cell function. The mechanism of this so called glucose toxicity has remained unclear. These studies on the mechanism of toxicity of streptozotocin may provide some insight into the role of O-GlcNAc in glucose toxicity in the β cell. The metabolic pathway for protein modification by O-GlcNAc is summarized in Figure 7. In normal fasting individuals, UDP-GlcNAc availability in the β cell
would be restricted because the β cell glucokinase (36), with its relatively low affinity for glucose, would provide little substrate (fructose-6-phosphate) for glucosamine synthesis. Since O-GlcNAc transferase activity appears to be driven by the level of UDP-GlcNAc, the fasting β cell should exhibit very low rates of protein modification by O-GlcNAc despite the high level of expression of O-GlcNAc transferase. Conversely, in the postprandial state, when the blood glucose transiently exceeds 5 mM, substrate would be provided to GFAT to allow the synthesis of glucosamine and hence, UDP-GlcNAc. Because the β cell is well equipped with O-GlcNAc transferase, these cells would be predicted to display a marked difference in the rate of intracellular protein modification by O-GlcNAc depending on whether the blood glucose is above or below 5 mM. This glucose-induced alteration in protein O-glycosylation could subserve an as yet unidentified normal role in β cell function. However, exaggeration of this pathway as occurs when streptozotocin blocks the O-GlcNAcase, appears to result in the attainment of a threshold level of O-GlcNAc modified proteins that is cytotoxic.

While the temporary and physiological elevations in blood glucose that occur following a meal in normal individuals might increase the rate of protein modification by O-GlcNAc, accumulation of O-GlcNAc-modified proteins would normally be insufficient to reach cytotoxic levels. Indeed, it was unable to detect a change in the RL2 signal in islets of normal rats in the post-prandial state. However, it remains possible that the sustained elevations of blood glucose that occur in uncontrolled type 2 diabetes could drive the level of O-GlcNAc-protein modification in the β cells to levels approaching those seen
following streptozotocin treatment, thereby resulting in cytotoxicity. Repeated episodes of hyperglycemia could ultimately result in sufficient loss of β cell mass or function such that the insulin requirements could no longer be met, thereby accelerating this process of glucose toxicity.

Glucosamine has also been proposed to contribute to insulin resistance (37-41) and the vascular complications of diabetes (13). Increased O-GlcNAc modification of nuclear and cytoplasmic proteins in blood vessels, muscle and fat may also play a role in these aspects of diabetes. However, the association of O-GlcNAc with the β cell toxicity of streptozotocin is the first potentially direct connection between this protein modification and the development of diabetes.

**EXAMPLE 16**

Glucose and Glucosamine Infusion and STZ Treatment in Rats

Male Sprague-Dawley rats (Charles River laboratory, body weight 150-175 g) were fasted overnight. Catheterization was performed under anesthesia. The experimental rats were infused through a catheter in the right femoral vein with either dextrose (0.042-0.125 mg.g⁻¹.min⁻¹, Sigma) or glucosamine (0.025 mg.g⁻¹.min⁻¹, Sigma) by a electronically-controlled syringe pump (KD Scientific) while control rats were infused with PBS solution (PH 7.4). Blood glucose was determined before and during the infusion at ten minute intervals on blood derived from a tail bleed using an AAdvantage® blood glucose monitor (Boehringer Mannheim). Human insulin (Eli Lilly) was dissolved in 0.9% saline and administrated intravenously. The streptozotocin (200 mg.kg⁻¹ freshly dissolved in 100 mM citrate buffer, pH 4.2) was injected
intravenously 15 minutes before the glucose infusion. At the various time points and blood glucose levels, the pancreas was removed and formaldehyde-fixed immediately. Animal treatments were performed in an approved manner in a certified animal care facility.

**EXAMPLE 17**

**Tissue Immunohistochemistry**

Formaldehyde-fixed and paraffin-embedded pancreas sections were deparaffinized in xylene and rehydrated through graded alcohol concentrations to water. To facilitate immunostaining, the sections were pretreated with 2 N HCl for 20 minutes at 37°C and exposed to 0.01% trypsin at 37°C for 5 minutes. The sections were immunostained with RL2 monoclonal antibody (ascites, diluted 1:20) or anti-insulin polyclonal antibody (1:50; NCL-INS, Novocastra) using the Vectastain Elite ABC kit (Vector). 3,3’-Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, and sections were counterstained with Gill’s Hematoxylin (Vector).

**EXAMPLE 18**

**Treatment SJLXB6 Mice with STZ in Combination with Glucose or Glucosamine**

74 overnight fasted male SJLXB6 mice (body weight 19.6 to 27.4 g) were divided into three groups. The mice in the first group (n=26) were injected intraperitoneally with streptozotocin (50 mg/kg) and PBS (pH 7.4, 30 min after the treatment of STZ). The mice in the second group (n=23) were injected intraperitoneally with streptozotocin (50 mg/kg) followed
in 30 min by glucose (8.76-10.0 mg/g). The mice in the third group (n=25) were injected intraperitoneally with streptozotocin (50 mg/kg) followed in 30 min by glucosamine (4.98 mg/g). Additionally, 50 mice were divided into five groups and injected intraperitoneally with streptozotocin at dose of 55, 60, 65, 75, 80 mg/kg respectively. The blood glucose was monitored frequently prior to sacrifice 26 hours later.

**EXAMPLE 19**

**In situ Hybridization**

The paraffin and frozen sections of mouse pancreas were prepared and hybridized as described by Hanahan, D., *Nature* **315**, 115-22 (1985). The antisense insulin $^{35}$S-cRNA riboprobe was synthesized from the 350 bp rat insulin I cDNA in pBlueScript KS (Stratagene), using T3 polymerase. The sense $^{35}$S-cRNA riboprobe, to detect the transgenic antisense mouse GFAT mRNA, was synthesized from the 2.1 kb mouse GFAT cDNA in pT7T3 (Pharmacia), using T7 RNA polymerase. After hybridization, photographic emulsion dip, exposure, and developing, the sections were counterstained with hematoxylin and eosin and were subjected to microscopic examination under bright field and dark field illumination.

**EXAMPLE 20**

**Terminal Deoxynucleotide Transferase-Mediated dUTP Nicked End-Labeling (TUNEL) Assay**

The TUNEL assay was performed in paraffin-embedded sections of the pancreases using an *in situ* cell death detection kit (Boehringer Mannheim). The endogenous
peroxidase activity was blocked by immersing the sections in 0.3% H$_2$O$_2$ in methanol for 30 min prior to cell permeabilization. Non-specific binding of the peroxidase-coupled anti-fluorescein antibody was blocked with PBS containing 3% BSA for 20 min. Positive cells were visualized using peroxidase substrate enhancer and DAB substrate (Boehringer Mannheim) and sections were counterstained with Hematoxylin.

**EXAMPLE 21**

Generation of Transgenic Mice with β-Cell-Specific Expression of Mouse GFAT Antisense Gene

The 2.2 kb mouse GFAT cDNA$^{22}$, consisting of 150 b p 5'UTR and complete coding sequence, was inserted in the antisense direction into an XbaI-XbaI site between the rat insulin II promoter (RIP)$^{41}$ and the SV40 small T-antigen intron and polyadenylation sequences. The entire 4.4 kb fragment, containing the RIP-mGFAT (antisense)-SV40 construct, was excised from the cloning vector with KpnI, purified by QIAquick system (Qiagen) and microinjected into fertilized eggs from SJLXB6 mice. Of the 29 living births, seven founder mice were identified by PCR analysis of tail tip DNA using oligonucleotide primers hybridizing to the RIP and mGFAT sequence. The resulting 1.28 kb PCR product spans the RIP-mGFAT junction. Gene dose was determined by Slot-Blot analysis and the transgenic mouse line, termed 3-4, with the highest gene dose was used for the majority of studies. For consistency, 3 to 4 month-old male mice from this line were used for the subsequent studies, although similar results were obtained with the other lines, females and animals over 5 months old. *In situ* hybridization with a sense-oriented mouse
GFAT [\[^{35}S\]-cRNA probe was performed to determine the β-cell-specific expression of the antisense transgene in islets as before. Immunohistochemical staining with RL2 monoclonal antibodies was performed as before in the transgenic mice and their wild type littermates 1.5 hour after intraperitoneal injection with streptozotocin (50 mg/kg) and glucose (10.0 mg/g, 30 min after the treatment of STZ).

**EXAMPLE 22**

**Treatment of RIP-mGFAT (AS) Mice with Mutiple Low Doses of STZ**

Multiple low doses of streptozotocin treatment (5 consecutive days, 40 mg/kg) was performed in 14 transgenic mice and 16 littermates (from 3 different lines). The diabetes was assessed by blood glucose measurements every 3 days and histological analysis 14 and 28 days after the last injection of streptozotocin.

**EXAMPLE 23**

**Treatment of RIP-mGFAT (AS) Mice with STZ in Combination with Glucose or Glucosamine**

The transgenic mice and their littermates were injected with either streptozotocin (50 mg/kg) and glucose (8.76-10 mg/g, n = 41, 21 transgenic mice and 20 wild type littermates), or streptozotocin (50 mg/kg) and glucosamine (4.98 mg/g, n=35, 19 transgenic mice and 16 wild type littermates). The blood glucose was monitored frequently and the mice were sacrificed for histological analysis, immunohistochemical staining with anti-insulin polyclonal antibody, in situ hybridization for insulin transcripts, TUNEL assay 26 hours later. All values are expressed
as mean ± S.E. Statistical analysis is carried out with unpaired t

test and χ² test. Differences are considered statistically

significantly at p<0.05 or p<0.01.

EXAMPLE 24

O-GlcNAc Level in β-cells is Rapidly Responsive to the Blood

Glucose in vivo

For the glucose concentration to be transduced into a

change in the O-GlcNAc content in β-cells requires that glucose

first be converted to glucosamine, and secondly, that the increased

availability of UDP-GlcNAc as a substrate would drive increased

protein modification by OGT. The rate limiting step in glucosamine

synthesis is the enzyme glutamine:fructose-6-phosphate

amidotransferase (GFAT). Provision of substrate for GFAT

requires a glucose concentration sufficient for phosphorylation by

the β-cell glucokinase. Furthermore, GFAT is known to be

regulated by feedback inhibition by UDP-GlcNAc. While in other

cell types, altered glucose concentrations can be reflected by

changes in the O-GlcNAc modification of intracellular proteins, the

pancreatic β-cell has not been shown to respond in this manner.

To determine the effect of changing plasma glucose

concentrations on β-cell O-GlcNAc content in vivo, hyperglycemia

was established in Sprague-Dawley rats by intravenous glucose

infusion and assessed β-cell O-GlcNAc by immunohistochemical

staining with the RL2 monoclonal antibody. This antibody has

been shown to bind specifically to the O-GlcNAc modification on

proteins. Elevation of the plasma glucose concentration to

approximately 20 mM resulted in a marked and β-cell-specific
increase in the level of intracellular O-GlcNAc modification within
30 minutes. No change in O-GlcNAc content was detected in the
exocrine pancreas. Conversely, fifteen minutes after the blood
glucose concentration was decreased to 4.0 ± 0.2 mM (Mean ± S.E.)
following intravenous treatment with human insulin, the O-GlcNAc
modification level had returned to the level seen in control fasted
rats (Figure 8). This observation indicates that the level of O-
GlcNAc in β-cells is rapidly responsive to the extracellular glucose
concentration. This specific O-GlcNAc response to hyperglycemia
in the β-cells likely results from both the gating effect of the
glucokinase, which restricts substrate for glucosamine synthesis
under fasting conditions, and the high abundance of the
transferase in these cells.

**EXAMPLE 25**

**STZ Causes O-GlcNAc Accumulation by Blocking its Removal from the Proteins**

While streptozotocin appears to be specifically toxic to
the β-cell because it can block the O-GlcNAcase *in vitro* and OGT is
selectively abundant in β-cells, other mechanisms for this specific
toxicity have also been proposed. Streptozotocin has been shown
to be a DNA alkylating agent and nitric oxide (NO) donor, and its
specificity to the β-cell was suggested to result from the
preferential ability of the GLUT2 to transport this agent into β-
cells.

To determine whether streptozotocin behaves as an O-
GlcNAcase inhibitor in β-cells in intact animals, Sprague Dawley
rats were pretreated with streptozotocin before the initiation of
the glucose infusion. The islets were again examined by immunohistochemical staining with the RL2 antibody. In the absence of hyperglycemia, streptozotocin did not alter the O-GlcNAc content of β-cells. This observation is in agreement with the determination that streptozotocin itself is not incorporated into β-cell proteins. However, when hyperglycemia was achieved after the streptozotocin dose, the increase in β-cell O-GlcNAc was again observed. In contrast, when normoglycemia (3.9 ± 0.2 mM) was re-established, even for one hour in the streptozotocin-treated animal, the O-GlcNAc content failed to decrease in the islets in response to the falling blood sugar, indicating that the removal of the O-GlcNAc modification was blocked by streptozotocin (Figure 8). Thus, streptozotocin inhibits the O-GlcNAcase in β-cells in vivo.

**EXAMPLE 26**

**Glucose and Glucosamine Potentiate STZ Toxicity and Cause β-cell Apoptosis**

To further correlate the accumulation of O-GlcNAc in β cells with streptozotocin toxicity, the diabetogenic effect of a single dose of streptozotocin was tested in SJLXB6 mice. This strain of mice exhibits reduced sensitivity to this drug relative to the rat. While a single dose lower than 75 mg/kg of streptozotocin was insufficient to cause β-cell death, treatment with either glucose or glucosamine following an injection of 50 mg/kg of streptozotocin resulted in extensive β-cell death within 26 hours in 78.3% and 76.0% of treated mice respectively (Table 1).
TABLE 1

The effect of glucose and glucosamine on the β-cell toxicity of STZ in RIP-GFAT (AS) transgenic and wild type SJLXB6 mice.

<table>
<thead>
<tr>
<th>Mice Type</th>
<th>Treatment</th>
<th># Mice Examed</th>
<th># Mice Affected</th>
<th>X² test</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJLXB6</td>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1) STZ (50 mg/kg)</td>
<td>26</td>
<td>0 (0)</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2) STZ (55 mg/kg)</td>
<td>10</td>
<td>0 (0)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3) STZ (60 mg/kg)</td>
<td>10</td>
<td>0 (0)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4) STZ (65 mg/kg)</td>
<td>10</td>
<td>0 (0)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5) STZ (70 mg/kg)</td>
<td>10</td>
<td>2 (20)</td>
<td>8</td>
<td>p=0.020 vs.(1)</td>
</tr>
<tr>
<td></td>
<td>(6) STZ (75 mg/kg)</td>
<td>10</td>
<td>8 (80)</td>
<td>2</td>
<td>p&lt;0.0001 vs.(1)</td>
</tr>
<tr>
<td></td>
<td>(7) STZ (80 mg/kg)</td>
<td>10</td>
<td>8 (80)</td>
<td>2</td>
<td>p&lt;0.0001 vs.(1)</td>
</tr>
<tr>
<td></td>
<td>(8) STZ (50 mg/kg)+Glu</td>
<td>23</td>
<td>18 (78.3)</td>
<td>5</td>
<td>p&lt;0.0001 vs. (1)</td>
</tr>
<tr>
<td></td>
<td>(9) STZ (50 mg/kg)+GlcN</td>
<td>25</td>
<td>19 (76.0)</td>
<td>6</td>
<td>p&lt;0.0001 vs. (1)</td>
</tr>
<tr>
<td>Transgenic Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(10) STZ (50 mg/kg)+Glu</td>
<td>21</td>
<td>6 (28.6)</td>
<td>15</td>
<td>p=0.0010 vs.(8)</td>
</tr>
<tr>
<td></td>
<td>(11) STZ (50 mg/kg)+GlcN</td>
<td>19</td>
<td>15 (78.9)</td>
<td>4</td>
<td>p=0.0015 vs.(10)</td>
</tr>
<tr>
<td>Wild Type Littermates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12) STZ (50 mg/kg)+Glu</td>
<td>20</td>
<td>16 (80.0)</td>
<td>4</td>
<td>p=0.0009 vs.(10)</td>
</tr>
<tr>
<td></td>
<td>(13) STZ (50 mg/kg)+GlcN</td>
<td>16</td>
<td>13 (81.3)</td>
<td>3</td>
<td>p=0.8653 vs.(11)</td>
</tr>
</tbody>
</table>

*: extensive β cells apoptosis; STZ: streptozotocin; Glu: glucose; GlcN: glucosamine. The experimental methods are described (26,32). Statistical analysis was carried out with Chi test in MicroExcel Statistics Software. Difference were considered statistically significant at P<0.01.
Cell death was evident on histological sections by the appearance of the pyknotic nuclei and was accompanied by a diminution of insulin mRNA as determined by in situ hybridization (Figure 9). To determine if the β-cell death was apoptotic in nature, sections from the same pancreases were stained using the terminal deoxynucleotidyl transferase-mediated dUTP nicked end-labeling (TUNEL) assay. The islets in the animals treated with streptozotocin alone showed no evidence of nicked DNA whereas the islets from those animals treated with streptozotocin followed by glucose or glucosamine showed numerous apoptotic cells (Figure 9).

It has been observed that glucose potentiates the toxic effect of streptozotocin on β-cells, however, the mechanism has not been understood. This potentiation of streptozotocin toxicity with both glucose and glucosamine is compatible with the notion that the sustained accumulation of O-GlcNAc caused by the combination of streptozotocin and glucose administration is behind this toxicity. Furthermore, a high concentration of glucose has recently been shown to induce apoptosis in cultured islets and hyperglycemia induces β-cell apoptosis in certain rodent strains. However, there was no significant islet pathology or apoptosis following an 8 hour intravenous glucose infusion in Sprague Dawley rats. In contrast, an 8 hour intravenous infusion of glucosamine in these rats did result in selective islet pathology. Islets from the glucosamine-infused rats exhibited nuclear pyknosis (Figure 10) with several cells being positive for DNA nicking by the TUNEL assay (not shown). There were fewer apoptotic cells after the 8 hours of glucosamine exposure than
were seen 24 hours after streptozotocin plus glucose or glucosamine. Depletion of immunoreactive insulin in the β-cells in the glucosamine-treated rats was also observed. No significant pathological changes were found in the exocrine pancreas.

**EXAMPLE 27**

**Trangenic Mice with Impaired Glucosamine Synthesis are Resistant to the Diabetogenic Effect of STZ**

To establish a causal link between β-cell glucosamine metabolism and streptozotocin toxicity, a transgenic mouse line was developed in which an anti-sense GFAT construct was expressed in β-cells that blocks the glucose-stimulated increase in O-GlcNAc modification in cultured mouse cells. Integration of the transgene was detected by PCR in DNA extracted from the mouse tails (Figure 11) and by Southern blot analysis (not shown). *In situ* hybridization confirmed the β-cell-specific expression of the anti-sense transgene in the islets (Figure 11). As shown previously in cultured cells, this anti-sense construct was able to blunt the streptozotocin and glucose-induced O-GlcNAc accumulation in the β-cells of the transgenic mice as compared to the non-transgenic littermates (Figure 11).

A multiple low dose streptozotocin protocol has been developed to induce diabetes in mice. Application of this protocol to the mice expressing the anti-sense GFAT transgene failed to induce diabetes while their wild type littermates developed hyperglycemia and islet destruction within 21 days after the same treatment (Figure 12). To prove that it is impaired glucosamine synthesis in the anti-sense GFAT transgenics that confers this streptozotocin resistance, the observation that both glucose and
glucosamine potentiate the diabetogenic effect of a single dose of streptozotocin (Figure 9) was used.

Concurrent treatment with glucose and streptozotocin (50 mg/kg) failed to cause β-cell apoptosis in 71.4% of the transgenic mice, but caused extensive β-cell apoptosis in 80.0% of their wild type littermates. In contrast, when the mice were administered concurrent treatment with glucosamine and streptozotocin, extensive β-cell apoptosis was observed in 78.9% of the transgenic mice and 81.3% of their wild type littermates (Table 1). This observation strongly supports the idea that glucose must be metabolized by GFAT to glucosamine to potentiate the toxicity of streptozotocin. The glucosamine administered with the streptozotocin bypassed the block in glucose metabolism induced by the anti-sense GFAT transgene thereby implicating the transgene in the streptozotocin resistance. Furthermore, this effect of the anti-sense transgene was observed in three independently developed transgenic lines, ruling out an integration-site specific mechanism for the streptozotocin resistance.

The β-cell as a glucose sensor has adapted to respond both instantaneously and more chronically to changes in the nutritional load. The long-term adaptation that switches the β-cell from the famine mode with limited insulin-secretory capacity to the surplus mode with increased capacity is poorly understood. In insulin resistant animal models or after partial pancreatectomy, the rate of β-cell proliferation increases. However, in those animal models susceptible to the development of type 2 diabetes, this proliferative response is ultimately offset by an increase in
apoptosis that results in a failure of the net β-cell mass to increase sufficiently to fulfill the insulin demand. Both the proliferative and apoptotic response appear to be driven by hyperglycemia. Indeed, the apoptotic response may be part of the glucose toxicity that results in reduced insulin secretion following prolonged hyperglycemia.

The present invention demonstrates the link between the metabolism of glucose to O-GlcNAc with β-cell apoptosis. The normal β-cell, like no other known cell, has a markedly accelerated on and off rate of O-GlcNAc, streptozotocin arrests this cycle resulting in the stable and β-cell-specific accumulation of the modification in vivo. The enhancement of the apoptotic response to streptozotocin plus glucose is blunted in transgenic animals with impaired glucosamine synthesis from glucose, indicating that it is this pathway of glucose metabolism that is required for the β-cells-specific toxic and apoptotic effect of STZ.

Until now, no in vivo role for the O-GlcNAc modification of proteins has been ascertained. These results linking the O-GlcNAc modification of proteins to β-cell apoptosis will now open up the search for the protein targets of OGT that lead to the initiation of this process. Candidate targets may arise from the idea that the O-GlcNAc response in β-cells to hyperglycemia likely serves some physiological role besides causing apoptosis. OGT contains nuclear localization signals and is enriched in the nucleus and there is evidence that the O-GlcNAc modification may play an important role in the control of gene transcription. Many transcription factors are modified with O-GlcNAc. Sp1, an O-GlcNAc modified transcription factor, has been
found to be regulated in a complex manner by O-GlcNAc. First, the
stability of Sp1 has been shown to be regulated by the
proteasome. Under condition of O-GlcNAc depletion, Sp1 is rapidly
degraded while glucosamine treatment of cells results in the
stabilization of Sp1 through the inhibition of proteasomal
degradation in vivo\textsuperscript{16} and in vitro. These changes in the
abundance of Sp1 or perhaps other transcription factors, may alter
the transcription of a wide variety of genes including those TATA-
less genes that encode the housekeeping proteins. Thus, the O-
GlcNAc response to glucose could upregulate the expression of
many β-cell genes leading to cellular hypertrophy. However, it
also remains possible that this inhibition of proteasomal
degradation of Sp1 may extend to other proteins known to be
degraded by the proteasome and involved in apoptosis. Another
effect of O-GlcNAc on Sp1 is in the control of the Sp1 protein
interactions involved in transcriptional activation. This
observation raises the possibility that sustained O-GlcNAc
modification of a transcription activation domain of Sp1 could
preclude the protein interactions required for transcriptional
activation and lead to impaired gene transcription.

The studies reported here strongly support the notion
that STZ-induced β-cell apoptosis results from blockade of O-
GlcNAcase activity and the subsequent accumulation of O-GlcNAc
on certain proteins in β-cells. While glucose itself can cause β-cell
apoptosis in certain rodent models, the accumulation of O-GlcNAc
in β-cells in response to short term hyperglycemia alone, at least
in this strain of rat, was not sufficient to induce a detectable level
of apoptosis, while pushing this pathway with an 8 hour
glucosamine infusion did result in apoptosis, albeit, less extensive
than seen 24 hours after STZ. The difference in behavior of islets treated with glucose from those treated with glucose in combination with streptozotocin may relate to the cyclical nature of the O-GlcNAc modification. That is, the on-rate for the modification normally may be matched by the off-rate even though the steady-state level of modification may be high during hyperglycemia. Interruption of this cycle with streptozotocin may result in the prolonged accumulation of O-GlcNAc on the critical substrates that become involved in the apoptotic response. While delineation of the mechanism of O-GlcNAc-induced apoptosis awaits identification of these substrates, the anti-sense GFAT transgenic model described provides evidence that the O-GlcNAc pathway is a worthy target for intervention in the glucose toxicity that leads to β-cell failure and the pathogenesis of type 2 diabetes.

The following references were cited herein.


Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.
WHAT IS CLAIMED IS:

1. A method of treating an individual with type 2 diabetes, comprising the step of:
   contacting said individual with an effective dose of a drug, wherein said drug blocks a glucose metabolic pathway.

2. The method of claim 1, wherein said drug inhibits activity of one or more than one enzyme involved in said glucose metabolic pathway.

3. The method of claim 2, wherein said enzyme is selected from the group consisting of glutamine:fructose-6-phosphate amidotransferase and O-linked N-acetylglucosamine transferase.

4. The method of claim 1, further comprising the step of:
   administering insulin to said individual.

5. A method of treating an individual with type 2 diabetes, comprising the step of:
   contacting said individual with an effective dose of a drug, wherein said drug activates O-linked N-acetylglucosamine removal from intracellular proteins.

6. The method of claim 5, wherein said drug enhances the activity of O-GlcNAc-selective N-acetyl-β-D-glucosaminidase.
7. The method of claim 5, further comprising the step of:
administering insulin to said individual.

8. A method of screening for a drug which blocks production and/or accumulation of O-GlcNAc-modified proteins in an animal, comprising the steps of:
contacting the animal with a drug candidate;
staining pancreatic islets from treated and untreated animals with an antibody directed against said O-GlcNAc-modified proteins;

obtaining a distribution of said antibody in pancreatic β cells of said treated animal;

comparing said distribution in said treated animal and the distribution of said antibody in pancreatic β cells of the untreated animal, wherein if said distribution in said treated animal is less than the distribution in said untreated animal, said drug blocks the production and/or accumulation of O-GlcNAc-modified proteins.

9. A method of screening a compound which blocks production of O-GlcNAc in a cell, comprising the steps of:
contacting the cell with a test compound, wherein said cell is in a glucose-containing medium;

measuring the level of glucosamine in said cell;

comparing the level of glucosamine in the treated cell and that in an untreated cell, wherein if the level of glucosamine in the treated cell is less than that in the untreated cell, said compound blocks O-GlcNAc production.
10. A transgenic animal containing a transgene, wherein said transgene is mouse glutamine:fructose-6-phosphate amidotransferase (GFAT) antisense gene.

11. The transgenic animal of claim 10, wherein 150 bp 5' UTR and complete coding sequence of mouse GFAT cDNA is inserted in the antisense direction into an XbaI-XbaI site between the rat insulin II promoter (RIP) and the SV40 small T-antigen intron and polyadenylation sequences to form RIP-mGFAT (antisense)-SV40 construct in said transgene.

12. The transgenic animal of claim 11, wherein said construct is expressed in islet β-cells that blocks the glucose-stimulated increase in O-GlcNAc modification, resulting impaired β-cell glucosamine synthesis.

13. A method of screening for a drug useful for treating diabetes, comprising the steps of:

administering the transgenic animal of claim 10 with streptozotocin (STZ) in combination with glucosamine to obtain diabetic transgenic animal;

measuring blood glucose level in said diabetic transgenic animal;

administering said diabetic transgenic animal with a drug candidate; and

measuring blood glucose level in said drug-treated diabetic transgenic animal, wherein if the blood glucose level in said drug-treated diabetic transgenic animal is lower than the
blood glucose level in the diabetic transgenic animal without the
drug treatment, said drug is useful for treating diabetes.

treating diabetes, comprising the steps of:
administering streptozotocin in combination with
glucosamine to the transgenic animal of claim 10 to obtain diabetic
transgenic animal;
measuring β-cell apoptosis in said diabetic transgenic
animal;
administering a drug candidate to said diabetic
transgenic animal; and
measuring β-cell apoptosis in said drug-treated
diabetic transgenic animal, wherein if the β-cell apoptosis in said
drug-treated diabetic transgenic animal is reduced compared to
the β-cell apoptosis in the diabetic transgenic animal without the
drug treatment, said drug is useful for treating diabetes.
N-Acetylglucosamine  

Streptozotocin
FIG. 6

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SUBSTITUTE SHEET (RULE 26)
Protein O-Glycosylation

FIG. 7
Schematic of Transgene

1.28 kb

RIP  Antisense  SV40
mGFAT  Poly A

FIG. 11A

FIG. 11B

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SUBSTITUTE SHEET (RULE 26)
FIG. 12
### A. CLASSIFICATION OF SUBJECT MATTER

**IPC(6)**: A01N 37/18; A61K 38/00  
**US CL.**: 514/2, 866  
According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
**U.S.**: 514/2, 866

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

PHYSICIAN’S DESK REFERENCE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
WEST, STN, MEDLINE, BIOSIS, CAPLUS

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 4,952,568 A (K. SAWAI) 28 August, 1990, column 2, lines 3-8, claim 1</td>
<td>1</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.  
See patent family annex.

<table>
<thead>
<tr>
<th>*</th>
<th>Special categories of cited documents:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>document defining the general state of the art which is not considered to be of particular relevance</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>earlier document published on or after the international filing date</td>
</tr>
<tr>
<td><strong>L</strong></td>
<td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td>
</tr>
<tr>
<td><strong>O</strong></td>
<td>document referring to an oral disclosure, use, exhibition or other means</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>document published prior to the international filing date but later than the priority date claimed</td>
</tr>
</tbody>
</table>

| **T** | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| **X** | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| **Y** | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |

**Date of the actual completion of the international search**: 18 NOVEMBER 1999  
**Date of mailing of the international search report**: 23 DEC 1999

**Name and mailing address of the ISA/US Commissioner of Patents and Trademarks**:  
Box PCT  
Washington, D.C. 20231  
**Facsimile No.**: (703) 305-3230

**Authorized officer**:  
KRIS PELHAM MAYO  
**Telephone No.**: (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)*
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y,P</td>
<td>US 8,877,183 A (A.H. CINCOTTA) 02 March 1999, column 5, lines 20-30, lines 46-57.</td>
<td>2-7</td>
</tr>
</tbody>
</table>
INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. 1-7

Remark on Protest  
[ ] The additional search fees were accompanied by the applicant’s protest.  
[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1992)
BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-7, drawn to a method of treating Type II diabetes.
Group II, claim(s) 8-9, drawn to an in vivo screening assay.
Group III, claim(s) 10-14, drawn to a transgenic animal and method of using the transgenic animal in a drug screening assay.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: a method of treating type II diabetes, an in vivo assay for screening for a drug, and a transgenic animal and its method of use are materially different and plurally independent. The methods involve different process steps, reagents and technical considerations, requiring divergent searches.