Properties of Native and In Vitro Glycosylated Forms of the Glucagon-Like Peptide-1 Receptor Antagonist Exendin(9-39)

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The intestinal hormone glucagon-like peptide-1-(7-36)-amide (GLP-1) has recently been implicated as a possible therapeutic agent for the management of type 2 non–insulin-dependent diabetes mellitus (NIDDM). However, a major difficulty with the delivery of peptide-based agents is their short plasma half-life, mainly due to rapid serum clearance and proteolytic degradation. Using a peptide analog of GLP-1, the GLP-1 receptor antagonist exendin(9-39), we investigated whether the conjugation of a carbohydrate structure to exendin(9-39) would generate a peptide with intact biological activity and improved survival in circulation. The C-terminal portion of exendin(9-39) was reengineered to generate an efficient site for enzymatic *O*-glycosylation. The modified exendin(9-39) peptide (exe-M) was glycosylated by recombinant UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 1 (GalNAc-T1) alone or in conjunction with a recombinant GalNAc α2,6-sialyltransferase (Sialyl-T), resulting in exe-M peptides containing either the monosaccharide GalNAc or the disaccharide NeuAcα2,6GalNAc. The nonglycosylated and glycosylated forms of exe-M competed with nearly equal potency (> 90% of control) with the binding of [^{125}I]GLP-1 to human GLP-1 receptors expressed on stably transfected COS-7 cells. In addition, each peptide was equally effective for inhibiting GLP-1–induced cyclic adenosine monophosphate (cAMP) production in vitro. Studies with rats demonstrated that the modified and glycosylated forms of exendin(9-39) could antagonize exogenously administered GLP-1 in vivo. Interestingly, glycosylated exendin(9-39) homologs were more than twice as effective as the nonglycosylated peptide for inhibiting GLP-1–stimulated insulin production in vivo, suggesting a longer functional half-life in the circulation for glycosylated peptides. Results from in vivo studies with ^{3}H-labeled peptides suggest that the glycosylated peptides may be less susceptible to modification in the circulation.

GLUCAGON-LIKE peptide-1-(7-36)-amide (GLP-1) is a hormone secreted from intestinal L cells that has been implicated in the endocrine control of metabolism and the regulation of body weight.[1] In vivo, GLP-1 is released from the gut into the circulation following ingestion of a carbohydrate-rich meal. Once in the circulation, this peptide acts as a potent stimulator of glucose-induced insulin secretion and plays an important role in metabolic control (reviewed in Holst[2] and Fehmann et al[3]).

Due to the powerful action of GLP-1 to improve postprandial insulin release (incretin effect), this peptide and its analogs have attracted interest as potential therapeutics for the management of non–insulin-dependent diabetes mellitus (NIDDM) in man.[4-7] Recent studies with GLP-1 in normal human subjects and patients with NIDDM demonstrated that GLP-1 is a potent agent for decreasing blood glucose without causing hypoglycemia.[8-11] Unfortunately, however, GLP-1 has significant limitations as a therapeutic, due to its short half-life in the circulation (measured at 3 to 11 minutes in man), as well as its rapid degradation in serum.[12-18]

Exendin-4(1-39) is a 39–amino acid peptide present in the venom of the lizard *Heloderma suspectum* that shares 53% amino acid sequence similarity with GLP-1.[19] This peptide has been shown to have equipotent or greater agonistic effects on the GLP-1 receptor in vitro, as well as to act as a potent insulin secretagogue in vivo.[20-23] An *N*-terminally truncated form of exendin-4, exendin(9-39), has been shown to antagonize the effects of GLP-1 and exendin-4 in vitro and to abolish the insulin-stimulatory effects of these peptides in vivo.[20-25]

We have used the antagonist exendin(9-39) peptide as a model to determine the feasibility of generating an antidiabetic peptide with a more prolonged effect in vivo, by selective glycosylation. We first modified the amino acid sequence of exendin(9-39) such that a site for *O*-glycosylation was created near the carboxy terminus. Subsequently, the modified peptide was glycosylated using recombinant glycosyltransferases. Initial experiments were performed to address whether the modification of the C-terminal residues of exendin(9-39), as well as the addition of monosaccharides or disaccharides to the modified exendin(9-39) peptide (exe-M), altered the binding of the peptide to the GLP-1 receptor in vitro. Thereafter, studies were performed to analyze whether peptide modification and glycosylation altered the biological activity and half-life of the GLP-1 receptor antagonist in vivo.

MATERIALS AND METHODS

Synthesis of Exendin Peptides

Exendin(9-39) and exe-M were synthesized by stepwise solid-phase methods[26] on an Applied Biosystems Peptide Synthesizer (Foster City, CA). The exendin(9-39) peptide was synthesized on a 0.25-mmol scale. The 9-fluorenylmethyloxycarbonyl (Fmoc) group was used as the N^{a}-amino protecting group, and the temporary side-chain protecting groups were as follows: Arg(Pmc), Asn(Trt), Asp(OtBu), Gln(Trt), Glu(OtBu), Lys(Boc), and Ser(tBu). Side chains of Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val were unprotected. Each residue was single-coupled using a O-Benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU)/*N*-methyl-z-pyrrolidinone (NMP) protocol and then capped with acetic anhydride before the next synthesis cycle. After removal of the *N*-terminal Fmoc group, temporary side-chain protecting groups were removed and the peptide was cleaved from the resin by treatment with 95% trifluoroacetic acid (TFA)/5% scavengers (ethyl methyl sulfide:anisole:1,2-ethanedithiol 1:3:1) at room temperature for 2 hours. The crude peptide was precipitated from the cleavage solution with cold diethyl ether and collected on a sintered

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Submitted June 19, 1998; accepted December 17, 1998.

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0026-0495/99/4806-0008$10.00/0

glass funnel, washed with diethyl ether, dissolved in dilute acetic acid, and evaporated to dryness under reduced pressure, and the residue was redissolved and lyophilized from glacial acetic acid. The crude peptide was purified by preparative reverse-phase chromatography on a Phenomenex (Rancho Palos Verdes, CA) C-18 column (22.5 × 250 mm) using a water/acetonitrile gradient, with each phase containing 0.1% TFA. Clean fractions as determined by analytical high-performance liquid chromatography (HPLC) were pooled, the acetonitrile was evaporated under reduced pressure, and the aqueous solution was lyophilized. The purified peptide was characterized by compositional analysis and time-of-flight mass spectroscopy, which yielded the anticipated $(M + H)^+$.

The exe-M peptide was synthesized on a 0.5-mmol scale. The *t*-butyloxycarbonyl (Boc) group was used as the N^a-amino protecting group, and temporary side-chain protecting groups were as follows: Arg(Tos), Asp(OBzl), Glu(OBzl), Lys(Cl-Z), Ser(Bzl), Thr(Bzl), and Trp(CHO). Side chains of Ala, Asn, Gln, Ile, Leu, Met, Phe, Pro, and Val were unprotected. Each residue was double-coupled and then capped before the next double-couple cycle. After removal of the *N*-terminal Boc group and before HF cleavage, the temporary side-chain group on the Trp residue was removed using TFA:trifluoromethane sulfonic acid 5:1 with 1,2-ethanedithiol:*m*-cresol:dimethyl sulfide as scavengers at −5°C for 3 hours. The resin was washed with cold diethyl ether and dried. Remaining temporary side-chain protecting groups were removed, and the peptide was cleaved from the resin by treatment with HF:dimethylsulfide:anisole 10:1:1 for 1 hour at −5°C. The crude peptide was purified and characterized as before.

Production and Isolation of [^{3}H]Exe-M

To prepare radioactively labeled exendin(9-39), an analog of exe-M, exe_1-M, which contains a 3,4-dehydroproline unit in place of the C-terminal proline of exe-M, was produced. Exe_1-M was synthesized essentially as described for exe-M, except the 3,4-dehydroproline residue was coupled manually using benzotriazol-l-yloxyltris(dimethylamino)phosphonum hexafluorophosphate (BOP) in dimethylformamide (DMF), and the resin was deformylated before HF cleavage using DMF:piperdine 1:10 at 0°C for 2 hours. To label this peptide with ^{3}H, 2 mg purified exe_1-M was reduced with tritium gas on a Tri-Sorber (Inus Systems, Tampa, FL). This produced greater than 3.4 mCi exe_1-M, which was purified by preparative reverse-phase HPLC (> 98% pure), resulting in exe_1-M with a specific activity of 1.7 mCi/mg (6.5 Ci/mmol). The tritium label positions were confirmed by ^{3}H-nuclear magnetic resonance.

Preparation of Glycosyltransferases

Recombinant soluble bovine UDP-GalNac:polypeptide *N*-acetylgalactosaminyltransferase 1 (GalNAc-T1) was prepared as previously described.[27] The chicken recombinant GalNAc α2,6-sialyltransferase (Sialyl-T) was expressed in Sf9 cells using a recombinant baculovirus containing the soluble Sialyl-T cDNA sequence previously described.[28] Serum-free culture medium from infected Sf9 cells containing the soluble recombinant Sialyl-T was collected (~100 mL), supplemented with protease inhibitors (10 mL/mL soybean trypsin inhibitor, 0.5 mg/mL pepstatin, 0.25 mg/mL leupeptin and antipain, and 30 mg/mL aprotinin), and concentrated 10-fold at 4°C using an Amicon (Beverly, MA) Ultrafiltration Cell with a YM-10 membrane. The concentrated Sialyl-T preparation was used without further purification for in vitro glycosylation experiments. Both recombinant glycosyltransferases were stored at −20°C in 50% glycerol.

Glycosylation of Exe-M Peptide

Batch glycosylation of exe-M peptides was performed in vitro using either recombinant bovine GalNAc-T1 alone or bovine GalNAc-T1 in combination with Sialyl-T. For the production of GalNAc-exendin(9-39) (exe-GN), a reaction mix (40 μL) containing 10 mmol/L exe-M, 1 mmol/L UDP-GalNAc (Sigma, St Louis, MO), 4 mmol/L $MnCl_2$, 50 mmol/L imidizole buffer (pH 7.5), and 100 ng purified bovine GalNAc-T1 was incubated at 37°C for greater than 2 hours. To produce NeuAcα2,6GalNAc-exendin(9-39)(exe-GN-SA), a 50-μL reaction mix containing 10 mmol/L exe-M, 75 μmol/L UDP-GalNAc, 1 mmol/L CMP-NANA (Sigma), 50 mmol/L imidizole buffer (pH 7.0), 100 ng purified bovine GalNAc-T1, and 10 μL concentrated chicken Sialyl-T was incubated at 37°C overnight, followed by a boost with 10 μL CMP-NANA (5-mmol/L), 25 μL 50-mmol/L imidizole buffer (pH 7.0), and 10 μL concentrated Sialyl-T for 6 hours at 37°C. The radiolabeled glycosylated exendins, exe_1-GN and exe_1-GN-SA, were also produced as outlined before, except the reaction mixtures contained 10 μCi lyophilized exe_1-M (0.39 μCi/mL) and 4 mmol/L exe_1-M. All peptides were purified by HPLC as outlined in the following section.

Purification and Characterization of Glycosylated Peptides

Exe-M, exe_1-M, and the glycosylated exendin peptides exe-GN, exe_1-GN, exe-GN-SA, and exe_1-GN-SA were purified by HPLC on a C18 reverse-phase column (Vydac, Hesperia, CA) using a gradient (0% to 48%) of acetonitrile in 0.1% TFA. The peptides were eluted at a flow rate of 1 mL/min using the following conditions: a 1-minute linear increase in the acetonitrile concentration to 28%, followed by an additional linear increase in acetonitrile to 48% over 50 minutes, and a final 10-minute linear decrease to 0% acetonitrile. A 10-minute wash with 100% TFA 0.1% followed each chromatographic determination. Fractions (30-second) of 0.5 mL were collected between the time points T_{20} and T_{30} of the program. Under these conditions, exe-GN-SA eluted at approximately 27.5 minutes, exe-GN at approximately 28.6 minutes, and exe-M at approximately 29.9 minutes. The ^{3}H-labeled peptides had identical retention times. Fractions containing eluted peptides were pooled, lyophilized, reconstituted in 10 mmol/L HEPES buffer (pH 7.2), and stored frozen at −20°C. The concentration of each peptide solution was determined by measuring the absorption at 280 nm and by compositional analysis.

Exoglycosidase Digestion

Approximately 1 mg of the purified glycosylated peptides exe-GN and exe-GN-SA was subjected to digestion with either *Patella vulgata* α-*N*-acetylgalactosaminidase (V Labs, Covington, LA), *Arthrobacter ureafaciens* sialidase (Oxford Glycosystems, Bedford, MA), or Newcastle disease virus sialidase (Oxford Glycosystems). Conditions were as outlined by the supplier. Reaction products were analyzed by reverse-phase HPLC using the protocol already described.

Expression of Human GLP-1 Receptor in COS-7 Cells

Green monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (vol/vol), 2 mmol/L glutamine, 1% MEM nonessential amino acids, 100 U/mL penicillin, and 100 U/mL streptomycin. Transfected cells were maintained in this medium supplemented with 500 mg/mL neomycin sulfate (G418). COS-7 cells (1×10^6) were transfected with 10 mg of the vector pHGLPR-1 (containing a human GLP-1 receptor cDNA[29]) using 40 mg Lipofectase (GIBCO BRL, Gaithersburg, MD) as outlined by the manufacturer. Cells were placed under selective pressure by growth in medium containing G418 for 10 to 14 days. Upon foci formation, cell clones were isolated and expanded. Cell clones were screened for GLP-1 receptor expression by competitive binding assays (description follows). Five positive cell clones were further expanded (clone 2, 7, 15, 16, and 18), and cell clone 16 was subsequently used for binding assays and receptor coupling analyses.

Competitive Binding Studies

[^{125}I]GLP-1 (specific activity, 1,600 to 2,000 Ci/mmol) was obtained from Peninsula Laboratories (Belmont, CA). Displacement of [^{125}I]GLP-1 binding from the human GLP-1 receptor expressed in COS-7 clone 16 cells was measured in the presence of varying concentrations of either GLP-1 (Sigma), exendin(9-39), exe-M, exe-GN, or exe-GN-SA. For the binding studies, COS-7 clone 16 cells were plated in 12-well dishes and cultured to approximately 60% confluency. The cells were then treated in triplicate as follows: (1) one wash at room temperature with Hanks balanced salt solution (HBSS) containing 20-mmol/L HEPES, pH 7.4, 0.5 mL/well; (2) incubation at 4°C overnight with ice-cold HBSS containing 20 mmol/L HEPES (pH 7.4), 0.5% bovine serum albumin, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.07 μCi/mL [^{125}I]GLP-1, and varying concentrations (0 to 1,000 nmol/L) of unlabeled competing peptide, 1 mL/well; (3) two washes with ice-cold 0.9% NaCl solution, 0.5 mL/well; and (4) lysis at room temperature with 1% sodium dodecyl sulfate/0.2N NaOH for 2 hours, 0.5 mL/well. Radioactivity associated with the lysates (0.25 mL/sample) was determined using a gamma counter. Data were analyzed by plotting radioactivity associated with the cells versus the concentration of the cold peptide added. Dissociation binding constants (K_d) and inhibition constants (K_i) were calculated essentially as outlined by Yamaoka et al.[30]

Determination of Receptor-Coupled Production of Cyclic Adenosine Monophosphate

COS-7 clone 16 cells plated in 12-well dishes and cultured to approximately 60% confluency were treated with supplemented DMEM containing 1 mmol/L 3-isobutyl-1-methylxanthine ([IMBX] Sigma) for 15 minutes at 37°C (1 mL/well). To duplicate wells, either saline (control), 5 μL GLP-1 (final concentration, 3 nmol/L) alone, or GLP-1 (3 nmol/L) and 5 μL of each respective exendin peptide (final concentration, 250 nmol/L) were added in the presence of IBMX for 15 minutes at 37°C. The medium was removed, and the cells were washed twice with unsupplemented DMEM followed by cell lysis with 50 mmol/L HCl (0.5 mL/well) for 1 hour on ice. Cell lysates were neutralized with 50 mmol/L NaOH (0.5 mL/well) and then analyzed (100 μL) in duplicate for cyclic adenosine monophosphate (cAMP) using the nonacetylation assay of the Amersham (Arlington Heights, IL) SPA kit (Biotrak RPA 538). Data are expressed as percent cellular cAMP produced, normalized to treatment with GLP-1 alone.

Incretin Action of Intravenously Infused Peptides in Fasted Anesthetized Rats

Male Sprague-Dawley rats (160 to 240 g) were obtained from Charles River (Portage, MI), acclimated to standard rat chow and water ad libitum, and then fasted overnight. Rats were lightly anesthetized by ether inhalation prior to collection of blood samples from the retroorbital sinus and injection of materials into the tail vein. The animals received peptide injections at time points T_0 and T_{30}: T_0 injections contained a 0.3-nmol/kg dose of GLP-1 (7-36 amide) with either saline, exendin(9-39), exe-M, exe-GN, or exe-GN-SA (all exendin peptide doses 5 nmol/kg); T_{30} injections contained a 0.3-nmol/kg dose of GLP-1 alone. Blood samples were collected in heparinized tubes at the indicated post-T_0 time points (3, 15, 30, 33, 45, and 60 minutes) from the retroorbital sinus under light ether anesthesia. Circulating insulin levels were measured in duplicate serum samples (50 μL) using a rat insulin immunoassay (Linco, St Charles, MO). Data are reported as the mean ± SE for values from the individual experiments; n refers to the number of rats used for each data point.

Fate of [^{3}H]Exendin Peptides in Rat Serum

[^{3}H]-labeled exendin peptides were purified by HPLC on the day before the experiment (outlined earlier). On the day of the study, rats were anesthetized by ether inhalation followed by a retroorbital sinus bleed to acquire a sample for the T_0 time point. The animals subsequently received tail vein injections of peptide at time point T_0. Injections contained a 5-nmol/kg dose of either exe$_1$-M, exe$_1$-GN, or exe$_1$-GN, or exe$_1$-GN-SA in 20 mmol/L HEPES buffer (pH 7.4). Blood samples were collected by retroorbital bleeds into heparinized tubes at the time points indicated (2.5, 5, 8, 11.5, 15, 30, 45, and 60 minutes postinjection. All serum samples (75 μL) were analyzed in duplicate for tritium by scintillation counting.

HPLC Analysis of Serum Samples

Serum samples were separated by reverse-phase HPLC. A Brownlee (Santa Clara, CA) RP-18 Spheri-5, 4.6-mm × 10-cm column with a 2-cm guard was used. Detection was made with a Flo-One HS detector from Radiomatic (Tampa, FL) fitted with a 250-μL flow cell. The mobile phase was 35% acetonitrile in water containing 0.02% (vol/vol) TFA and 0.02% *N,N*-dimethyloctylamine. The flow rate was maintained at 1 mL/min with a scintillant (Flo-Scint II) to column effluent ratio of 4:1. Under these conditions, exe$_1$-M, exe$_1$-GN, and exe$_1$-GN-SA had a retention time of 12.3, 6.8, and 6.0 minutes, respectively.

Rat serum samples were frozen prior to analysis. Early time point samples of 100 μL (2.5 to 8 minutes) were thawed, diluted with water to produce a final volume of 400 μL, and injected directly onto the HPLC system for analysis. The 11.5-, 15-, and 30-minute serum samples were thawed and purified on C-18 SEP-PAK cartridges (Waters, Milford, MA). The 45- and 60-minute time points, serum samples from three animals for each respective time point, were pooled and applied to C-18 SEP-PAK columns; following elution of the adsorbed radioactivity with methanol, the samples were dried under nitrogen and analyzed by HPLC as outlined before.

RESULTS

Glycosylation and Characterization of Exe-M Peptide

To produce a glycosylated exendin(9-39) molecule, an analog of the peptide was required that contains a site for enzymatic *O*-glycosylation. Results from structure-activity studies on GLP-1 and exendin-4 have shown that the residues critical for GLP-1 receptor binding and activation reside predominantly in the *N*-terminal half of the peptides.[31-36] Although the C-terminal half of GLP-1 contains several residues that contribute to selective recognition of the GLP-1 receptor,[32,33] studies have indicated that the extreme C terminus is not critical for GLP-1 receptor affinity.[31,33-35] In addition, exendin-4, which contains a nine-residue C-terminal extension in relation to GLP-1, is a potent GLP-1 receptor agonist.[20] Therefore, with the goal of synthesizing a bioactive exendin(9-39) analog that could be glycosylated, the C-terminal portion of exendin(9-39) was selected for the introduction of an *O*-linked glycosylation site. The resulting exe-M contained an altered C-terminal amino acid sequence of PPASTSAPG (Fig 1), which was previously determined to be an efficient in vitro acceptor sequence for GalNAc-T1.[37] Using either recombinant soluble bovine GalNAc-T1 or GalNAc-T1 in combination with recombinant soluble chicken Sialyl-T, this peptide was glycosylated overnight to greater than 90% under the conditions outlined in the Methods. The resulting glycosylated peptides, exe-GN and exe-GN-SA (Fig 1), were purified by HPLC and characterized by compositional analysis, glycosidase digestion (specific for the saccharide linkages), and electrospray mass spectroscopy (data not shown). Together, these analyses indicated that the exe-M core peptide was intact, and that both exe-GN and

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HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR                    GLP-1 (7-36)

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPSS            Native Exe-4 (1-39)

DLSKQMEEEAVRLFIEWLKNGGPSSGAPSS                    Exendin (9-39)

DLSKQMEEEAVRLFIEWLKNGGPPASTSAPG                   Exe-M

DLSKQMEEEAVRLFIEWLKNGGPPASTSAPG                   Exe-GN
                          |
                      α GalNAc

DLSKQMEEEAVRLFIEWLKNGGPPASTSAPG                   Exe-GN-SA
                          |
                      α GalNAc
                          |
                     α2,6 NeuAc
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Fig 1. Reengineering and glycosylation of exendin(9-39). An exendin(9-39) peptide was synthesized in which C-terminal residues 32-39 were modified to contain an acceptor site for *O*-linked glycosylation. Either GalNAc-T1 or GalNAc-T1 in combination with soluble chicken Sialyl-T were used to generate the glycosylated forms of exe-M in vitro. All peptides were purified by reverse-phase HPLC, lyophilized overnight, and stored at −20°C.

exe-GN-SA contained carbohydrate structures identical to those found in nature.

In Vitro Activity of Native Exendin(9-39) and Exe-M

GLP-1 receptor binding studies were used to determine the binding kinetics and the GLP-1–inhibitory effect of exendin-(9-39) and its modified, nonglycosylated and glycosylated analogs. Although exendin(9-39) is an antagonist of the GLP-1 receptor, it is able to bind to the receptor with an affinity similar to GLP-1.[21,22,24,36,38,39] A COS-7 cell clone (clone 16) constitutively expressing the GLP-1 receptor was prepared and shown to display high-affinity binding of GLP-1 ($K_d = 0.56 \pm 0.3$ nmol/L). The native COS-7 cells displayed no detectable GLP-1 binding. Preliminary studies with exendin(9-39) found that this peptide displaced [^{125}I]GLP-1 binding from clone 16 cells with a potency ($K_i = 1.97$ nmol/L) in the range previously reported.[21,22,24,38] Exe-M, exe-GN, and exe-GN-SA all had binding activity similar to native exendin(9-39) ($K_i = 2.29$, 3.96, and 2.58 nmol/L, respectively). These results suggest that modifying the amino acid residues in the carboxyl terminus of exendin, as well as adding monosaccharides or disaccharides to exe-M, does not significantly alter the binding affinity of the peptide for the GLP-1 receptor (Fig 2).

The binding of GLP-1 to the GLP-1 receptor induces receptor coupling to adenylate cyclase, resulting in cAMP production.[40] It has been shown previously that although exendin(9-39) can bind to the GLP-1 receptor, it does not cause receptor coupling. Furthermore, exendin(9-39) can act as an antagonist of GLP-1–stimulated cAMP production in cells expressing the GLP-1 receptor.[21,22,38,39] Using COS-7 clone 16 cells, a study was performed to determine the ability of exendin(9-39) analogs to inhibit GLP-1–induced cAMP production. To establish the assay parameters, clone 16 cells were initially treated for 15 minutes with increasing concentrations of GLP-1 alone. This resulted in a dose-dependent increase in cAMP production, with an apparent 50% effective dose of 3 nmol/L that is essentially identical to the level reported previously for COS-7 cells expressing the recombinant GLP-1 receptor.[29,40] Subsequently,

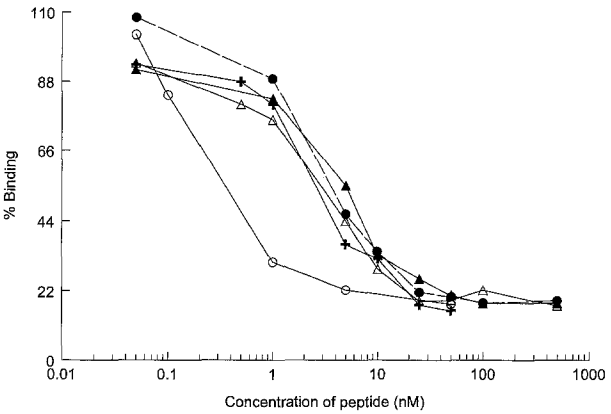

Fig 2. Displacement of [^{125}I]GLP-1 binding to its receptor by cold GLP-1 and exendin peptides. Transfected COS-7 cells stably expressing the human GLP-1 receptor were incubated in the presence of radioiodinated GLP-1 and increasing concentrations of either GLP-1 (○), exendin(9-39) (+), exe-M (△), exe-GN (▲), or exe-GN-SA (●) for 15 hours at 4°C. The cells were then washed, and radioactivity was measured in a gamma counter. GLP-1 displaced radioactive tracer binding with $K_d = 0.56$ nmol/L, exendin(9-39) with $K_i = 1.97$ nmol/L, exe-M with $K_i = 2.29$ nmol/L, exe-GN with $K_i = 3.96$ nmol/L, and exe-GN-SA with $K_i = 2.58$ nmol/L.

using a half-maximal concentration of GLP-1 (3 nmol/L), the antagonistic effects of exendin(9-39), exe-M, and glycosylated exe-M peptides were assessed by their ability to decrease the GLP-1–induced elevation of cAMP in clone 16 cells. It has been previously demonstrated that the maximal inhibition of cAMP production requires a 50- to 100-fold higher concentration of exendin(9-39) in relation to GLP-1.[21,38] Using an antagonist concentration of 250 nmol/L, exendin(9-39) and its analogs displayed between 86% and 91% inhibition of GLP-1–induced cAMP production in cells cotreated with 3 nmol/L GLP-1 (Fig 3). These data, in addition to the receptor binding data presented, suggest that exe-M, exe-GN, and exe-GN-SA have

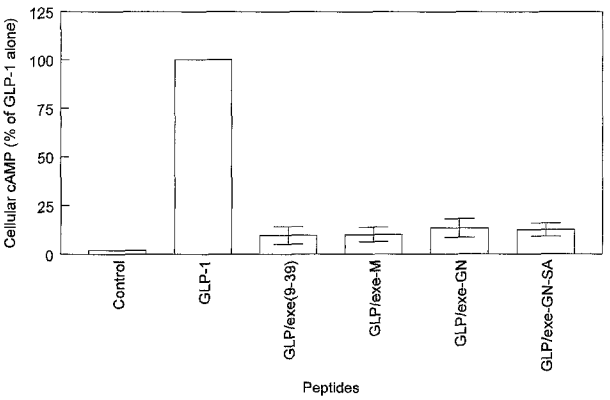

Fig 3. Inhibition of GLP-1–induced cAMP production by exendin(9-39), exe-M, exe-GN, and exe-GN-SA. COS-7 cells (clone 16) stably expressing the human GLP-1 receptor were incubated for 15 minutes in the absence or presence of 3 nmol/L GLP-1 alone or in the presence of GLP-1 (3 nmol/L) and 250 nmol/L of each of the following antagonists: exendin(9-39), exe-M, exe-GN, and exe-GN-SA. Data are the percent of cellular cAMP produced, normalized to cells treated with GLP-1 alone.

in vitro activity and potency similar to that of native exendin-(9-39).

Analysis of the Antagonistic Effect of Exendins on Insulin Release In Vivo

To address the in vivo biological activity of modified and glycosylated exendin(9-39) peptides, we investigated whether a single injection of nonglycosylated or glycosylated exe-M peptides, in rats could antagonize the insulintropic effect of two injections of GLP-1 given 30 minutes apart. Fasted rats were anesthetized and bled prior to injection. The dose of GLP-1 and exendin(9-39) peptide analogs was chosen to achieve maximal inhibition of the GLP-1 insulintropic effect based on previously reported results.[24] Rats were injected with either a 0.3-nmol/kg dose of GLP-1 alone or GLP-1 (0.3 nmol/kg) in combination with exe-M, exe-GN, or exe-GN-SA (each at 5 nmol/kg). Rats injected with saline at both time points served as the negative control. At 3 minutes postinjection, plasma insulin levels were 150% higher than with the saline control in rats dosed only with GLP-1, as compared with 74%, 75%, and 20% in rats receiving GLP-1 coadministered with exe-M, exe-GN, and exe-GN-SA, respectively (Fig 4). All rat groups had plasma insulin near control levels after 15 minutes. With the exception of the saline control, all rat groups received a second injection of GLP-1 (0.3 nmol/kg) at 30 minutes. This resulted in an immediate elevation of plasma insulin to 280% of the saline control in rats receiving only GLP-1. By contrast, rats that were originally injected with exe-M and exe-GN displayed more moderate increases of plasma insulin (160% and 110%, respectively), while rats that received exe-GN-SA exhibited an increase of only 30% (Fig 4).

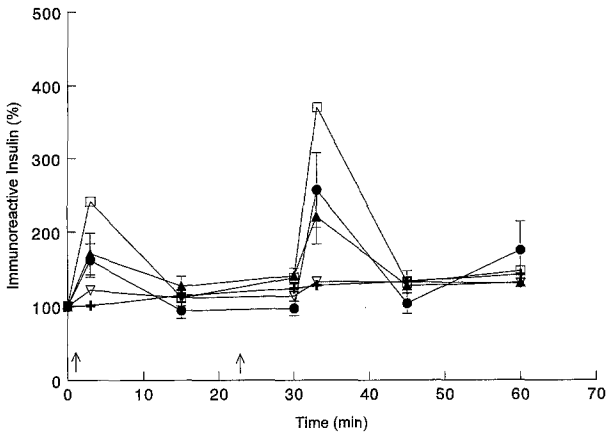

Fig 4. In vivo antagonistic effects of exendin(9-39) and modified nonglycosylated and glycosylated exendin(9-39) homologs on exogenously administered GLP-1. Five rat groups were used. All groups received tail vein injections at time points T_0 and T_{30} (30 minutes apart, arrows). Group 1 (+) received 2 injections of saline. Group 2 (□) received 2 injections of GLP-1 (0.3 nmol/kg). Group 3 (●) received a mixture of GLP-1 (0.3 nmol/kg) and exe-M (5 nmol/kg) at T_0 and GLP-1 (0.3 nmol/kg) at T_{30}. Group 4 (▲) received a mixture of GLP-1 (0.3 nmol/kg) and exe-GN (5 nmol/kg) at T_0 and GLP-1 (0.3 nmol/kg) at T_{30}. Group 5 (▽) received a mixture of GLP-1 (0.3 nmol/kg) and exe-GN-SA (5 nmol/kg) at T_0 and GLP-1 (0.3 nmol/kg) at T_{30}. Serum samples were collected at 0, 3, 15, 30, 33, 45, and 60 minutes after the initial injection. The circulating plasma insulin level was measured by rat insulin-specific immunoassay (Linco) and is expressed as a percent of the level at time zero. Data represent the mean of 9 rats per group.

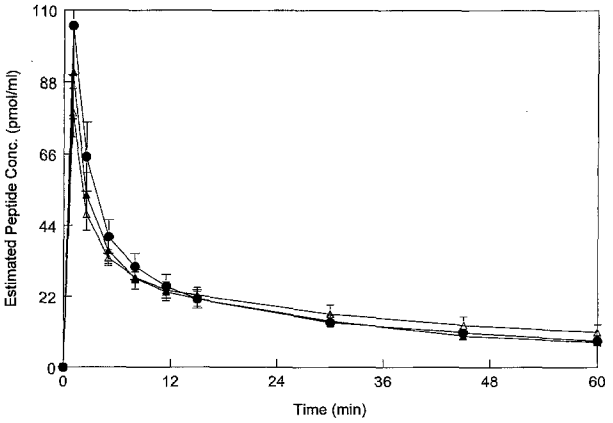

Fig 5. Measurement of serum radioactivity following injection of [^{3}H]-labeled exe-M and glycosylated exe-M. Three rat groups were used. All groups received tail vein injections at time point T_0. Group 1 (△) received exe$_1$-M (5 nmol/kg), group 2 (▲) exe$_1$-GN (5 nmol/kg), and group 3 (●) exe$_1$-GN-SA (5 nmol/kg). Blood samples were taken at 0, 1, 2.5, 5, 8, 11.5, 15, 30, 45, and 60 minutes postinjection. For each time point, radioactivity in 75 μL serum was analyzed by scintillation counting. Data represent the mean of 6 rats per group.

These results demonstrate that all exendin(9-39) derivatives are capable of inhibiting the GLP-1–induced release of insulin in vivo. The data also strongly suggest that glycosylated exe-M peptides have a more prolonged inhibitory effect than the nonglycosylated peptide. Interestingly, the disaccharide-containing peptide (exe-GN-SA) inhibited the elevation of plasma insulin, following the second administration of GLP-1 nearly four times more efficiently than the peptide containing only the monosaccharide (exe-GN).

Fate of Native and Glycosylated Exendin(9-39) in Serum

The outcome of the in vivo study suggested that the disaccharide-containing exendin (exe-GN-SA) may have a longer half-life in serum than either exe-M or exe-GN. Previous studies have shown that glycosylated peptides and proteins can have a longer serum half-life than their nonglycosylated counterparts.[41-44] Additionally, the presence of terminal sialic acid residues on carbohydrate side chains has been shown in some cases to further extend the circulatory properties of glycoconjugates in vivo (Saxena et al[44] and references therein and Szkudlinski et al[45]). To examine the half-life of exendin(9-39) analogs in vivo, a tritium-labeled form of exe-M was prepared (exe$_1$-M) and used for synthesis of the glycosylated derivatives exe$_1$-GN and exe$_1$-GN-SA. Using these labeled peptides, serum tritium levels in rats were measured over time following an intravenous bolus of 5 nmol/kg of either exe$_1$-M, exe$_1$-GN, or exe$_1$-GN-SA. The measured half-life of radioactivity associated with the three peptides was approximately 1.0, 1.3, and 1.4 minutes for exe-M, exe$_1$-GN, and exe$_1$-GN-SA, respectively (Fig 5). The data from this experiment suggest that the plasma half-life of the three exendin derivatives is similar. This result was somewhat unexpected, based on the outcome of the bioactivity study (description follows). However, the rank order of the duration of action of the peptides in vivo correlated with the half-life results.

Thus, to further analyze the fate of the peptides in serum,

samples were subjected to fractionation on reverse-phase HPLC such that intact peptide[5] could be separated from truncated metabolites. Serum samples from early time points (2.5 through 8 minutes) containing either exe_1-M, exe_1-GN, or exe_1-GN-SA all displayed retention times, based on radioactivity, that appeared identical to the native intact standards. Only very small amounts of radioactivity were detected in the void volume, indicating the presence of a minimal amount of ^3H_2O in the samples (data not shown). Direct analysis of the later time points in the study (15 to 60 minutes) was not possible, due to limited volumes and low radioactivity levels present in the collected samples. For the 15- and 30-minute time points, the samples were extracted on C_{18} solid-phase cartridges (C_{18} SEP-PAK). This procedure effectively separated ^3H_2O and any other polar radioactive metabolites from the parent peptides. By comparing the relative amount of unretained (water elute) and retained (methanol elute) radioactivity, the percent of unmetabolized peptide in the serum was determined. The results of this analysis for serum samples from 0 (control), 15, and 30 minutes are presented in Table 1. At the 15- and 30-minute time points for all three peptides, a significant portion (> 70%) of serum-associated radioactivity is still retained on the C_{18} cartridges. The material not retained presumably represents polar metabolites. The relative distribution of retained and unretained radioactivity was similar for the three peptides.

To analyze the 45- and 60-minute time points, these serum samples were pooled (see the Methods) and initially separated on C_{18} SEP-PAK cartridges. Thereafter, to determine whether the retained radioactivity on C_{18} cartridges was in fact associated with the respective parent peptide and not with apolar metabolic fragments, the adsorbed radioactive fractions from pooled 45- and 60-minute serum samples were examined by HPLC. Analysis of each of the glycosylated peptide fractions (exe_1-GN and exe_1-GN-SA) showed single well-defined radioactive peaks eluting with retention times similar to those of the native peptides. By contrast, separation of exe_1-M yielded a broad asymmetric peak, indicating the presence of several peptide species with comparable chromatographic properties (Fig 6). The low level of radioactivity recovered from these samples precluded any further analysis. In summary, these data suggest that in relation to the nonglycosylated exe_1-M, the glycosylated analogs are less susceptible to degradation in rat serum for up to 60 minutes. Furthermore, the addition of a carbohydrate side chain to exendin(9-39) may aid in the protection of this peptide from clearance or modification in vivo.

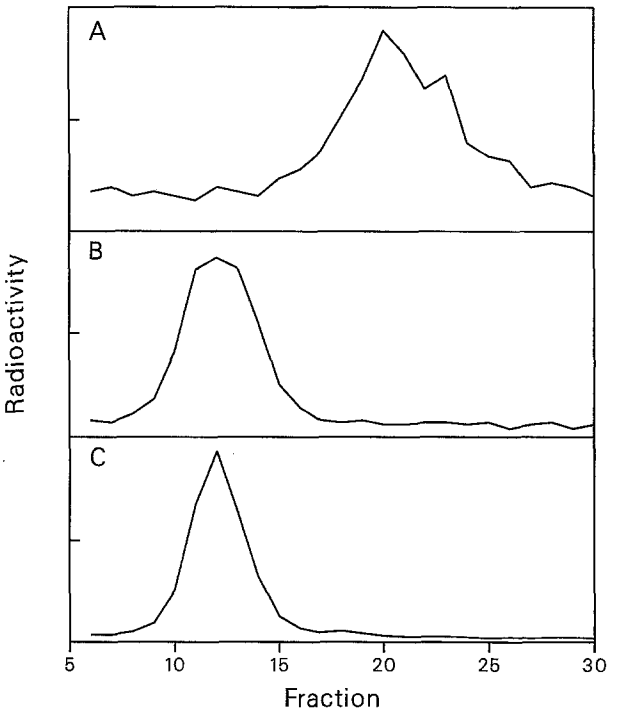

Fig 6. Separation of metabolites generated between 45 and 60 minutes after intravenous administration of exe_1, exe_1-GN, and exe_1-GN-SA. Rats received a bolus tail vein injection of either exe_1-M, exe_1-GN, or exe_1-GN-SA. At 45 and 60 minutes postinjection, serum samples were collected, pooled, and separated on C_{18} SEP-PAK cartridges. The eluted adsorbed radioactivity from SEP-PAK cartridges for each exendin(9-39) analog was analyzed by HPLC. (A) Exe_1, (B) exe_1-GN, and (C) exe_1-GN-SA, show reverse-phase HPLC separation profiles of SEP-PAK adsorbed radioactivity.

DISCUSSION

NIDDM is a metabolic disease that affects up to 5% of the worldwide population and is currently considered a major unmet medical need. Existing therapies for the treatment of this disease are not very effective, and complications contribute substantially to healthcare costs.[46] The antidiabetic peptide GLP-1 and analogs like exendin-4 represent alternatives for NIDDM therapy. However, these molecules have significant shortcomings due to their short duration of action[12,13] and susceptibility to rapid proteolytic degradation and inactivation in serum.[14-18]

Peptides in general have often been described as poor therapeutics, due to factors such as low aqueous solubility, poor oral availability, and short serum half-life.[47-49] Several strategies aimed at circumventing the bioavailability problems of peptide therapeutics have been devised, such as the use of solubility-enhancing formulations, polymer encapsulization, and synthesis of peptide analogs that are resistant to proteolytic degradation. Recent studies have shown that the glycosylation of

Table 1. Relative Serum Radioactivity Retained (methanol-eluted) and Unretained (water-eluted) After Application to Solid-Phase Extraction Columns

Time (min)	Exe_1-M		Exe_1-GN		Exe_1-GN-SA	
	H_2O	Methanol	H_2O	Methanol	H_2O	Methanol
0	3	97	—	—	8	92
15	17 ± 13	83 ± 15	17 ± 8	83 ± 8	15 ± 13	89 ± 5
30	24 ± 11	76 ± 14	23 ± 12	77 ± 12	26 ± 8	74 ± 8

NOTE. Values are the percent of total counts from an average of 3 animals (mean ± SD).

peptides can, to some extent, alleviate the problems associated with the use of these molecules as therapeutics.[41,42,50,51]. Glycosylated peptides appear less susceptible to liver clearance via the bile, thereby gaining an increased serum half-life. Additionally, glycosylated peptides are often more resistant to proteolytic digestion and typically acquire an increased aqueous solubility (Harrison et al[42] and references therein).

Previous studies on glycosylated bioactive peptides used chemical techniques to conjugate the sugar moiety to the peptide.[41,50-52] However, these procedures have notable limitations in that they involve multiple reaction steps with varying product recovery, and often require considerable product purification and characterization efforts at each reaction step. In addition, exact anomeric configurations and linkage positions of the saccharide, which often are absolute requirements in biological applications, further enhance the complexity of chemical synthesis techniques. Lastly, due to the complexity of these procedures, the final yields of glycosylated product are frequently low.

Enzymatic glycosylation procedures circumvent the majority of the difficulties found with chemical glycosylation (reviewed in Ichikawa et al[53]). For example, glycosylation by enzymatic means produces carbohydrate structures with anomeric and linkage configurations identical to those found in nature. In this study, the carbohydrate side chains conjugated to the exe-M peptide are identical to structures found on several characterized mammalian glycoproteins (reviewed in Schachter and Brockhausen[54]). Conversely, studies on in vitro glycosylated bioactive peptides that relied on chemical glycosylation methods produced glycoconjugates of a type not normally found on mammalian secreted and cell-surface proteins.[41,42,51] A second advantage of enzymatic glycosylation is that peptides or proteins can be rapidly (within hours) and efficiently glycosylated with a specific glycosyltransferase or set of glycosyltransferases, using a simple "one-pot" reaction without generating side reactions and byproducts. In the investigation reported here, the typical yields were greater than 90%. Lastly, glycosylated peptide products can be rapidly purified on reverse-phase HPLC with high yields (> 60%). Limitations previously associated with the enzymatic glycosylation of peptides, such as the requirement for specific glycosyltransferases and identification of efficient acceptor sites, have largely been overcome by the cloning and expression of peptide glycosylating enzymes and the description of sequence features associated with high-efficiency glycosylation acceptors.[37,55,56]

The experiments presented in this report represent the first attempt to use enzymatic *O*-glycosylation as a means of altering the in vivo properties of a bioactive peptide. Results from the in vivo studies suggest that the glycosylated forms of exe-M, particularly the disaccharide-containing peptide, have longer-lasting inhibitory effects on GLP-1 action in vivo. Given the apparent unaltered functional properties of the glycosylated exendins, a likely explanation for this would be that glycosylated exe-M peptides have a longer serum half-life than the nonglycosylated exe-M. However, for measurements of the half-life of radioactivity in serum following injection of exe_1-M, exe_1-GN, and exe_1-GN-SA, only small differences were detected between the three peptides. Additionally, analysis of serum samples from time points up to 30 minutes postinjection suggested a similar rate of conversion to hydrophilic metabolites for all three peptides, again inconsistent with a significantly longer serum half-life for the glycosylated peptides. A comparison of HPLC profiles from underivatized exe_1-M to the two glycosylated forms of the molecule after 60 minutes in circulation suggests that while the glycosylated peptides appear largely intact, the underivatized exe_1-M is not (Fig 6). Thus, the difference in the duration of GLP-1 receptor antagonism between the exendin derivatives appears to be due in part to a greater susceptibility to modification of exe-M, as compared with the two glycosylated derivatives. It is unknown whether glycosylation in this case might also affect the extravascular distribution or half-life of the exe-M peptides. The longer duration of action of the disaccharide-containing exendin(9-39) analog compared with the analog containing a monosaccharide raises the possibility that the sialic acid residue on exe_1-GN-SA protects the peptide from more rapid liver clearance. Sialic acid "caps" are believed to regulate the clearance of plasma glycoproteins by the asialoglycoprotein receptor in the liver, and this type of substitution may also be of importance for interaction with the *N*-acetylgalactosamine/galactose receptor in Kupfer cells (Meijer and Ziegler[48] and references therein). Still, additional investigation is clearly required to determine unequivocally the reason(s) for the longer therapeutic effect of glycosylated exendins.

In this context, it should also be noted that an important process in the clearance of the intact hormone, GLP-1(7-36)amide, involves proteolytic cleavage by dipeptidyl dipeptidase IV (DPP4).[15] Inhibition of DPP4 has been shown to lead to an extended duration of action of GLP-1 in vivo.[57] Since exendin(9-39) lacks the sequence motif that is a substrate for DPP4, the extended activity provided to exendin(9-39) analogs by glycosylation may not apply to GLP-1(7-36)amide. On the other hand, glycosylation of either exendin-4 or one of several DPP4-resistant GLP-1 analogs[58] (reviewed in Drucker[59]) may well result in an extension of the (in vivo) biological activity of these peptides, analogous to the current findings for exendin-(9-39).

Presently, GLP-1 is being evaluated as a potential therapeutic for the treatment of NIDDM. Moreover, exendin-4 has been suggested as an alternative therapeutic for this disease, due to its greater avidity for the GLP-1 receptor and its increased metabolic stability in serum (ie, resistance to metabolism by DPP4 and endopeptidase 24.11) as compared with GLP-1.[17,19,20,59,60] The results presented in this study suggest that the enzymatic C-terminal glycosylation of exe-M appears to have little, if any, effect on the binding efficiency and potency of this peptide in vitro and in vivo. Furthermore, the addition of an oligosaccharide results in a prolonged inhibitory effect of the peptide at the GLP-1 receptor in vivo, possibly by protecting the peptide from proteolytic degradation in serum. Preliminary data from our laboratory have shown that GLP-1 can be efficiently *O*-glycosylated and that the glycosylated peptides have activity and potency at the GLP-1 receptor nearly identical to native GLP-1. Hence, it is possible that glycosylation of either exendin-4 or a DPP4-resistant GLP-1 analog may result in a more efficacious therapeutic with increased metabolic stability and a longer duration of action in vivo.

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