Control of Blood Glucose by Novel GLP-1 Delivery Using Biodegradable Triblock Copolymer of PLGA-PEG-PLGA in Type 2 Diabetic Rats

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Purpose. The incretin hormone glucagon-like peptide-1 (GLP-1) is a promising candidate for treatment of type 2 diabetes mellitus. However, plasma half-life of GLP-1 is extremely short, thus multiple injections or continuous infusion is required for therapeutic use of GLP-1. Therefore, we investigated a new delivery system as a feasible approach to achieve sustained GLP-1 release for a 2-week period.

Methods. A water-soluble, biodegradable triblock copolymer of poly [(DL-lactide-co-glycolide)-b-ethylene glycol-b-(DL-lactide-co-glycolide)] (ReGel) was used in this study as an injectable formulation for controlled release of GLP-1. GLP-1 was formulated into ReGel as insoluble zinc complex to stabilize GLP-1 against aggregation and slow down release. The GLP-1 release profile was monitored *in vitro* and *in vivo*. Zucker Diabetic Fatty rats were administered subcutaneously with the GLP-1 formulation. The concentration of GLP-1, insulin, and glucose was monitored every day after the GLP-1 administration.

Results. The GLP-1 release from ReGel formulation *in vitro* and *in vivo* showed no initial burst and constant release for 2 weeks. Animal study demonstrated that the plasma insulin level was increased, and the blood glucose level was controlled for 2 weeks by one injection of ReGel/ ZnGLP-1 formulation.

Conclusions. It is concluded that one injection of zinc-complexed GLP-1 loaded ReGel can be used for delivery of bioactive GLP-1 during a 2-week period. Because this new delivery system is biocompatible and requires twice-a-month injection, it can improve patient compliance and cost-effectiveness.

KEY WORDS: biodegradable; drug-delivery; glucagon-like peptide-1; hydrogel; type 2 diabetes.

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is a 30-amino-acid peptide hormone secreted from the intestinal L-cells in response to nutrient ingestion (1). GLP-1 administration has been shown to reduce hyperglycemia with type 2 diabetic patients (2). GLP-1 has a number of advantages, which makes it an extremely desirable candidate for type 2 diabetes treatment. First, GLP-1 stimulates insulin secretion in a glucose-dependent manner (3). GLP-1 potentiates glucose-induced insulin secretion, but it does not have any effect on unstimulated insulin secretion. Therefore, it is unlikely to cause hypoglycemia. Second, it stimulates not only insulin gene transcription, but also all steps of insulin biosynthesis (4). Thus, it helps to provide continuous supply of insulin for secretion. Third, GLP-1 strongly inhibits glucagon secretion. In addition, GLP-1 has been shown to be capable of inducing new β-cells in subjects with insufficient number of these cells (5). Further effects of GLP-1 include inhibition of gastrointestinal motility, especially gastric emptying (6). The slower rate of gastric emptying may be beneficial in diabetic patients, because it reduces rapid postprandial increase of glucose level. All these effects render GLP-1 very promising as a therapeutic agent for type 2 diabetes.

However, there is a problem limiting the usefulness of GLP-1 in diabetes treatment. The major drawback for the use of GLP-1 as a therapeutic agent is the extremely short half-life due to rapid degradation. The rapid initial degradation is due to the presence of a ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV) (7). It cleaves off the two N-terminal amino acid residues. The conversion of intact, biologically active GLP-1 to its inactive metabolites occurs with an apparent half-life of 1–1.5 min. Even after subcutaneous injection, the plasma half-life has been assessed to be around 60 min (8). It has been shown that improved control in type 2 diabetic patients with sulfonylurea failure can be obtained by 24-h GLP-1 exposure (9). Therefore, continuous infusion or multiple injections are required to achieve therapeutic efficacy.

During the past few years, there have been growing numbers of publications on delivery of therapeutic proteins or peptides using polymers, as increasing numbers of recombinant proteins are available for therapeutic applications. One of the popular methods for protein or peptide delivery is to entrap those in biodegradable polymers to achieve sustained and controlled release rates (10). Poly (lactic-co-glycolic acid) is the most representative example of biodegradable polymers used in drug delivery. However, those polymers are not generally soluble in water, and fabrication of protein-loaded implants or microspheres using such polymer requires use of water-immiscible organic solvent such as methylene chloride. As a result of employing harsh preparation condition as well as the organic solvent, loaded proteins or peptides can undergo conformational change to possess decreased structural integrity and compromised biological activity. One way to overcome these problems is to use polymer hydrogels formed by sol-gel transition in water (11), such as Poloxamers (PEO-PPO-PEO). However, such non-biodegradable polymer exhibits moderate toxicity *in vivo*.

In this study, a thermosensitive and biodegradable low-molecular-weight PLGA-PEG-PLGA that exhibits sol-gel transition property in water (12) was used as a depot for the delivery of GLP-1. This triblock copolymer undergoes sol-gel transition above its transition temperature involving increase in viscosity by several orders of magnitude. Upon subcutaneous injection, the *in situ*–formed gel can maintain integrity up to several weeks.

As a consequence of poor oral bioavailability and current lack of alternative delivery routes, GLP-1 is presently administered parentally. In this study, we designed a sustained release system, which provides GLP-1 release for over a period of 2 weeks by one injection.

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ABBREVIATIONS: DPP-IV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1.

MATERIALS AND METHODS

Materials

Glucagon-like peptide-1 (7-37) (GLP-1) was synthesized at the Emory University Microchemical Facility (Atlanta, GA, USA). Poly [(DL-lactic acid-co-glycolic acid)-b-ethylene glycol-b-(DL-lactic acid-co-glycolic acid)] triblock copolymer (ReGel) was supplied by MacroMed, Inc. (Sandy, UT, USA). The weight-averaged molecular weight of the triblock copolymer was approximately 4200 with polydispersity index of 1.3, with its central PEG being 1450. The molar ratio of lactide: glycolide in the PLGA block was 4:1.

In Vitro Release Study

The PLGA-PEG-PLGA triblock copolymer was dissolved in an isotonic 30 mM phosphate buffer, pH 7.4, at room temperature to make a 23% solution. For the first group, GLP-1 was dissolved in 1 mM HCl at concentration 5 mg/ml and pH adjusted to 7.0 with 0.1 M NaOH. The formed suspension was allowed to stay at 4°C overnight, and mixed with ReGel solution prepared as described above. The final GLP-1 concentration was 10 mg/ml. For the second group, same amount of GLP-1 was precipitated in the presence of zinc prior to loading essentially as described by Gappa *et al.* (13). After GLP-1 dissolution, zinc chloride was added (final pH was ~3.5). Then, pH was adjusted to 7.0 with 0.1 M NaOH. The GLP-1 solution was mixed with ReGel solution as described above.

Then, 1-ml amount of each formulation was placed in vials, incubated at 37°C until forming gels, and 20 ml of 10 mM PBS, pH 7.4, solution was added as release medium. Samples were withdrawn from the release medium, and PBS was replaced daily. Samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) to measure the concentration of GLP-1. Shimadzu SCL-10Avp liquid chromatograph was equipped with a C_4 column (Vydac, Columbia, MD, USA), which was previously equilibrated. The mobile phases were water and acetonitrile containing 0.1% trifluoro acetic acid (TFA) with a gradient 2% B/min, and the flow rate was 1.2 ml/min.

Animal Experiments

Twenty male Zucker Diabetic Fatty (ZDF) rats (410–460 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) at 11 weeks of age. All animals used in this study were housed according to the principles established for care and use of laboratory animals (21–23°C, 12-12 h light-dark cycle). They were fed *ad libitum* with Purina 5008 (6.5% fat). The experimental protocols concerning the use of laboratory animals were reviewed and approved by the Institutional Animal Care and Use Committee of University of Utah.

The experimental animals, male ZDF rats (12 weeks), were divided into two groups of five animals each. Both groups were fasted overnight, and anesthesia was induced by intramuscular injection of Pentobarbital (60 mg/kg). The first group was injected with 1 ml of blank triblock copolymer aqueous solution. The second group was administered subcutaneously 1-ml formulation containing 10 mg ZnGLP-1. At designated times (day 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, and 15), 600 μl of blood samples were obtained from the tail vein of ZDF rats.

GLP-1 concentration was determined by using an ELISA kit (Linco Research, St. Charles, MO, USA). The samples were treated with dipeptidyl peptidase IV inhibitor (Linco Research). The serum insulin levels were determined by using insulin radioimmunoassays kit (ICN Pharmaceuticals, Costa Mesa, CA, USA). The blood glucose levels of the ZDF rats were monitored using Accucheck-Instant (Boehringer Mannheim, Indianapolis, IN, USA).

Statistical Analysis

The statistical program SPSS for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. Data for the effects of GLP-1 in the ZDF rats were analyzed by a general linear model. In all cases, p values of less than 0.05 were taken as statistically significant. All data are presented as means ± SEM.

RESULTS

The *in vitro* release of GLP-1 from the PLGA-PEG-PLGA polymer depot was investigated, and the data are presented in Fig. 1. The graph shows the % cumulative amount of released GLP-1 during the period of 20 days. The release of GLP-1 from the triblock copolymer hydrogel without using zinc complex shows a big burst with more than 60% of initial loading released in day 1 and 80% released in 3 days. As compared to the first group, the release of zinc-complexed GLP-1 exhibited no initial burst and displayed constant rate and almost linear release profile over 2 weeks to reach 90% of the initial loaded amount.

Animal study using ZDF rats was performed with ZnGLP-1/ReGel formulation. Figure 2 shows the plasma concentration of GLP-1 after subcutaneous injection as determined by ELISA. It should be noted that the detection is limited only to biologically active form of GLP-1. After injection of the formulation, GLP-1 concentration in plasma reached 200 ng/L initially (day 1 and day 2) and later significantly higher GLP-1 levels (>50 ng/L) were maintained compared to that of the control group (treated with blank polymer) with duration of at least 14 days.

Figure 3 shows the insulin levels in the plasma samples over 15 days. Injection of blank polymer hydrogel shows the

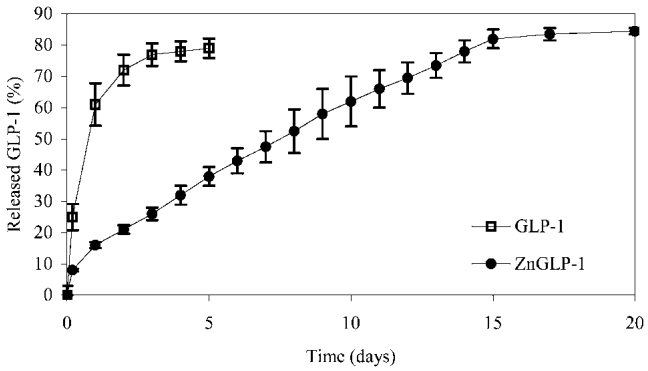

Fig. 1. Cumulative amount of released GLP-1 from ReGel formulation *in vitro.* Released amount of GLP-1 from GLP-1/ReGel (open circle) and ZnGLP-1/ReGel (closed square) was measured for the indicated times. The graph represents the average ± SEM; n = 3.

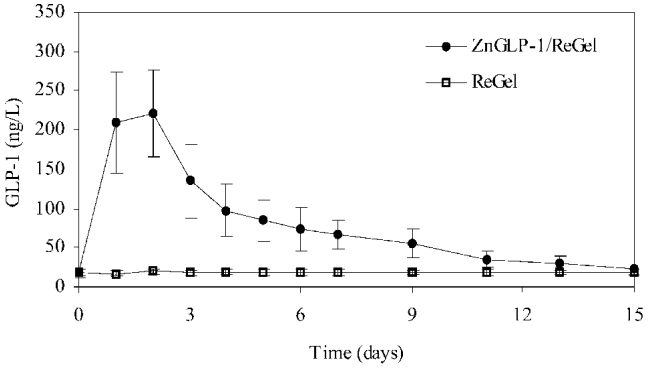

Fig. 2. Plasma GLP-1 level in ZDF rats after injection. Plasma GLP-1 levels in rats treated with ZnGLP-1/ReGel formulation (closed circle) and ReGel (open square) were assayed for the indicated times. The graph represents the average ± SEM; each group was composed of five rats.

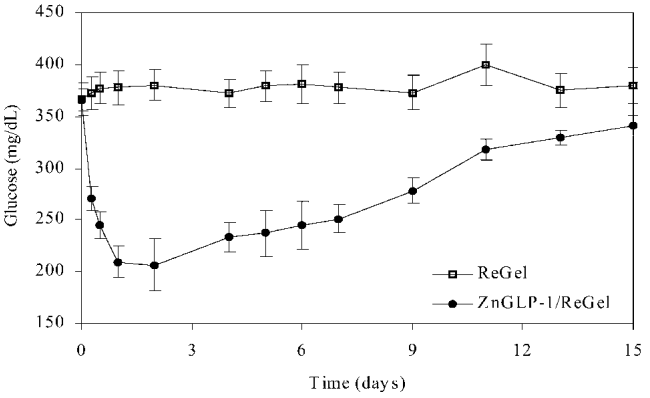

Fig. 4. Blood glucose level in ZDF rats after injection. Blood glucose levels in rats treated with ZnGLP-1/ReGel formulation (closed circle) and ReGel (open square) were assayed for the indicated times. The graph represents the average ± SEM; each group was composed of five rats.

constant basal insulin levels of ~1.8 μg/ml. The injection of zinc-complexed GLP-1/ ReGel induced increased insulin levels about 3 μg/ml initially and which were maintained over several days with subsequent gradual decrease over 15 days. The significant difference between control and positive groups was found up to day 11.

Blood glucose profile as a result of GLP-1 release is depicted in Fig. 4. Whereas the injection of blank polymer resulted in no impact on the blood glucose level that was maintained at 350~400 mg/dl, GLP-1/ReGel demonstrated significant glucose lowering activity over the 15-day period in type 2 diabetic animals. Also, the change in blood glucose level over time is consistent with the plasma insulin profile (Fig. 3).

Finally, subcutaneous injection of GLP-1/ReGel was well tolerated, and there was no sign of chronic inflammation around the injection sites during the experiment.

DISCUSSION

The current results show that the elevated blood glucose level in type 2 diabetic rat can be controlled by single injection of ReGel formulation with zinc-complexed GLP-1 for 2 weeks. The clinical application of GLP-1 has been limited by its extremely short half-life of 2–3 min (14). Although many practical problems still remain to be solved, a therapy based on the GLP-1 is one of the most promising approaches to the treatment of type 2 diabetes. A number of new approaches are currently under investigation for using GLP-1 as therapeutic agent (15–17). To overcome its instability, different analogs of GLP-1 (16,18) and inhibitors of dipeptidyl peptidase IV (19) are currently being tested in clinical trials. Even though the use of DPP IV-resistant analogs of GLP-1 shows considerable promise, it still requires at least every-day injection to achieve therapeutic effect. Moreover, a complete normalization of glucose concentrations has only been demonstrated with the intravenous infusion of native GLP-1 (2,9,20).

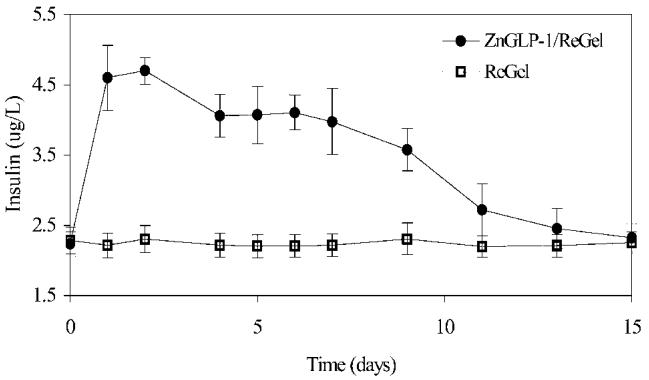

Fig. 3. Plasma insulin level in ZDF rats after injection. Plasma insulin levels in rats treated with ZnGLP-1/ReGel formulation (closed circle) and ReGel (open square) were assayed for the indicated times. The graph represents the average ± SEM; each group was composed of five rats.

The early stage of type 2 diabetes is characterized by a progressive loss of first-phase insulin secretion and a compensatory increased second-phase secretion (21,22). Delayed insulin secretion after meal ingestion causes postprandial hyperglycemia (23). Continuous release of GLP-1 can reduce postprandial glycemic excursions effectively. Due to its glucose-depending insulinotropic effects, GLP-1 actions do not cause hypoglycemia (2).

The thermosenstive, biodegradable triblock copolymer (ABA-type triblock copolymer composed of PLGA and PEG) in aqueous solution (ReGel) is a free-flowing sol at room temperature and becomes a gel at body temperature. At 37°C, the flow of the aqueous solution of the triblock copolymer decreases immediately and dramatically for the gel state to be established, holding its entire content. The first study that demonstrated the feasibility of using this system as a carrier for controlled release of protein was performed by Kim *et al.* (24). In this study, we have shown that this system can also be used as a drug delivery carrier for the continuous release of GLP-1. The stimulating effect of zinc-complexed GLP-1 on the secretion of insulin from pancreatic islets was demonstrated by Gappa *et al.* (13). Although GLP-1 is a very attractive therapeutic agent for type 2 diabetes, GLP-1 is very unstable in the bloodstream, requiring continuous infusion or frequent subcutaneous injections. Thus, the use of ReGel as a controlled release depot for GLP-1 in type 2 diabetic patients will result in improved patient compliance.

The release of zinc-complexed GLP-1 (Zn-GLP-1) from ReGel showed no initial burst and constant release rate as

demonstrated by *in vitro* release study. It is evident that zinc complexation provided a means to stabilize the peptide physically and chemically and to achieve dramatic reduction of initial burst as compared to GLP-1 release from the same hydrogel without zinc. Uncomplexed GLP-1 is highly soluble in water, and thus, the majority of GLP-1 stays in the hydrophilic domain of ReGel and is quickly released via water channels, exhibiting burst release in 3 days.

It should be emphasized, however, that the sustained release of Zn-GLP-1 from the triblock copolymer hydrogel did not rely on the fact that Zn-GLP-1 was poorly water-soluble. Release of GLP-1 from Zn-GLP-1 crystalline suspension alone *in vitro* revealed that the release was complete within 1–2 days (data not shown). This means that the sustained release of GLP-1 from ReGel is due almost entirely to the entrapment of Zn-GLP-1 in the triblock copolymer network, which eliminates the possibility of solubility-dependent release of GLP-1 from the poorly water-soluble Zn-GLP-1.

Animal studies using ZDF rats have been performed to study the bioactivity of the released GLP-1. The ZDF rats used were 12 weeks of age with fully developed type 2 diabetes. As shown in Fig. 2, the plasma GLP-1 levels were maintained *in vivo* for about 2 weeks at concentrations significantly higher than that of control group. This was manifested in increased plasma insulin and decreased blood glucose levels over the same time period. In Fig. 3, insulin level reached and stayed at higher levels over 2 weeks as long as there was a significant GLP-1 level in plasma. This means that the continuously released GLP-1 from the subcutaneously injected ReGel demonstrated insulinotropic effect. As observed in the *in vitro* release of GLP-1 from Zn-GLP-1 entrapped in the triblock copolymer, the continuous *in vivo* release of GLP-1 from the hydrogel was not due to the Zn-GLP-1 solubility dependent release. This is well supported by the fact that a single subcutaneous injection of Zn-GLP-1 crystalline suspension (1 equivalent Zn, as used in our study) resulted in >90% absorption of GLP-1 within 1 day, even for such a protracted GLP-1 formulation (25), and we have confirmed the same observation with the ZDF rats. Blood glucose level was accordingly maintained to the near normalized level as shown in Fig. 4. The glucose level dropped to the level significantly lower (~200 mg/dl) than the control (~400 mg/dl) as long as the insulin level was maintained. In contrast, the control group in Fig. 3 shows endogenous insulin levels yet this has no effect on lowering blood glucose, signifying insulin resistance that is characteristic of the type 2 diabetes.

As shown by the data, steady amount of GLP-1 released from ReGel formulation depot more than 10 days after single subcutaneous injection. It is evident that the GLP-1 released from the thermosensitive biodegradable hydrogel (ReGel) formulation is bioactive as it stimulates insulin secretion *in vivo*. Also, GLP-1 released from ReGel formulation depot results in the improved glucose tolerance. Therefore, we conclude that it is feasible to use ReGel formulation with zinc-complexed GLP-1 as a 2-weeks-delivery system, making it a twice-a-month injection depot. Dose may be further adjusted by loaded amount of GLP-1 and gel concentration for normalizing blood glucose levels completely.

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