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A novel exendin-4 human serum albumin fusion protein, E2HSA, with an extended half-life and good glucoregulatory effect in healthy rhesus monkeys

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) has attracted considerable research interest in terms of the treatment of type 2 diabetes due to their multiple glucoregulatory functions. However, the short half-life, rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) and excretion, limits the therapeutic potential of the native incretin hormone. Therefore, efforts are being made to develop the long-acting incretin mimetics via modifying its structure. Here we report a novel recombinant exendin-4 human serum albumin fusion protein E2HSA with HSA molecule extends their circulatory half-life *in vivo* while still retaining exendin-4 biological activity and therapeutic properties. *In vitro* comparisons of E2HSA and exendin-4 showed similar insulinotropic activity on rat pancreatic islets and GLP-1R-dependent biological activity on RIN-m5F cells, although E2HSA was less potent than exendin-4. E2HSA had a terminal elimation half-life of approximate 54 h in healthy rhesus monkeys. Furthermore, E2HSA could reduce postprandial glucose excursion and control fasting glucose level, dose-dependent suppress food intake. Improvement in glucose-dependent insulin secretion and control serum glucose excursions were observed during hyperglycemic clamp test (18 h) and oral glucose tolerance test (42 h) respectively. Thus the improved physiological characterization of E2HSA make it a new potent anti-diabetic drug for type 2 diabetes therapy.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a naturally occurring peptide hormone that is released from intestinal L cells in response to nutrient ingestion [1]. It has attracted considerable research interest in terms of the treatment of type 2 diabetes due to their multiple glucoregulatory functions.

The natural incretin hormone GLP-1 supports glucose homeostasis by suppressing inappropriately elevated postprandial glucagon secretion from α cells and enhancing glucose-dependent insulin secretion from β cells. In addition, GLP-1 has been demonstrated to reduce appetite and food intake and inhibit gastric emptying, which may facilitate weight management [2,3]. However, a short half-life (2–3 minutes), rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) and excretion, limits the therapeutic potential of the native GLP-1 hormone [4,5]. Thus, long-acting DPP-IV-resistant formulations of analogues have been developed. For example, exenatide (synthetic exendin-4), approved by the US Food and Drug Administration, is the first clinically available mimetic sharing many of the beneficial effects of GLP-1 in therapeutic use. Although exenatide effectively lower blood glucose levels in type 2 diabetic patients, the requirement for twice daily administration of this agents, has focused on the development of long-acting and/or sustained-release incretin mimetics. Among the several approaches, the structural modifications of incretin mimetics appear to be the most effective approaches. Specific approaches target the prevention of rapid renal clearances by increasing molecular size. The strategies include modifications of GLP-1 receptor agonists either by attaching fatty acids or PEG moieties to facilitate binding to blood proteins (albumin) or by direct fusion with a blood protein such as albumin, transferring or immunoglobulin G (IgG4) Fc fragment [6–9].

Human serum albumin (HSA) fusion technology is based on the fact that fusing the heterogeneous proteins with full-length HSA molecule extends their circulatory half-life *in vivo* while still retaining their biological and therapeutic properties. One such

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agent is E2HSA, a genetic fusion protein consisting of two tandem exendin-4 molecules that has been covalently bonded to recombinant HSA via a peptide linker.

To determine whether the considerably larger Exendin-4-albumin hybrid E2HSA retains the identical spectrum of biological actions exhibited by the much smaller GLP-1R peptide agonist Exendin-4, we studied the effects of E2HSA on GLP-1R-dependent actions *in vitro* and pharmacokinetics and biological activity in healthy rhesus monkeys.

1.1. Amino acid sequences of exendin-4 and E2HSA

- Exendin-4: H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
- E2HSA: Exendin-4 – Exendin-4-GGGGS-HSA

2. Materials and methods

2.1. Materials

Cell culture medium, serum were purchased from Invitrogen-Gibico. Exendin-4 and E2HSA was obtained from Huayang Pharmaceutical Co., Ltd. (Zhejiang, China). Insulin EIA kit was purchased from Mercodia (Uppsala, Sweden). cAMP EIA kit was purchased from R&D systems. All other reagents, unless indicated, were purchased from Sigma–Aldrich.

2.2. Animals

Male Sprague Dawley (SD) rats were purchased from the Experimental Animal Center, Academy of military Medicine Science, Beijing, China. Male rhesus monkeys were purchased from Xieerxin biotic resources Institutes, Beijing, China. Thirty individually housed adult male rhesus monkeys, weighing between 4 and 6 kg, were used in this study. Monkeys were maintained under a 12-h light-dark cycle (lights on at 7 AM and off at 7 PM) in an environmentally controlled room with ad libitum access to food and water, except where noted. All animals were handled in accordance with National Institutes of Health guidelines. The rhesus monkeys were randomly assigned to five different groups.

2.3. Cell culture

RIN-m5F cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.4. cAMP assay

RIN-m5F cells (3.0×10^5 cells) were seeded and plated in 96-well plate. After 48 h, the media was replaced by RPMI 1640 containing 500 μM IBMX (3-isobutyl-1-methylxanthine, an inhibitor of cAMP phosphodiesterase). Thereafter, cells were incubated with increasing concentrations of exendin-4 or E2HSA for 15 min at 37 °C. All reactions were carried out in triplicate and terminated by the addition of ice-cold absolute lysate. The intracellular cAMP concentration was measured using a cAMP EIA kit.

2.5. Plasma stability

E2HSA (final concentration 100 nM) were added to fresh rat plasma and incubated at 37 °C for 0, 1, 2, 4, 8, 12, 24, 48 h, respectively. Thereafter, samples were incubated with RIN-m5F cells for 15 min at 37 °C. The cells were lysated, and intracellular cAMP concentration was measured.

2.6. Insulinotropic activity test

After common bile duct cannulation in male Sprague-Dawley rats, the pancreas was distended with Hank buffer (8 mL, containing 2% BSA and 0.5 mg/mL Sigma type V collagenase). Subsequently, tissues were digested in Hank buffer at 37 °C for 15 min. Purified islets (Ficoll 400, 20 min at 3000 rpm) were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After a 2 days maintenance period, islets were washed and incubated in Krebs-Ringer bicarbonate (KRB) buffer, seeded at 10 islet/well on 48-well plate in 1 ml of KRB buffer containing 16.8 mM glucose, and incubated with various concentrations (1, 10 and 100 nM) of either exendin-4 or E2HSA for 2 h. Insulinotropic activity was evaluated by measuring the amount of insulin released to media using insulin EIA kit.

2.7. Pharmacokinetics in vivo

The pharmacokinetic profiles of E2HSA administered by subcutaneous injection at doses of 0.3, 0.9, or 2.7 mg/kg were studied in male rhesus monkeys ($n = 6$). Blood samples were collected predose and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 288 h postdose into ice-cold polyethylene tubes. Plasma samples were obtained by centrifugation and stored at −70 °C until required for assay. Plasma concentrations of E2HSA were determined using a sandwich ELISA method for the quantification of fusion protein E2HSA in monkey plasma.

2.8. Acute and long time-course test of E2HSA in rhesus monkeys

After overnight fasting, venous blood samples were collected from lower extremity. Monkeys ($n = 6$) received an subcutaneous injection of saline vehicle, E2HSA or Exenatide 1 h before the onset of food access. Doses of E2HSA were 0.3, 0.9 or 2.7 mg/kg, saline vehicle was 0.1 ml/kg, whereas Exenatide dose was 0.4 μg/Kg. The whole blood glucose was measured predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h after injection using an automated analyzer (Accu-Chek Active Blood Glucose Meter from Roche; Germany). And then, fasting blood glucose was measured for consecutive 5 days.

2.9. Feeding study

Accompanied with pharmacokinetics studies, we investigated the food intake changes after single subcutaneous injection. Food in the form of nutritionally complete standard diet was provided for 6 h/day beginning at 10:00 AM. The mean food intake of the three consecutive days saline baseline levels for each monkey served as the saline control values. Water intake was not measured in these studies. Subcutaneous injection was 1 h before the onset of food access. Total food intake for each monkey was recorded for 8 consecutive days. To facilitate comparisons across monkeys with different levels of baseline intake, daily intakes in response to drug treatments were expressed as a percentage of their saline baseline intake (percent saline) measured as the mean intake of the 3 days prior to beginning a dose on which saline was administered.

2.10. Behavioral assessments

All monkeys were observed behaviorally 0.25, 0.5, 1 and 3 h after each injection. In particular, monkeys were assessed for

changes in alertness or activity and the presence of excessive salivation, gagging, vomiting, or evidence of vomiting.

2.11. Hyperglycemic clamp test

After 10 half-life period when E2HSA was completely eliminated *in vivo*, we started the hyperglycemic clamp test. After a single subcutaneous E2HSA injection at hour 18, exenatide was injected half an hour before clamp test, following an overnight fasting, a hyperglycemic clamp test was performed to determine insulin secretory capacity [10]. During the clamp test, 20% glucose solution was infused to maintain serum glucose levels of 6.3 mM above baseline by measuring Glucose Analyzer (Roche) every 5 min. Serum insulin levels were measured at 0, 2, 4, 6, 8, 10, 20, 30, 60, 90 and 120 min.

2.12. Oral glucose tolerance test (OGTT)

On hour 42, following an overnight fasting, an oral glucose tolerance test (OGTT) was performed. Exenatide was injected half an hour before test, Monkeys received a 2-g oral glucose dose and blood samples were collected at 0, 10, 20, 30, 60, 80, 120 and 180 min following ingestion of glucose. Blood glucose levels at those times were measured.

2.13. Statistical analysis

All data are presented as means ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni post hoc test using Prism version 5 software. A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. E2HSA increases cAMP production in vitro

To determine whether fusion of a large molecule like HSA impaired the ability of the exendin-4 moiety within E2HSA to activate the GLP-1R, we compared the potency of E2HSA *vs* exendin-4 *in vitro*. Both E2HSA and exendin-4 increased cAMP levels in insulinoma RIN-m5F cells in a dose-dependent manner with half maximal effective concentration values for E2HSA and exendin-4 is 2.68×10^{-8} and 5.1×10^{-11} mol/L, respectively. The biological activity of E2HSA is lower than exenatide *in vitro* (Fig. 1A).

3.2. Plasma stability

E2HSA showed a good stability *in vitro* and *vivo* condition. There was approximately no change on the cAMP production in fresh rat plasma among 48 h incubation. Combining the pharmacokinetics study in monkeys, we could detect the E2HSA for 288 h after injection (Figs. 1B, 2C).

3.3. Insulinotropic effects of E2HSA in vitro

Because the most obvious biological effect of GLP-1 receptor agonists is to stimulate insulin secretion by pancreatic β-cells, glucose-induced insulin secretion tests were performed using isolated rat islets in the presence of various concentrations of E2HSA. Insulin secretion from isolated rat islets was potently enhanced by the concentration of 10 nM E2HSA in the extracellular medium at high (16.8 mM) glucose (Fig. 1C).

3.4. Pharmacokinetics in vivo

The pharmacokinetic profile of E2HSA in rhesus monkeys was presented in Fig. 2C and Table 1. The terminal half-life of E2HSA in monkey was approximately 54 h after a single subcutaneous administration. E2HSA was absorbed slowly and the maximum plasma concentrations occurred between 11 and 15 h in monkeys.

3.5. E2HSA reduces postprandial glucose excursion and controls fasting glucose

E2HSA could significantly decrease plasma glucose concentration after food ingestion in healthy monkeys in a dose-dependent manner. A subcutaneous injection 1 h before the onset of food access, the postprandial glucose level was significantly reduced compared with control group (Fig. 2A). The glucose lowering effect was lasted for 8 h compared with control group. After a single subcutaneous administration, the fasting blood glucose was measured for consecutive 5 days. E2HSA lowered fasting plasma glucose concentration to below preinjection values at certain doses and time. E2HSA resulted in a slight decrease of fasting plasma glucose and this glucose lowering effect of E2HSA can last up to 4 days after injection (Fig. 2B). No episodes of hypoglycemia occurred in the monkeys. Observation of the monkeys following each injection did not reveal any outward signs of nausea or malaise. During the observed period, monkeys displayed normal activity.

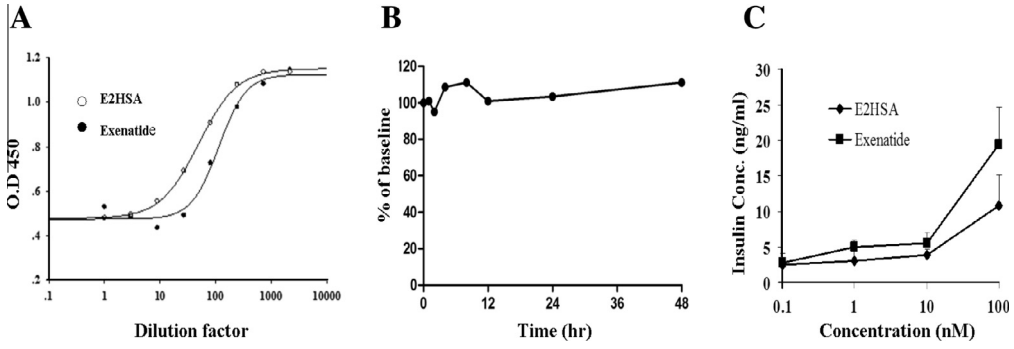

Fig. 1. *In vitro* biological activities of E2HSA. (A) E2HSA exhibits potent cAMP activation in RIN-m5F cells *in vitro*. (B) Stabilization study of E2HSA *in vitro*. E2HSA was added to fresh rat plasma and incubated at 37 °C for different period. The mixed samples were added in RIN-m5F cells and intracellular cAMP concentration was measured. (C) Insulinotropic activity of E2HSA on isolated rat islets. Islets were isolated and then incubated in the presence 16.8 mM of glucose for 2 h at 37 °C. The amount of insulin released to media was measured.

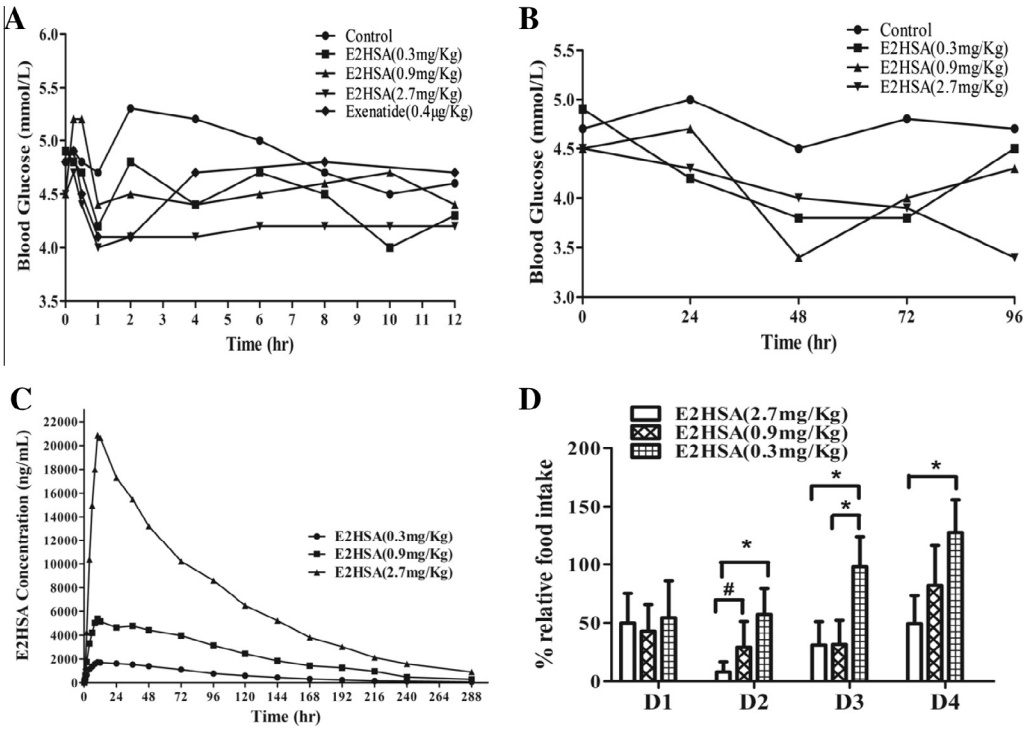

Fig. 2. Pharmacokinetics and acute and long time-course test of E2HSA in rhesus monkeys. (A) Glucose lowering effects of E2HSA on postprandial glucose. (B) The effect of the single injection with E2HSA on fasting glucose. (C) The plasma concentrations were determined with a sandwich ELISA recognizing of the exendin-4 and HSA following single subcutaneous administration of E2HSA in healthy rhesus monkeys. (D) Daily food intake following E2HSA administration over 4 consecutive days. $^{\#}p < 0.05$ compared with E2HSA 0.9 mg/kg, $^{*}p < 0.05$ compared with E2HSA 0.3 mg/kg.

Table 1
Pharmacokinetics parameters in rhesus monkeys.[a]

Sample	(h)	CL/F (mL h^{-1} kg^{-1})	Vd (mL kg^{-1})	C_{max} (ng/mL)	AUC (ng h mL^{-1})	MRT (h)
Exendin-4	0.58 ± 0.09	861.23 ± 164.0	1534.8 ± 415.88	0.2 ± 0.06	0.39 ± 0.12	1.39 ± 0.15
E2HSA	53.4 ± 8.0	1.66 ± 0.27	125.5 ± 8.8	1810.1 ± 198.7	179182 ± 27148	78.3 ± 6.2

[a] Data are means ± SD ($n = 6$). $t_{1/2}$, elimination half-life; CL/F, clearance; Vd, volume of distribution; C_{max}, maximum plasma concentration; AUC, area under the curve; MRT, mean residence time.

3.6. E2HSA reduces food intake

Food intake was reduced significantly for all doses of E2HSA at the first 2 days in a dose dependent manner compared with baseline level. The 0.9 and 2.7 mg/kg dose produced partial suppressions that were maintained for 4 days ($p < 0.01$), with the exception of the exenatide. On the days following E2HSA induced feeding suppressions, intake returned to baseline levels in all cases. There were no decreases in food intake over baseline levels on the days following exenatide single dose administration (Fig. 2D).

3.7. E2HSA enhances glucose-elicited insulin secretion in healthy monkeys

Insulin secretion in response to hyperglycemic clamp test was evaluated in monkeys 18 h after one subcutaneous dose of 0.9 and 2.7 mg/kg E2HSA and after an overnight fasting to determine insulinotropic capacity of E2HSA. Administration of E2HSA demonstrated significant enhancement of glucose-elicited insulin release with time (Fig. 3A). But exenatide didn't show so strong effect as E2HSA did.

3.8. Plasma glucose lowering effect at single dose in healthy monkeys

42 h after one subcutaneous dose of 0.3 mg/kg E2HSA and after an overnight fasting, E2HSA could significantly decrease the plasma glucose level after an oral glucose loading in healthy monkeys (Fig. 3B). The area under the blood glucose curve (AUC) between 0 and 3 h in E2HSA treated groups also significantly lowered at the dose of 0.3 mg/kg compared to the control group (Fig. 3C). The data show that E2HSA confers resistance to glucose challenge for at least 42 h.

4. Discussion

Although GLP-1-based strategies have attracted much interest for the treatment of type 2 diabetes [11–13], its short half-life in the circulation has posed practical limitations on the clinical use. Exendin-4 (also named exenatide) is the first DPP-IV resistant GLP-1R agonist approved by FDA for the treatment of type 2 diabetes. However, the need for twice daily injections of exendin-4 has continued to fuel attempts to develop GLP-1R agonists with long-acting pharmacodynamic and pharmacokinetic properties.

For this purpose, albumin binding has been utilized as a novel strategy to prolong GLP-1R agonist action *in vivo*, as exemplified by Albiglutide, a recombinant human GLP-1-albumin protein, resulting in a circulating half-life of ~5 days in humans, which was significantly higher than that of native GLP-1 (<5 min) [14,15]. Comparing to other methods, the production of long-acting albumin fusion can avoid complicated chemical modification or formulation process [16].

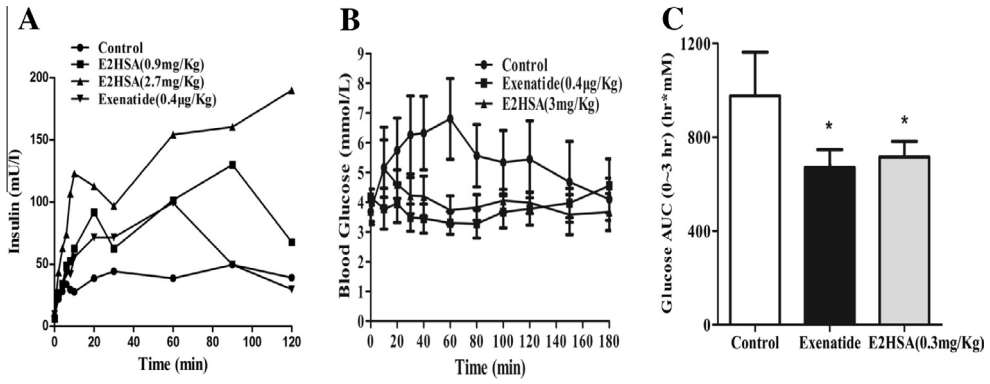

Fig. 3. *In vivo* efficacy of E2HSA following hyperglycemic clamp test and an oral glucose test in healthy monkeys. (A) Insulin levels were plotted *vs* time following hyperglycemic clamp in healthy monkeys. (B and C) Curves of plasma glucose and the AUC after an oral glucose loading (2 g/Kg). Oral glucose tolerance in healthy monkeys following subcutaneous administration 0.3 mg/kg dose of E2HSA 42 h before glucose loading and exenatide was subcutaneous administration 30 min prior to glucose loading. $^{*}p < 0.05$ compared with control group.

E2HSA is a novel genetic fusion protein consisting of two tandem exendin-4 molecules that has been covalently bonded to recombinant human serum albumin (HSA) via a linker, for the potential treatment of type 2 diabetes. The exendin-4 dimer was used to avoid potential reductions of the interaction of the exendin-4 moiety of the monomer with its receptor in the presence of albumin. E2HSA may be an available substitute for exendin-4. Despite increased molecular weight, E2HSA exhibits activities similar to exendin-4. In the present study, E2HSA bound to the GLP-1 receptor, dose-dependently increased cAMP production *and* augmented glucose dependent insulin secretion *in vitro*, demonstrated glucoregulatory activity and enhancement of glucose dependent insulin secretion *in vivo*.

RIN-m5F cells retain many of the differentiated function of pancreatic β cells including insulin secretion associated with activation of cAMP and expression of the GLP-1 receptor [17]. Exendin-4 exerts its effects by binding and activating a specific receptor, named GLP-1 receptor and structurally related to G-protein coupled receptor class 2 family, which is predominantly coupled to stimulation of cAMP activity [18]. The conjugation of exendin-4 to high molecular weight HSA modifies the physicochemical properties of the conjugated complexes maintaining, totally or in part, the biological function of the original non-conjugated molecule, such as the capability of receptor recognition and activation. Concomitantly, the conjugated HSA chain may hamper physical contacts between the peptide and receptor binding site, thus preventing or reducing the capability of receptor recognition and activation [19]. Hence, the data are consistent with E2HSA has lower biological activity than exendin-4 *in vitro*.

In this research, the stability of E2HSA was significantly increased *in vitro* and *in vivo*. We demonstrated that E2HSA was stable in fresh rat plasma for at least 48 h *in vitro* and E2HSA was detected for 288 h after injection in monkey. These results suggested that E2HSA was also resistant to cleavage by DPP-IV, which was similar to exendin-4. E2HSA also has a relatively longer half-life *in vivo* as shown in monkey pharmacokinetic study. The biological half-life of E2HSA was 54 h following subcutaneous administration in monkeys, whereas exendin-4 has a somewhat shorter half-life of 60 min [20]. The remarkable prolonged half-life of E2HSA was of great importance and might reveal the potent long-acting anti-diabetic property of E2HSA. E2HSA also exhibited a distinguished physiological characteristic of insulin secretion and glucose clearance. In the present study, we have shown that a single dose of E2HSA displayed glucoregulatory effects including reduce postprandial glucose excursion and control fasting glucose. In addition, we demonstrated prolonged activity of E2HSA in the hyperglycemic clamp test in monkeys 18 h after single subcutaneous administration. Additionally, the oral glucose tolerance test illuminated that E2HSA was capable of regulating the blood glucose level for 42 h after single subcutaneous administration, unmodified exendin-4 showed no efficacy at this point.

Besides insulinotropic activity, slowing of gastric emptying and food intake inhibition is believed to contribute to the glucoregulatory and weight loss effects of exendin-4 [21]. In this study, one interesting result was the inability of exendin-4 to inhibit food intake after healthy monkey were fed with the stander diet. The failure of exendin-4 to inhibit food intake may be the result of an insufficient dose, which is prescribed at doses of 4 μg/Kg for once daily subcutaneous administration to the monkey in this study. But for E2HSA, it elicited a dose-dependent trend in inhibiting food intake, the effect of which is sustained over 4-day period. For clinical use of E2HSA, duration of action and safety must be considered. Here, even at the higher doses that potently prevented food intake, we did not observe any signs of nausea, vomiting, or malaise in the monkeys. They simply seemed uninterested in acquiring food.

In conclusion, the exendin-4 and human serum albumin fusion protein E2HSA has longer plasma residence than exendin-4, with significant pharmacologic effects: a dose-dependent increase in the glucose-stimulated insulin secretion and does-dependent suppression food intake and other glucoregulatory effects. The ability of E2HSA to exert significant insulinotropic activity and glucose regulation effect for days after single dose, supports clinical development as a potential long-acting GLP-1 receptor agonist for type 2 diabetes management.

Contributions of authors

Ling Zhang: Be in charge of each experiment and the paper writing, Lin Wang: Technological assessor of the whole subject, Zhiyun Meng: Participate in the subject design, Hui Gan: Participate in the cell culture experiment, Ruolan Gu: Participate in the cell culture experiment, Zhuona Wu: Participate in the cell culture experiment, Lei Gao: Animal sample analysis, Xiaoxia Zhu: Animal sample analysis and data analysis, Wenzhong Sun: Managing animal studies, Jian Li: Managing animal studies, animal sample analysis, data analysis, Ying Zheng: Animal sample analysis, Guifang Dou: The organizer and designer of this subject.

Conflict of interest

None declared.

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