The anorexic effect of Ex4/Fc through GLP-1 receptor activation in high-fat diet fed mice

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Exendin-4 (Ex4), a peptide initially found in the saliva of the Gila monster, can activate the signaling pathway of the incretin hormone glucagon-like peptide-1 (GLP-1) through the GLP-1 receptor (GLP-1R). We previously reported that a chimera protein consisting of Ex4 and mouse IgG heavy chain constant regions (Ex4/Fc) can exert biological effects of GLP-1, such as improving glycemic control and ameliorating manifestations in diabetic mice. The aim of this study was to determine whether Ex4/Fc is effective in modulating energy homeostasis in mice. Our results showed that in vivo expression of Ex4/Fc by intramuscular injection of the plasmid encoding Ex4/Fc followed by local electroporation effectively decreased food intake in the mice on high-fat diet (HFD) feeding. In addition, the reduced energy intake was associated with the decreased excrements from the Ex4/Fc-treated HFD mice but not the Fc control mice. Remarkably, the Ex4/Fc-treated HFD mice displayed significantly lower triglyceride (TG) levels when compared with the control mice. Interestingly, while the leptin levels were not changed, the circulating ghrelin levels were higher in Ex4/Fc mice than those in the Fc control mice. These results suggested that Ex4/Fc can improve energy metabolism and lipid metabolism through GLP-1R in mice under excessive nutrition conditions.

Keywords
exendin-4 (Ex4); glucagon-like peptide-1 (GLP-1); high-fat diet (HFD); obesity; diabetes

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Introduction

Glucagon-like peptide-1 (GLP-1) is a peptide hormone released from intestinal L-cells in response to nutrient ingestion. GLP-1 exerts various biological effects such as enhancing glucose-dependent insulin secretion, suppressing inappropriately elevated glucagon secretion, slowing gastric emptying, reducing food intake, and stimulating satiety [1,2] through activation of the GLP-1 receptor (GLP-1R), a G-protein-coupled receptor expressed in a variety of tissues throughout the body. GLP-1 has a short plasma lifetime of 1–2 min due to a rapid degradation by dipeptidyl peptidase-IV (DPP-IV), which motivated scientists’ great efforts in developing long-lasting GLP-1R agonists as therapeutic agents in the treatment of the hyperglycemia of type 2 diabetes.

Exendin-4 (Ex4) is a 40-aa peptide produced in the salivary gland of the Gila monster and it has a 53% amino acid sequence overlap with mammalian GLP-1 [3]. Ex4 is resistant to DPP-IV degradation and acts through the GLP-1R to exert GLP-1-like actions [4]. A synthetic Ex4 has been approved and used clinically as adjunct therapy for type 2 diabetes. However, an immunological response to Ex4 was detected in some patients on Ex4 therapy [5] due to its protein sequence heterology, as xenoproteins are generally immunogenic. We previously reported that a fusion protein consisting of Ex4 and mouse IgG1 heavy chain constant regions (Ex4/Fc) produced less immunogenicity in mice compared with Ex4 alone, likely through its binding to the inhibitory Fc receptor FcγRIIB expressed by B-lymphocytes [6]. The present study was undertaken to evaluate the ability of Ex4/Fc fusion protein to improve energy homeostasis in vivo. Our in vitro data showed that the recombinant Ex4/Fc fusion protein produced by mammalian cells promoted the proliferation of MIN6 beta-cells via a GLP-1R agonism sensitive fashion. In vivo expression of Ex4/Fc by intramuscular gene transfer suppressed energy intake and improved hormone and metabolic profiles in high-fat diet (HFD) fed mice.

Materials and Methods

Materials
Cell culture reagents, 5-bromo-2′-deoxyuridine (BrdU), Lipofectamine 2000, TRITol, and leptin enzyme-linked...
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Dingguo Bio. (Shanghai, China). Ghrelin enzyme immunoassay (ELISA) kits were purchased from Life Technologies (Carlsbad, USA). Ghrelin enzyme immunoassay (EIA) kit was from Ray Biotech Inc. (Atlanta, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), GLP-1, and Exendin-9-39 (Ex9) were from Sigma Chemical (St Louis, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody and anti-BrdU monoclonal antibody were purchased from Cell Signaling Transduction (Boston, USA). All other chemicals were of analytical grade from Dingguo Bio. (Shanghai, China).

Plasmid construction
The Ex4/Fc/VRnew and Fc/VRnew recombinant plasmids were constructed as described previously [7]. Briefly, the sequence encoding Ex4 was chemically synthesized, ligated to a polymerase chain reaction (PCR)-amplified cDNA fragment encoding mouse IgG1-Fc (hinge-ch2-ch3) and inserted into the EcoRV and BamHI sites of the VRnew vector. An IgK signal peptide was used to permit the secretion of the fusion protein. The recombinant plasmids were verified by DNA sequencing.

Cell culture
Human embryonic kidney (HEK) 293 cells and MIN6 cells (passage 25–35) were cultured as previously described [7,8]. In brief, HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) calf serum and MIN6 cells were cultured in DMEM with 25 mM glucose, 15% (v/v) fetal bovine serum (FBS), and 5 ml/l beta-mercaptoethanol at 37°C and 5% (v/v) CO2.

Mini gene transfection and expression
HEK293 cells maintained in DMEM containing 10% (v/v) calf serum were transfected with Ex4/Fc/VRnew or Fc/VRnew using Lipofectamine 2000 according to the manufacturer’s manual. At 24 h after transfection, total RNA was extracted and subjected to reverse transcriptase-PCR (RT-PCR). The primers used were: forward: 5′-atgggtactgctgctc-3′; reverse: 5′-agccttcggtctgcctttggt-3′. For immunostaining, the transfected cells were fixed and permeabilized, and the Ex4/Fc fusion protein was detected by FITC-conjugated anti-mouse IgG antibody (1:300) followed by nuclear DAPI counterstaining.

Conditioned medium and proliferation of MIN6 cells
After transfection, HEK293 cells were incubated in the transfection medium containing 0.1% bovine serum albumin (BSA) for 24 h. The conditioned medium was prepared by diluting the transfection medium into DMEM at a volume ratio of 1:1, 1:2, and 1:5. Final concentrations of BSA and glucose were adjusted to 0.1% and 1.0 g/l, respectively. At 60% confluence, MIN6 cells were washed twice with DMEM without FBS, kept in a serum-free medium for 14 h, and then induced in conditioned medium for proliferation in the absence or presence of the Ex9 for 24 h. Cell proliferation was determined using MTT colorimetric assay according to the manufacturer’s manual.

BrdU incorporation assay
After being fasted for 16 h, MIN6 cells were treated with or without conditioned medium (1:1) for 24 h, and then incubated with 10 μM BrdU for 2 h and fixed with Carnoy’s fixative (3 parts methanol:1 part glacial acetic acid) for another 20 min. Subsequently, cells were denatured by 2 M HCl and stained with anti-BrdU monoclonal antibody. FITC-conjugated anti-mouse IgG antibody was added followed by nuclear DAPI counterstaining.

Animal study
Mice (Slac Laboratory Animal, Shanghai, China) were housed under controlled temperature conditions and a 12 h light/12 h dark cycle with free access to food and water except where noted. At the initiation of DNA injection, HFD (calorie density: 45% total kilocalories from fat and 35% from carbohydrate; Research Diets, New Brunswick, USA) was provided to replace the chow diet. All procedures were conducted according to protocols and guidelines approved by the institutional animal care committee.

In vivo expression of Ex4/Fc in mice
Intramuscular plasmid DNA injection and electroporation were carried out as described previously [6,9]. Briefly, each mouse received a total of 50 μg of DNA in 0.9% NaCl by intramuscular injection (25 μg in each tibialis anterior muscle) under anesthesia conditions. The injection was repeated on the seventh day to ensure that all injected mice expressed proteins encoded by the constructs. After the intramuscular DNA injection, electrical current was applied locally with caliper electrodes (BTX, San Diego, USA) to skin covered with conducting gel (BTX) at 150 V/cm, pulse length 20 ms, eight pulses at 1 s intervals.

Blood biochemistry and hormone profile
Blood samples obtained from the saphenous vein were centrifuged at 2000 g to obtain serum; for the plasma preparation, K3-EDTA was used during sampling. Serum and plasma were stored at −80°C. Circulating levels of leptin and ghrelin levels were measured by ELISA and EIA using the relevant kits as described in the Materials and Methods section. Triglyceride (TG) was analyzed using Full-Automatic Clinical Chemistry Analyzer (Bio-Rad Laboratories, Irving, USA).

Metabolic study and body composition
Body weight and food intake of the mice were measured twice weekly. At the end of experiment, metabolic cages
were used to measure water consumption, feces weight, and urine output in the mice fed with HFD.

**Immunohistochemistry**
For histochemistry analysis, muscle tissue was isolated, and the tissue fixation and paraffin embedding were conducted as described previously [6]. The expression of Ex4/Fc in skeletal muscle at DNA-injecting sites was detected by immunohistochemistry using FITC-labeled anti-mouse IgG antibody (1 : 300) with nuclear DAPI counterstaining.

**Statistical analysis**
The data were presented as mean ± SEM. Comparisons between groups were carried out by unpaired Student’s t-test and in the case of multiple treatments by one-way analysis of variance. \( P < 0.05 \) is considered significantly different.

**Results**

**Expression and characterization of Ex4/Fc fusion protein in mammalian cells**
We first tested the recombinant constructs by transfection of Ex4/Fc/VRnew (or Fc/VRnew as control) into HEK293 cells. RT-PCR and immunostaining were used to determine the expression of the constructs. As shown in **Fig. 1A**, Ex4/Fc fusion and Fc control transcripts were readily detected in the transfected cells. No transcripts were detected in non-transfected HEK293 cells. The Ex4/Fc fusion and Fc control transcripts could be distinguished by their different mobilities in the agarose gel (**Fig. 1A**). The fusion protein expression in the HEK293 cells was assessed by immunostaining using FITC-conjugated anti-mouse antibodies. As shown in **Fig. 1B,C**, the Ex4/Fc fusion and Fc alone protein could be detected in the transfected cells, but not in the non-transfected cells. These results indicated that these constructs were well processed in the mammalian cells for the expression and production of Ex4/Fc fusion protein.

**Ex4/Fc fusion protein promotes pancreatic beta-cell proliferation via activating GLP-1R**
Native GLP-1 enhances beta-cell mass via decreasing cell apoptosis and increasing cell proliferation [10,11]. To confirm the presence of Ex4/Fc fusion protein in the medium, MIN6 cells were incubated with the conditioned medium from transfected HEK293 cells for 24 h. A significant 1.17-fold promotion was first seen at 1 : 2 dilution ratio and a maximal 1.24-fold enhancement of cell proliferation appeared at 1 : 1 dilution ratio of conditioned medium (**Fig. 2A**). To further investigate that Ex4/Fc promoted the beta-cell growth via GLP-1R, MIN6 cells were treated with Ex9, a specific GLP-1R antagonist that has been shown to completely block GLP-1R binding of Ex4 at a concentration of 5 nM [12–14]. The data in **Fig. 2A** showed that Ex9 completely blocked the increased proliferation of MIN6 cells induced by the transfected conditioned medium. The promotion of MIN6 cell proliferation induced by Ex4/Fc was further confirmed by BrdU incorporation assay (**Fig. 2B**).

![Figure 1](image1.png)

**Figure 1. Expression of Ex4/Fc fusion transcript and protein in mammalian cells**  
(A) Schematic diagram of recombinant construct of Ex4/Fc. HEK293 cells were transfected with Ex4/Fc for 24 h. Ex4/Fc fusion and Fc control transcripts and proteins were detected by RT-PCR (B) using gene-specific primers and immunocytochemistry (C) using FITC-conjugated anti-mouse antibodies 24 h after transfection.
These data suggested that Ex4/Fc induces GLP-1R activation to exert its biological effects under in vitro condition.

The anorexic effect of Ex4/Fc fusion protein in mice challenged with HFD

To assess the biological effect of Ex4/Fc fusion protein in vivo, we delivered the plasmids by intramuscular injection followed by electroporation. At 10 weeks after the injection, Ex4/Fc fusion protein was detected in the muscle from the mice with plasmid injection, but not in the muscle from control mice as analyzed by immunohistochemistry, using FITC-conjugated anti-mouse IgG antibodies (Fig. 3A). Native GLP-1 decreases gastric motility and induces satiety, leading to reduced body weight. We investigated the effect of persistent expression of Ex4/Fc fusion protein on the body weight and food intake of mice challenged with HFD. The body weight gains of the two groups were similar (Fig. 3B). The mean energy intake of the two groups was similar at the initiation of the treatment, and then a 10% reduction was seen at 1–2 weeks. The decline was continued thereafter and a 14% reduction was observed at 6 weeks after the Ex4/Fc treatment in the HFD mice compared with the control group (Fig. 3C).

Effects of Ex4/Fc fusion protein on hormone profile

The reduced energy intake by Ex4/Fc treatment was associated with a 46% reduction in TG levels, compared with that of the control mice (Fig. 4A). We further detected the circulating hormone levels which regulate food intake. HFD feeding resulted in elevated circulating leptin and decreased ghrelin levels in the Fc control mice. The ghrelin level decreased at 4 weeks, while thereafter increased to control level at 10 weeks after treatment with the Ex4/Fc fusion protein (Fig. 4B). In addition, Ex4/Fc treatment had no effect on the leptin level (Fig. 4C).

Improvement of metabolic profile in Ex4/Fc-treated mice

There was no difference in water consumption between the two groups of mice (Fig. 5A). The mice receiving Ex4/Fc treatment showed lower urine output (Fig. 5B) and feces weight (Fig. 5C), in comparison with the Fc control mice. These data indicated that the reduction of energy intake in the treated mice contributes to reduced metabolic profile.

Discussion

In the present study, we demonstrated that Ex4/Fc fusion protein improved the energy homeostasis in the HFD-fed mice through reducing the food intake. This fusion protein was well processed for expression in mammalian cells and promoted the proliferation of pancreatic MIN6 cells via activating GLP-1R. Intramuscular injection of construct followed by electroporation resulted in the persistent in vivo expression of Ex4/Fc in the HFD-fed mice. This leaded to improved energy homeostasis and lipid metabolism, exemplified by decreased food intake and reduced circulating TG levels in the HFD mice.

The delivery of protein drugs often give rise to the production of neutralizing antibodies in the recipient, which may decrease or abolish the drug activity [15,16]. We demonstrated previously that the Ex4/Fc fusion protein has reduced immunogenicity compared with Ex4 alone, likely through its binding to the inhibitory Fc receptor FcγRIIb expressed by B-lymphocytes [6], suggesting a potential option of GLP-1 mimetics as therapeutic agents.

Consistent with our previous finding that demonstrated that a gene therapy with GLP-1/Fc fusion constructs [8], our present observations that in vivo expression of Ex4/Fc reduced food intake and improved lipid metabolism in HFD mice, which is in a good agreement with other previous...
studies in type 2 diabetes patients [17–19] and in obese, non-diabetic individuals as well [20]. However, it is interesting to mention that, in the current study setting, we did not observe any simultaneous reduction in body weight gain in the Ex4/Fc-treated HFD mice during the study duration. Although the underlying mechanism is not clear, it is possible that the effect of the degree of anorexia by Ex4/Fc which affects the food intake may be inadequate to influence the body weight in the recipient mice. This explanation is, at least in part, supported by other recent studies that demonstrated that a chronic calorie restriction (e.g. animals receiving 70% calorie of the control) significantly improved insulin sensitivity and glucose tolerance without obvious changes in the body weight of the experimental animals [21]. Nevertheless, our previous studies along with the in vitro and in vivo evidence suggested that the therapeutic effects of Ex4/Fc are through GLP-1R signaling pathway.

It has been previously shown that GLP-1-induced suppression of food intake was mediated by GLP-1R-dependent excitation of vagal nodose ganglia and the ghrelin-sensitive neurons in the hypothalamic arcuate (ARC) nucleus [22]. It is likely that the in vivo expression of Ex4/Fc induced reduction of food intake in the HFD-fed mice is, in part, due to activation of GLP-1R-dependent central pathways. A previous study demonstrated that GLP-1, in an IgG fusion format, could cross through the blood–brain barrier and stimulate the central c-FOS expression [23]. Further research is needed to delineate the signaling pathways that mediate the Ex4/Fc’s central effects.

A previous study showed that infusion of Ex4 increased the glomerular filtration rate and suppressed the proximal re-absorption in rats [24]. Furthermore, a single bolus injection of long-acting GLP-1 analog inhibited overnight food and water intake in a dose-dependent manner and increased diuresis in both normal and obese rats. However, these effects were found to be transient as they were normalized within a few days of treatment [25]. In our study, the treatment of Ex4/Fc decreased output of urine and feces, but it had no significant effect on water consumption. It is likely that the repression in food intake by Ex4/Fc contributed to the reduction in urine and feces output.

In vivo expression of Ex4/Fc significantly decreased the circulating ghrelin levels in the HFD mice. Ghrelin, a hormone peptide produced by ghrelin cells in the gastrointestinal tract, plays a role in regulating food intake and energy metabolism. It has been shown that central administration of ghrelin increased food intake primarily by reducing the time between meals. Interestingly, in the isolated stomach, GLP-1 exerted effects in decreasing ghrelin secretion [26]; in contrast, intravenously administered GLP-1 did not suppress ghrelin in healthy men [27]. However, when kept at supraphysiological plasma level, GLP-1 reduced postprandial ghrelin levels via increased insulin secretion [28].

In mice, the obese gene product leptin regulates food intake and energy balance through its receptor in which some of them are expressed in the hindbrain GLP-1 neurons [29]. In both normal and obese rats, administration of GLP-1 analog suppressed the plasma leptin levels, which was associated with a reduction in food intake and body weight [25]. In healthy volunteers, the plasma leptin levels were found to be unchanged either upon a meal ingestion or administration of physiological and pharmacological doses of GLP-1 [30]. However, the impaired ghrelin secretion and ghrelin sensitivity [31] accompanied with hyperleptinemia/leptin resistance

Figure 3. Persistent expression of Ex4/Fc fusion protein in mice undergoing HFD challenging  The plasmid of Ex4/Fc was intramuscularly injected followed by an electroporation. (A) Immunohistochemical staining was conducted in the plasmid DNA injected muscle (non-injected muscle as control) using FITC-conjugated anti-Fc antibody (green) and nuclear dye DAPI for nuclei staining (blue). Food intake (B) and body weight (C) in the mice were measured at the indicated timepoints.
was found in diet-induced obesity in mice, or in obese human. In diet-induced obesity models, the sensitivity to the anorexigenic effect of exogenous leptin [33] and orexigenic effect of ghrelin [31] was remarkably reduced, which could be, however, reversed upon body weight loss. In our study, the ghrelin level was decreased and leptin was unchanged in the HFD-fed mice, suggesting that Ex4/Fc might have direct effect on the ghrelin secretion, whereas the long-term regulation of ghrelin and leptin is more closely related to food intake and body weight change in a compensatory manner. Furthermore, in Ex4/Fc-treated HFD mice, the ghrelin level remarkably increased at 10 weeks treatment, which might counteract the weight-sparing effects of Ex4/Fc. Taken together, our results demonstrated that Ex4/Fc fusion protein exerted an anorexic effect in the HFD-fed mice via activating GLP-1R pathway. Furthermore, we present evidence that this effect is associated with decreased circulating level of TG and improved metabolic profile. In addition to the low immunogenicity and glucose-lowering effect reported previously, our study demonstrated that the Ex4/Fc fusion protein is a potential candidate as GLP-1R agonist for the treatment of hyperglycemia in the diabetes patients.

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