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Effects of tryptophan-selective lipidated glucagon-like peptide 1 (GLP-1) peptides on the GLP-1 receptor

Xuejing Lu¹, Norio Harada^{1,2}, Takuma Yasuda¹, Eri Ikeguchi-Ogura¹, Daishiro Kobayashi³, Masaya Denda³, Yohei Seno¹, Shunsuke Yamane¹, Daisuke Yabe¹, Akira Otaka³ and Nobuya Inagaki^{1,4}

¹Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan

²Department of Endocrinology and Metabolism, School of Medical Sciences, University of Fukui, Fukui, Japan

³Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University, Tokushima, Japan

⁴Medical Research Institute KITANO HOSPITAL, P.I.I.F. Tazuke-Kofukai, Osaka, Japan

Correspondence should be addressed to N Harada: nharada@g.u-fukui.ac.jp or to N Inagaki: inagaki@kuhp.kyoto-u.ac.jp

Abstract

Glucagon-like peptide 1 (GLP-1) receptor agonists (GLP-1 RAs) are widely used as antidiabetic and anti-obesity agents. Although conventional GLP-1 RAs, such as liraglutide and semaglutide, are acylated with fatty acids to delay their degradation by dipeptidyl peptidase-4, the manufacturing process is challenging. We previously developed selectively lipidated GLP-1 peptides at their only tryptophan residue (peptide A having one 8-amino-3,6-dioxaoctanoic acid (miniPEG) linker and peptide B having three miniPEG linkers). In this study, we evaluated their effects on the GLP-1 receptor *in vitro* and *in vivo*. Both novel peptides were shown to increase cyclic adenosine monophosphate production and insulin secretion similarly to that by GLP-1(7–37) and liraglutide *in vitro*. In addition, these novel peptides lowered blood glucose levels by increasing insulin levels after oral administration of glucose and they suppressed gastrointestinal motility as effectively as liraglutide. The effects of peptide A on activation of satiety-promoting neurons in the arcuate nucleus and the consequent suppression of food intake and body weight were also similar to those of liraglutide, while the effects of peptide B were less than those of liraglutide. Under high-fat diet feeding, both long-term administration of peptide A and peptide B improved glucose tolerance and insulin sensitivity similarly to liraglutide. Thus, tryptophan-selective lipidated GLP-1 peptides are as effective as conventional GLP-1 RAs in reducing plasma glucose levels and body weight and may represent a less demanding method of manufacture of GLP-1 RAs.

Keywords: glucagon-like peptide-1; insulin secretion; food intake; obesity; diabetes

Introduction

Obesity is increasing worldwide and is a severe global health challenge (NCD Risk Factor Collaboration 2017) due to its promotion of insulin resistance and onset of type 2 diabetes (T2D) (Kahn *et al.* 2006). Furthermore, obesity and T2D are both major risk factors for cardiovascular diseases (Petrie *et al.* 2018).

It is therefore necessary to treat obesity along with T2D to suppress the onset and progression of cardiovascular diseases.

Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted from enteroendocrine L-cells in response to nutrient ingestion and stimulates glucose-dependent

insulin secretion from pancreatic β -cells via the GLP-1 receptor (GLP-1R) (Joo *et al.* 2013, Suzuki *et al.* 2018). GLP-1 also reduces body weight by suppression of food intake (Harada & Inagaki 2022). GLP-1 can pass the blood–brain barrier (BBB) to elicit postprandial satiation signals directly from the arcuate nucleus (ARC) in the hypothalamus and area postrema (AP) in the medulla oblongata via GLP-1R (Harada & Inagaki 2022). GLP-1R is also expressed in the peripheral endings of vagal afferent nerves, allowing GLP-1 to indirectly stimulate the AP and nucleus tractus solitarius. In addition, central nervous system signaling via GLP-1R suppresses appetite by reducing activation of hunger-promoting agouti-related peptide (AgRP) neurons and promoting activation of satiety-promoting proopiomelanocortin (POMC) neurons (Secher *et al.* 2014, Bai *et al.* 2019, Trapp & Brierley 2022). However, the half-life of human GLP-1 is only about 2 min due to renal clearance and degradation by dipeptidyl peptidase-4 (DPP-4), which represents an obstacle to the use of GLP-1 therapy in clinical settings (Kieffer *et al.* 1995, Deacon 2004, Baggio & Drucker 2007).

GLP-1 receptor agonists (GLP-1 RAs) are designed and synthesized to extend the half-life of human GLP-1; liraglutide and semaglutide have acylation of the human GLP-1 peptide backbone with a spacer and a fatty acid chain to lysine at position 26, which mediates strong binding to albumin, reduces renal clearance and delays degradation by DPP-4 (Damholt *et al.* 2006, Marbury *et al.* 2017). Long-acting insulin analogs, such as degludec and detemir, are also acylated with fatty acids, resulting in longer half-life of GLP-1 (Heise *et al.* 2012, 2015). However, human GLP-1 has two ϵ -amino groups of lysine at positions 26 and 34 and an α -amino group of histidine at the N-terminal, which complicates the standard manufacturing process employing regioselective acylation of an amino group. In liraglutide and semaglutide, the lysine at position 34 of parent human GLP-1 is replaced by arginine, which is not reactive to acylation by fatty acid units. Construction of peptides A and B utilizes the acid-activated *p*-methoxybenzyl cysteine sulfoxide (Cys(MBzl)(O))-mediated conjugation of a fatty acid moiety to the indole side chain of tryptophan at position 31. The parent human GLP-1 peptide has one tryptophan residue, and the acid-activated sulfoxide-mediated reaction occurs only on the indole of tryptophan; thus, chemically synthesized human GLP-1 subjected to the tryptophan modification by Cys(MBzl)(O)-decorated fatty acid units under acidic conditions enables efficient production of Trp-lipidated GLP-1 analogs having a naturally occurring amino acid sequence (Kobayashi *et al.* 2022) (Supplemental Fig. 1 (see section on [Supplementary materials](#) given at the end of the article)).

In this study, we evaluated the effects of these tryptophan-selective lipidated GLP-1 peptides on

GLP-1R *in vitro* and their metabolic action related to T2D and obesity *in vivo*.

Materials and methods

Animals

Male C57BL/6J mice were used as wild-type mice in all experiments. The mice were maintained in a specific pathogen-free facility under conditions of controlled temperature and a 14h light:10h darkness cycle with free access to water and chow. Animal care and procedures were approved by Kyoto University Animal Care Committee (no. Med Kyo20217, approval date: 25 March 2020).

Plasmid construction, cell culture and transfection

The cDNA fragments of human GLP-1R were cloned into the pCMV-6c vector (Sigma-Aldrich Co. LLC, USA). COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Expression plasmids of human GLP-1R or pCMV-6c vectors (control) were transfected into COS-7 cells using Lipofectamine 2000 (Sigma-Aldrich Co. LLC, USA). Lipofectamine 2000 and plasmids (1.6 μ g/well) were mixed with the same amount of Gibco Opti-MEM Reduced Serum Medium (Cat No. 11058021; Thermo Fisher Scientific Life Sciences Solutions, USA), respectively. Solutions were incubated at room temperature for 5 min. The same amount of solution was mixed and incubated at room temperature for another 20 min. Finally, the plasmid solution (200 μ L) and COS-7 cells (800 μ L) were added into the 12-well plate and incubated at 37°C for 24 or 48 h. After 48-h incubation, the peptide mixtures were added to the COS-7 cells. MIN6 cells (2×10^5 cells/well) were cultured in DMEM with 10% FBS in a 24-well plate for 48 h at 37°C before peptide treatment.

Measurement of expression of GLP-1 receptor mRNA

Mice islets were isolated as described previously (Harada *et al.* 2008), and total mRNAs were extracted by RNeasy Mini Kit (Qiagen Inc, Germany). Human GLP-1R-expressing COS-7 cells and MIN6 cells were washed twice by PBS and were then collected. Total RNAs were extracted using TRIzol reagent (REF No. 15596018, Thermo Fisher Scientific Life Sciences Solutions, USA) and reverse transcribed by PrimeScript RT reagent Kit (Cat# RR037B; Takara Bio Inc., Japan). SYBR Green PCR Master Mix (Cat# 4334973; Applied Biosystems Inc., USA) was prepared for the real-time PCR (RT-PCR) run. All results are presented using the relative standard curve method normalized to a housekeeping gene (*PPIA*).

Primer pairs designed for evaluation of gene expression were as follows: *GLP-1R*: forward, 5'-CTACGTGAGCATAGGCTGGG-3'; reverse, 5'-ATGGGCAGCCGGATAATGAG-3'; and *PPIA*: forward, 5'-GAGCTGTTTGCAGACAAAGTTC-3'; reverse, 5'-CCCTGGCACATGAATCCTGG-3'.

Intracellular cyclic adenosine monophosphate (cAMP) production in human GLP-1 receptor-expressing COS-7 cells

COS-7 cells (5×10^5 cells/well) were transfected with human GLP-1R expression plasmid or control vector at indicated amounts and incubated in a 12-well plate. After 48-h incubation, the cells were washed twice with PBS. The reaction was started in 0.5 mL Krebs–Ringer bicarbonate buffer (KRBB) containing 0.1 mM 3-isobutyl-1-methylxanthine (no. 19624; Nacalai Tesque, Japan) with various concentrations of GLP-1(7–37), liraglutide or the lipidated GLP-1 peptides (peptides A and B). The cells were then incubated at 37°C for 30 min. Incubation buffers were removed, and the cells were lysed by 0.1 M HCl (109 μ L/well) to each well and incubated for 20 min at room temperature. The cell lysate was then scraped off and collected. The supernatant was stored at –80°C after centrifuge (1,000 g, 10 min). Intracellular cAMP levels were measured by enzyme-linked immunosorbent assay (ELISA) (no. 581001; Cayman, USA). Data are expressed as increments of GLP-1(7–37), liraglutide or the lipidated GLP-1 peptides (peptides A and B) from basal cAMP levels.

Insulin secretion from MIN6 cells

Mouse pancreatic cell line MIN6 cells were pre-incubated at 37°C for 30 min in KRBB containing 10 mM HEPES, 2.8 mM glucose and 0.2% (w/v) BSA. The cells were pre-incubated at 37°C for 30 min in 0.5 mL KRBB (2.8 mM glucose) and then incubated at 37°C another 30 min in 0.5 mL KRBB (11.1 mM glucose) with or without 10^{-6} M human GLP-1(7–37), 10^{-6} M liraglutide or the 10^{-6} M lipidated GLP-1 peptides (peptides A or B). Supernatants were collected by centrifugation (9,000 g, 1 min). To determine insulin content, the cells were homogenized in 500 μ L/well PBS with 0.2% Triton and frozen overnight at –20°C. The extracts were thoroughly mixed, and 20 μ L/well of extracts were diluted in 980 μ L borate buffer. Insulin levels and insulin content levels were measured by homogeneous time-resolved fluorescence assay using Insulin High Range Assay Kit (Code 62IN1PEG; CIS Bio International, France) and Synergy H1 (BioTek Instruments, USA) according to the manufacturers' instructions (Usui *et al.* 2019).

Oral glucose tolerance tests (OGTTs)

After a 16-h fasting period, wild-type mice were administered glucose (2 g/kg body weight)

by oral gavage. About 50 μ L blood samples were collected via tail vein at 0, 15, 30, 60, 120 and 150 min and centrifuged (9,000 g, 5 min) to collect plasma stored at –80°C. Blood glucose levels were measured using the glucose oxidase method (Sanwa Kagaku Kenkyusho Co., Ltd, Japan). Insulin levels were measured using ELISA kits for insulin (Cat. # AKRIN-031, FUJIFILM Wako Shibayagi Corporation, Japan).

Gastrointestinal transit

Transit through the stomach and small intestine was measured by oral administration of a nonabsorbed marker containing 10% (w/v) charcoal suspension in 5% (w/v) gum arabic (Ogawa *et al.* 2011) in saline solution immediately after intraperitoneal injection of PBS, liraglutide (500 μ g/kg body weight) or the lipidated GLP-1 peptides (A and B) (500 μ g/kg body weight). The 17-week-old mice were given 0.2 mL of the suspension by oral gavage. Twenty minutes later, the mice were sacrificed and the distance from the pylorus to the front of the charcoal bolus and the ileocecal junction was measured. The transit rate in the small intestine was determined as ((distance from the pylorus to the charcoal front)/(distance from the pylorus to the ileocecal junction)) $\times$ 100 (%) (Supplemental Fig. 2A).

Measurement of active GLP-1 levels

Wild-type mice were administered PBS, liraglutide (500 μ g/kg or 250 μ g/kg body weight) or the lipidated GLP-1 peptides (peptides A and B) (500 μ g/kg or 250 μ g/kg body weight) by intraperitoneal injection. The doses of the lipidated peptides were determined according to previous studies (Noyan-Ashraf *et al.* 2009, Sisley *et al.* 2014, Bugáňová *et al.* 2017). Blood samples were collected via tail vein at 0 (before injection), 12, 24, 48 and 72 h into 0.4 mL microcentrifuge tubes (Cat. # 500-Q; Quality Scientific Plastics, USA) containing 2 μ L DPP-4 inhibitor vildagliptin (100 μ mol/L) (FUJIFILM Wako Shibayagi Corporation, Japan) on ice and centrifuged (9,000 g, 5 min) to collect plasma stored at –80°C. Active GLP-1 levels were measured using active GLP-1 ELISA kit (Cat. # EGLP-35K; EMD Millipore Corporation, USA).

Measurement of food intake

Food intake was measured according to the protocol described in Supplemental Fig. 2B and C. Sixteen 24-week-old wild-type mice were divided into eight groups (2 mice/cage) for two days for adaptation before measurement. First, the amount of food intake in all eight groups was measured for two days (measurement [–2]) as the basic food intake. The food intake for the subsequent two days was also measured (measurement [0]). The mice were then intraperitoneally treated with PBS, liraglutide or the lipidated GLP-1 peptides (A and B) for two days at 500 μ g/kg body weight once a day

(Supplemental Fig. 2B) or 250 µg/kg body weight twice a day (Supplemental Fig. 2C). Food intake upon two-day treatment was evaluated (measurement [2]). Afterward, food intake was measured three times every two days (measurements [4], [6] and [8]). The results of food intake were calculated by subtracting the food amount of measurement [−2] from each result.

Immunohistochemistry

The protocol of immunohistochemistry was previously described (Jarvie *et al.* 2021). One group comprised six mice, and three slides were taken from one mouse. Brain samples were taken from the mice at 90 min after intraperitoneal injection of PBS, liraglutide or the lipidated GLP-1 peptides (peptides A and B). Wild-type mice were anesthetized and then cardiac-perfused with PBS, followed by 4% paraformaldehyde (PFA) (FUJIFILM Wako Shibayagi Corporation, Japan). The brain samples were fixed in 4% PFA at 4°C for 48 h and coronally dissected into sections including the ARC by ASI rodent brain matrix (adult mouse, 30 g, coronal) according to the Allen Mouse Brain Atlas. The dissections were then equilibrated in 10, 15 and 20% (w/v) sucrose buffer at 4°C for 12 h, respectively. Finally, the samples were placed into a tissue mold covered with cryoembedding media (OCT) and stored at −80°C before use. Tissue sample (20 µm) frozen sections from around the hypothalamus were imaged using Leica CM1900. The samples then were washed and blocked in PBST (PBS +0.3% v/v Triton X, 5% w/v BSA) and stained by anti-c-Fos antibody 1:2,000 (host: rabbit; No. ab190289: Abcam, USA) as the primary antibody overnight at 4°C. The samples were then washed three times with PBS and stained with Alexa Fluor 488 goat anti-rabbit IgG [H+L] secondary antibody 1:500 (no. A11034: Invitrogen, USA) for 3 h. The samples were then washed three times and counterstained with DAPI 1:100 (No. 340-07971: Dojindo, Japan) for 30 min. Finally, the slides were washed and covered with a coverslip. Images were taken using a fluorescence microscope Keyence BZ-9000 (Keyence, Japan).

Long-term high-fat diet (HFD) tolerance tests

Six-week-old wild-type mice were fed HFD (60% of fat and energy density of 5.24 kcal/g) (Cat# D12492; Research Diets Inc., USA) for ten weeks. PBS, liraglutide (250 µg/kg body weight) or the lipidated GLP-1 peptides (peptides A and B) (250 µg/kg body weight) were intraperitoneally administered twice a day. After body weight was measured every day for six weeks, insulin tolerance tests (ITTs), OGTTs and locomotor activity evaluations were conducted in 12-week-old, 14-week-old and 16-week-old mice, respectively. Locomotor activity was measured using SMART Video Tracking System (Panlab SL, Spain) (Kanemaru *et al.* 2020). Organ (liver) and tissue (white adipose tissue and

skeletal muscle) weight were measured in 16-week-old mice. Epididymal fat, inguinal fat and gastrocnemius muscle were labeled as visceral fat, subcutaneous fat and skeletal muscle, respectively.

ITTs

HFD-fed wild-type mice were fasted for 3 h before the start of the experiment. Blood glucose levels from the tail vein at 0 min (fasting levels), 30, 60, 90 and 120 min were measured by the glucose oxidase method after intraperitoneal administration of human insulin (0.5 U/kg body weight) (Eli Lilly and Co., USA). Results were calculated with blood glucose levels at 0 min being considered 100% (Yasuda *et al.* 2023).

Statistical analysis

The results are shown as mean ± standard error of the mean (SEM). Statistical significance was determined using the unpaired *t* test, Tukey's test and the Steel–Dwass test. Statistical analyses were performed using JMP Pro version 17.0.0 (SAS Institute, USA). *P*-values <0.05 were considered statistically significant.

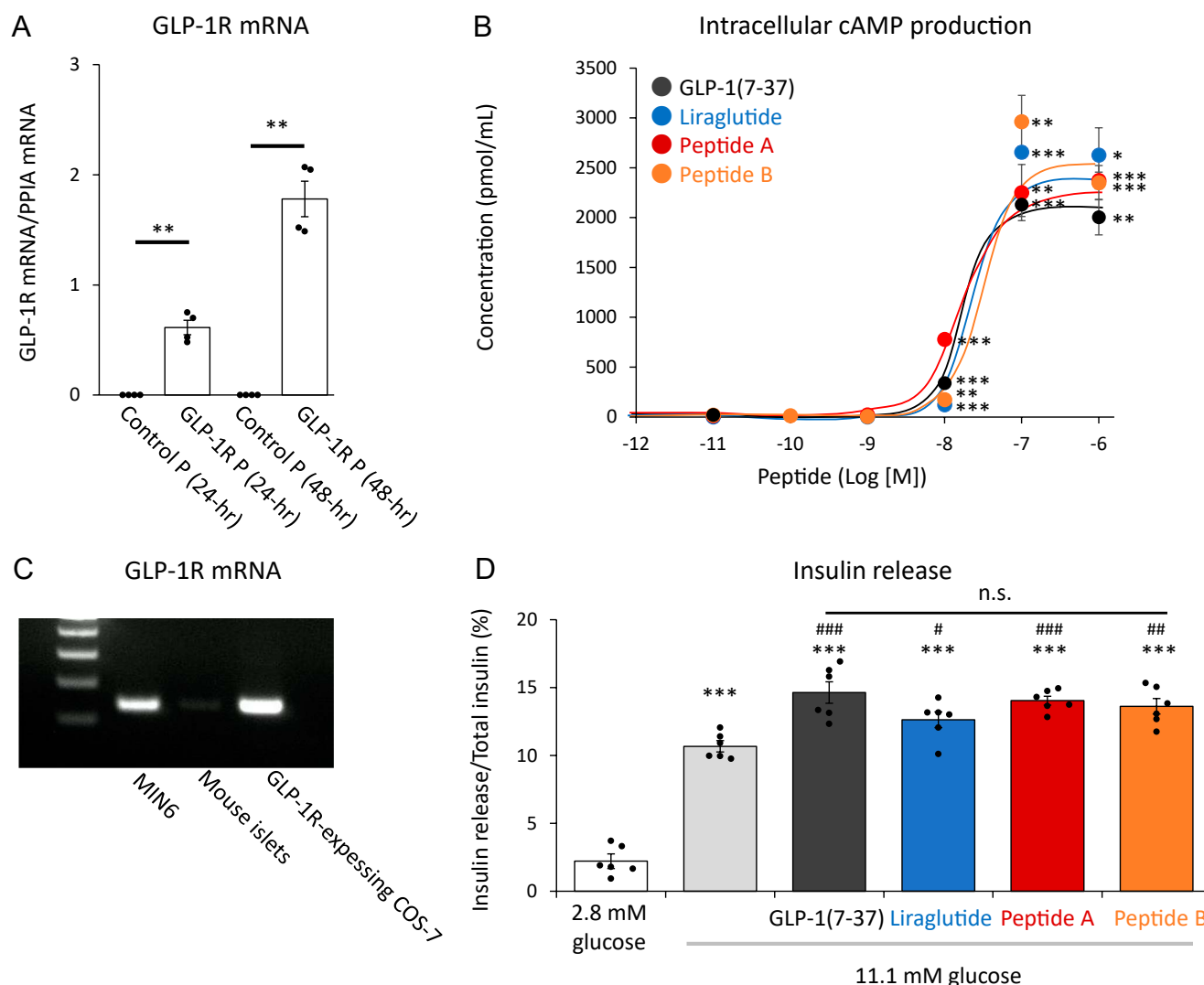
Results

Intracellular cAMP production and insulin secretion induced by the lipidated GLP-1 peptides *in vitro*

After GLP-1R transfection, expression levels of GLP-1R mRNA at 48 h were greater in COS-7 cells than those at 24 h (Fig. 1A). Intracellular cAMP production was measured using COS-7 cells at 48 h after transfection of human GLP-1R. GLP-1(7–37), liraglutide, peptide A and peptide B increased cAMP production in GLP-1R-expressing COS-7 cells similarly (Fig. 1B). Half maximal effective concentration (EC₅₀) (log) values of GLP-1(7–37), liraglutide, peptide A and peptide B were −7.86, −7.77, −7.8 and −7.82, respectively. Insulin secretion from MIN6 cells in which GLP-1R is expressed was then measured (Fig. 1C). Insulin secretion under 11.1 mM glucose condition was significantly higher than that under 2.8 mM glucose (Fig. 1D). GLP-1(7–37), liraglutide, peptide A and peptide B enhanced insulin secretion in the presence of 11.1 mM glucose similarly in all four groups.

Effect of the lipidated GLP-1 peptides on glucose tolerance and insulin secretion *in vivo*

OGTTs were conducted to evaluate the effects of lipidated GLP-1 peptides on insulin secretion *in vivo*. After PBS (control), liraglutide, peptide A or peptide B intraperitoneal injection to normal chow-fed

**Figure 1**

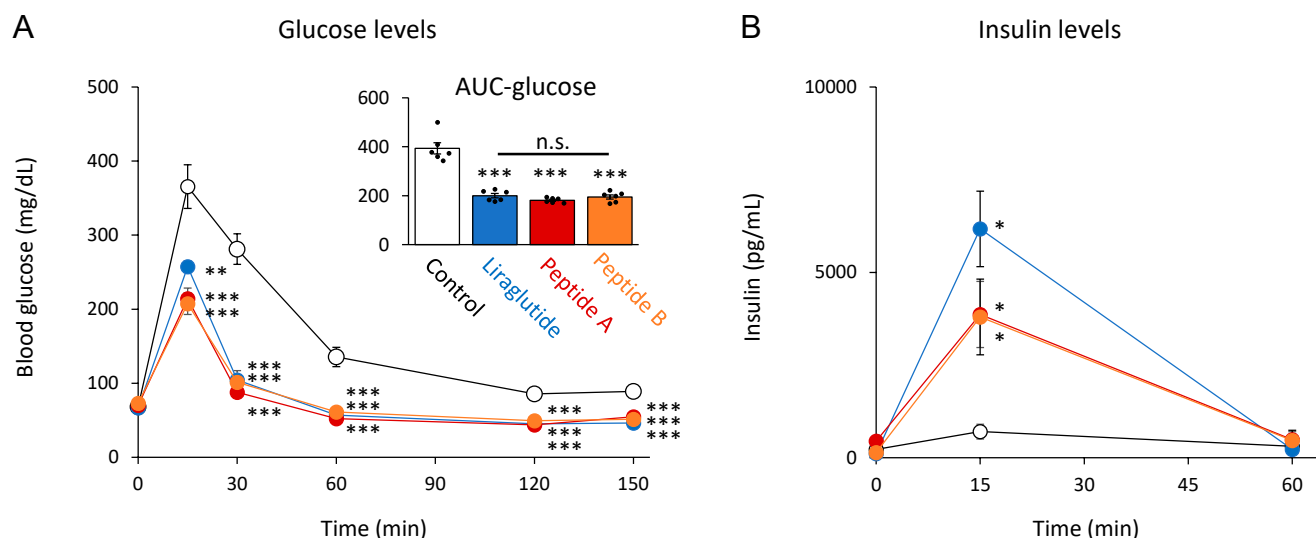
Effect of the lipidated GLP-1 peptides on GLP-1 receptor (GLP-1R) *in vitro*. (A) RT-PCR analysis of GLP-1R mRNA expression in COS-7 cells transfected with human GLP-1R expression plasmid or control plasmid (pCMV-6c) after incubation for 24 h or 48 h ($n = 4$). $^{**}P < 0.01$ vs control. (B) Intracellular cAMP production in GLP-1R-expressing COS-7 cells with GLP-1(7-37), liraglutide, peptide A or peptide B ($n = 4$). The black circles indicate GLP-1(7-37). The blue circles indicate liraglutide. The red circles indicate peptide A. The orange circles indicate peptide B. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs 10^{-12} M. (C) PCR analysis of GLP-1R mRNA expression in MIN6 cells, mouse islets and COS-7 cells transfected with GLP-1R expression plasmids. (D) Insulin release from MIN6 cells with glucose and GLP-1(7-37), liraglutide, peptide A and peptide B ($n = 6$). The white bar indicates 2.8 mM glucose. The light gray bar indicates 11.1 mM glucose. The dark gray bar indicates GLP-1(7-37) in 11.1 mM glucose. The blue bar indicates liraglutide in 11.1 mM glucose. The red bar indicates peptide A in 11.1 mM glucose. The orange bar indicates peptide B in 11.1 mM glucose. $^{***}P < 0.001$ vs 2.8 mM glucose; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs 11.1 mM glucose. n.s., not significant. A full colour version of this figure available at <https://doi.org/10.1530/JOE-24-0026>.

18-week-old mice, glucose was immediately administered (Fig. 2). Liraglutide, peptide A and peptide B significantly decreased blood glucose levels; their glucose-lowering effect continued similarly for 150 min during OGTT (Fig. 2A). The area under the curve (AUC) of the glucose levels did not differ among the liraglutide and the lipidated GLP-1 peptide groups. Insulin levels at 15 min during OGTT were higher in the liraglutide, peptide A and peptide B groups compared with controls (Fig. 2B).

There was no significant difference in insulin levels among the three GLP-1 peptide-treated groups.

Effect of the lipidated GLP-1 peptides on gastrointestinal motility

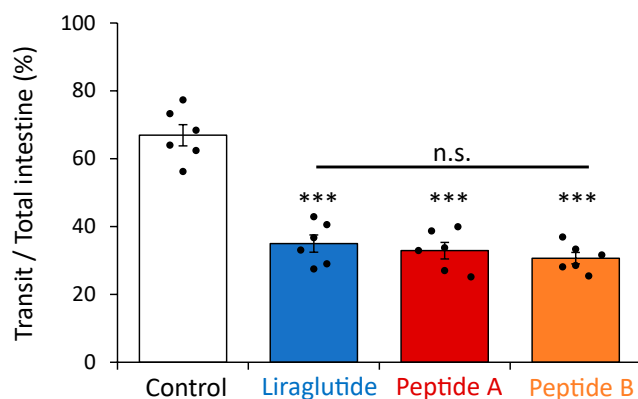
GLP-1R is expressed on sensory nerve endings (Bai *et al.* 2019); GLP-1 peptide and GLP-1 RAs are reported to

**Figure 2**

OGTT after intraperitoneal administration of GLP-1 peptides *in vivo*. (A) Glucose and (B) insulin levels during OGTT ($n = 6$). The white bar and circles indicate control. The blue bar and circles indicate liraglutide. The red bar and circles indicate peptide A. The orange bar and circles indicate peptide B. AUC indicates area under the curve. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control. n.s., not significant. A full colour version of this figure available at

<https://doi.org/10.1530/JOE-24-0026>.

suppress gastric emptying (Meier *et al.* 2003, Krieger 2020). We therefore compared gastrointestinal transit after administration of liraglutide and the lipidated GLP-1 peptides (peptides A and B) (Fig. 3). While liraglutide and the lipidated GLP-1 peptides reduced gastrointestinal transit compared to PBS control, there was no significant difference among the three groups.

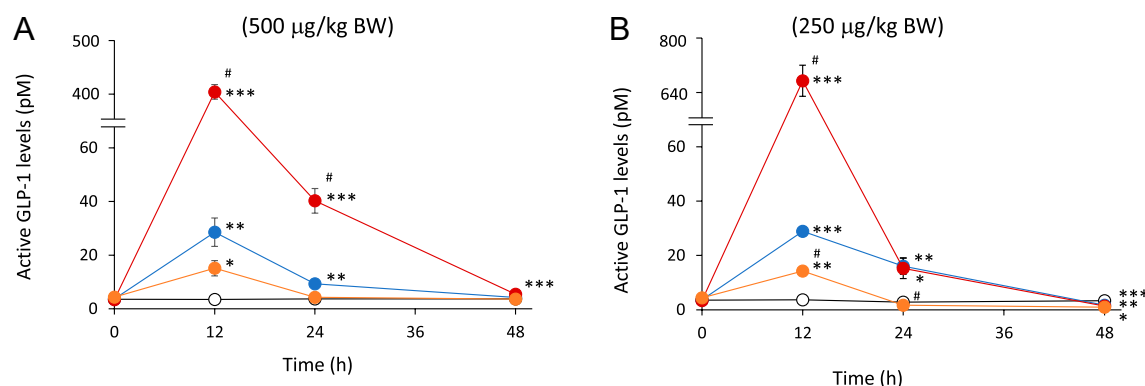
**Figure 3**

Gastrointestinal transit after administration of GLP-1 peptides. Charcoal suspension was orally administered immediately after intraperitoneal administration of PBS (control), liraglutide, peptide A or peptide B. Twenty minutes after the oral gavage, gastrointestinal transit was measured ($n = 6$). The white bar indicates control. The blue bar indicates liraglutide. The red bar indicates peptide A. The orange bar indicates peptide B. *** $P < 0.001$ vs control. n.s., not significant. A full colour version of this figure available at

<https://doi.org/10.1530/JOE-24-0026>.

Plasma active GLP-1 levels after treatment of the lipidated GLP-1 peptides

We are currently unable to evaluate the blood levels of the lipidated GLP-1 peptides precisely. Therefore, immunoreactive active GLP-1 levels in plasma were measured after two doses of the lipidated GLP-1 peptide administration (Fig. 4A and B). Active GLP-1 levels at 12 h after administration were significantly increased in the liraglutide, peptide A and peptide B groups compared to the values before administration. The active GLP-1 level of the peptide A group at 12 h was highest among the three groups. Twenty-four hours after administration, liraglutide and peptide A but not peptide B still increased the active GLP-1 levels in comparison with those before treatment. Active GLP-1 levels of the three peptide-treated groups at 48 h were slightly different from those before administration, but they returned to levels very close to baseline. In addition, to investigate the change of active GLP-1 levels by the frequency of injections, active GLP-1 levels were measured after the administration of each peptide for three days (500 μ g/kg body weight once a day or 250 μ g/kg body weight twice a day). The active GLP-1 levels after treatment once a day were highest at 12 h and returned to levels close to baseline at 48 h, with values similar to those after single injection (Supplemental Fig. 3A). On the other hand, the administration of peptide A and liraglutide twice a day increased the active GLP-1 levels markedly until 72 h after treatment, while the active GLP-1 levels in the peptide B group were close to those at baseline (Supplemental Fig. 3B).

**Figure 4**

Plasma active GLP-1 levels after administration of GLP-1 peptides. Immunoreactive plasma active GLP-1 levels by ELISA were measured after intraperitoneal administration of PBS (control), liraglutide, peptide A or peptide B ($n = 6$). Peptides were administered at (A) 500 µg/kg body weight (BW) and (B) 250 µg/kg BW. The white circles indicate control. The blue circles indicate liraglutide. The red circles indicate peptide A. The orange circles indicate peptide B. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs 0 h. # $P < 0.05$ vs liraglutide group. A full colour version of this figure available at <https://doi.org/10.1530/JOE-24-0026>.

Effect of the lipidated GLP-1 peptides on food intake

GLP-1 RA is reported to reduce food intake (Kanoski *et al.* 2011, Krieger 2020). Because plasma active GLP-1 levels of the peptide B group returned to baseline at 24 h while active GLP-1 levels of the peptide A and liraglutide groups were still higher than baseline (Fig. 4), peptide B might well have a shorter half-life in plasma than peptide A. Based on these results, we administered the peptides to wild-type mice using two dosing regimens to evaluate the effect of the peptides on appetite (Supplemental Fig. 2B and C). While PBS control administration did not affect food intake (Fig. 5A and E), liraglutide, peptide A and peptide B significantly reduced the amount of food intake at the measurement [2] period (Fig. 5B, C, D, F, G, H). Food intake of the liraglutide group (500 µg/kg body weight, once a day (SD), and 250 µg/kg body weight, twice a day (BID)) and peptide A group (500 µg/kg body weight, SD) increased at measurement [4] (Fig. 5B, C, F). Peptide B mildly suppressed food intake at measurement [2] (Fig. 5D and H). Food intake of the liraglutide, peptide A and peptide B groups at measurements [6] and [8] then returned to baseline.

The number of c-Fos-positive cells in the hypothalamus induced by lipidated GLP-1 peptides

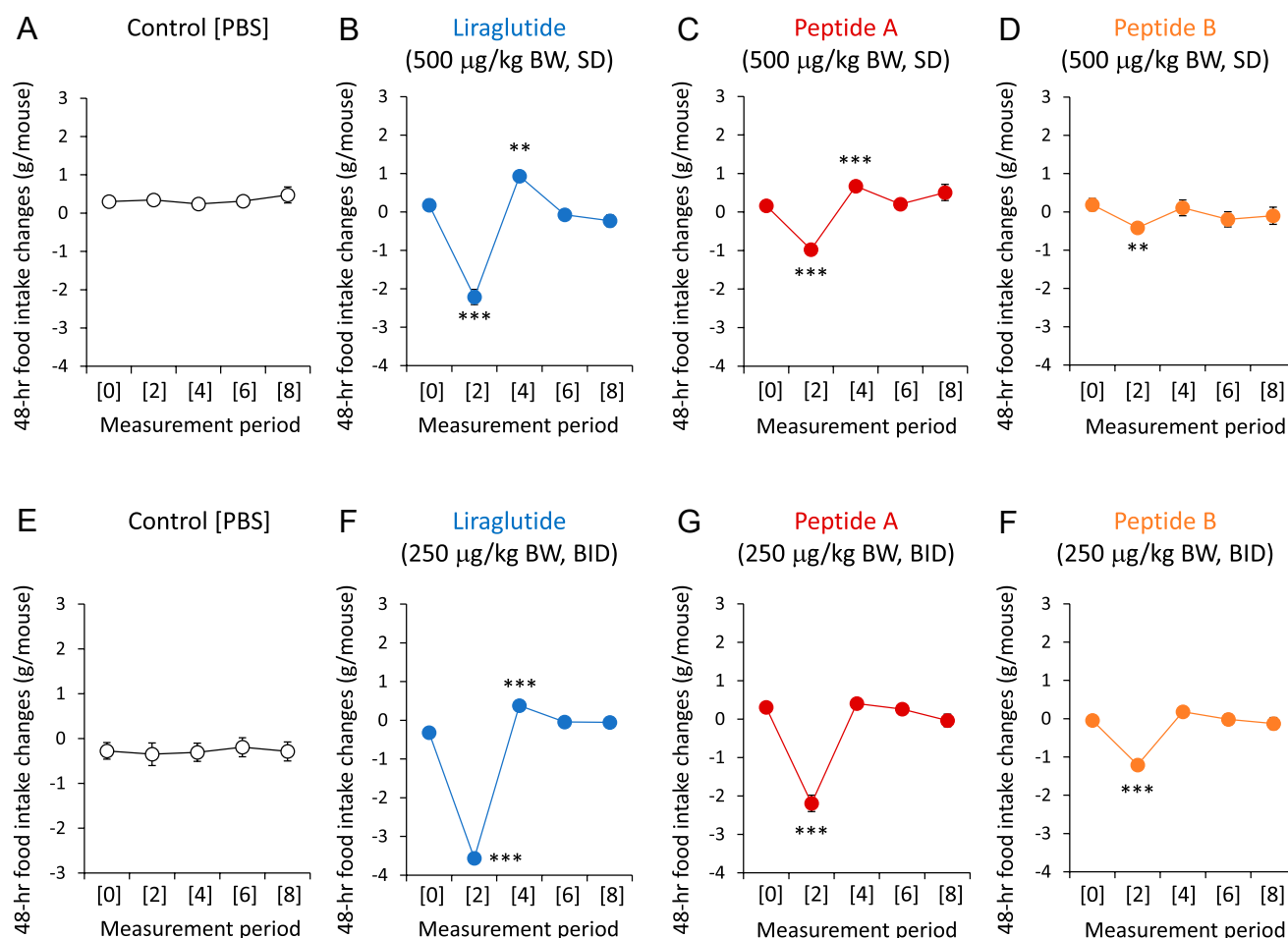
GLP-1R is widely expressed in the hypothalamus (Harada & Inagaki 2022). GLP-1 peptide and GLP-1 RAs directly activate GLP-1R in the hypothalamus to influence food intake (Secher *et al.* 2014, Bai *et al.* 2019, Trapp & Brierley 2022). GLP-1 and GLP-1 RAs are reported to increase transcriptional factor c-Fos, a marker used to assess the functional activity in the brain (Aja *et al.* 2006, Baraboi *et al.* 2011, Parker *et al.* 2013). Therefore, the number of c-Fos-positive cells in ARC, which is important for appetite

regulation, was measured. While c-Fos-positive cells were not observed in the ARC after administration of PBS control (Fig. 6A and B), liraglutide, peptide A and peptide B increased c-Fos-positive cells similarly. Interestingly, the number of c-Fos-positive cells was significantly less in the peptide B group than in the liraglutide and peptide A groups.

Effect of the lipidated GLP-1 peptides on body weight and insulin sensitivity under HFD feeding conditions

In the long-term HFD tolerance tests, each of the GLP-1 peptides was administered to wild-type mice twice a day (250 µg/kg body weight). Body weight was significantly reduced in the liraglutide, peptide A and peptide B groups compared to the control group (Fig. 7A). While body weight was more dramatically suppressed during the first week of liraglutide administration, it gradually increased after the second week; after two weeks, there was no significant difference between the liraglutide and peptide A groups. In addition, body weight was greater in the peptide B group than in the liraglutide and peptide A groups after one-week treatment. Locomotor activity was similar among the four groups (data not shown). ITT data showed that insulin sensitivity was increased similarly in the liraglutide, peptide A and peptide B groups (Fig. 7B).

Liver, visceral fat and subcutaneous fat weight were significantly lower in the liraglutide, peptide A and peptide B groups compared to those in the control group (Fig. 7C), but skeletal muscle weight did not differ among the four groups. Among the three GLP-1 peptide treatment groups, liver, visceral fat and subcutaneous fat weight were significantly greater in the peptide B group than in the liraglutide group, but there was no significant difference between the liraglutide and peptide A groups.

**Figure 5**

Food intake after administration of GLP-1 peptides. Food intake after intraperitoneal administration of lipidated GLP-1 peptides ((A, B, C, D) 500 $\mu\text{g/kg}$ body weight (BW), once a day (SD), and (E, F, G, H) 250 $\mu\text{g/kg}$ BW, twice a day (BID) ($n = 8$)). (A and E) PBS control, (B and F) liraglutide, (C and G) peptide A and (D and H) peptide B. The white circles indicate control. The blue circles indicate liraglutide. The red circles indicate peptide A. The orange circles indicate peptide B. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. [0]. A full colour version of this figure available at <https://doi.org/10.1530/JOE-24-0026>.

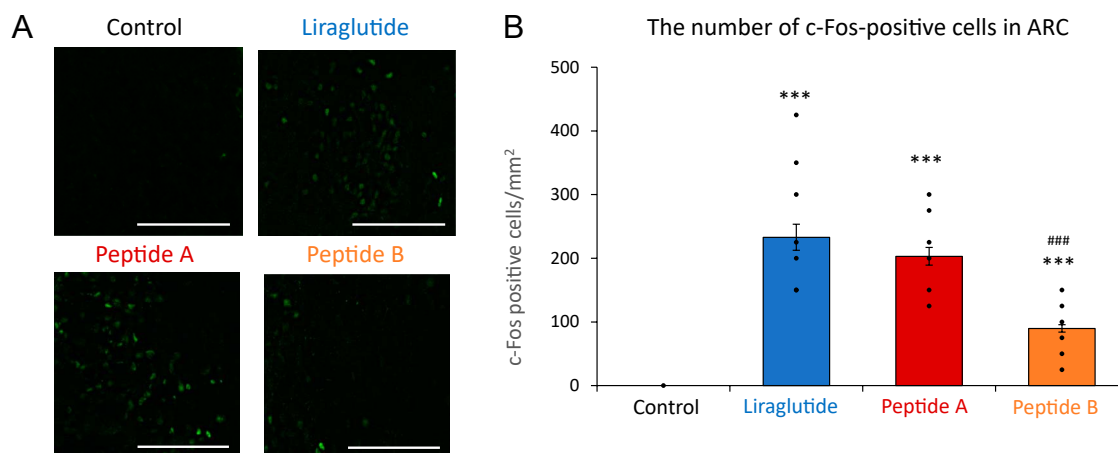
In the OGTTs, glucose and insulin levels were reduced similarly in the liraglutide, peptide A and peptide B groups compared to those in the control group (Fig. 7D and E).

Discussion

We evaluated the efficacy of tryptophan-selective lipidated GLP-1 peptides *in vitro* and *in vivo*. Both lipidated peptides A and B increased cAMP production and insulin release similarly *in vitro*. *In vivo*, the lipidated peptides lowered blood glucose levels, increased insulin levels and suppressed gastrointestinal motility as effectively as liraglutide. On the other hand, peptide A and liraglutide reduced food intake more strongly than peptide B, apparently through better enhancement of

functional activity in the ARC, resulting in greater loss of body weight, fat and liver weight under HFD feeding conditions.

Acylation with fatty acids changes the affinity of lipidated peptides for their receptors. For example, liraglutide and semaglutide are acylated with C16 or C18 fatty diacid, respectively, and their affinity for GLP-1R in the presence of albumin is affected by the fatty acid chain and linker when compared with native GLP-1 (Lau *et al.* 2015). We therefore measured cAMP production and insulin release *in vitro* to evaluate whether tryptophan-selective lipidation changes the affinity of lipidated GLP-1 peptides for GLP-1R. Peptide A and peptide B produced equivalent levels of intracellular cAMP production and insulin release in comparison with human GLP-1(7–37), indicating that tryptophan-selective lipidation of GLP-1 peptide does not affect its affinity for GLP-1R.

**Figure 6**

Immunohistochemical analysis of c-Fos-positive cells in ARC. (A) Immunohistochemical staining by anti-c-Fos antibody in ARC at 90 min after intraperitoneal injection of PBS (control), liraglutide, peptide A or peptide B. (B) The numbers of c-Fos-positive cells ($n = 6$). The white bar indicates control. The blue bar indicates liraglutide. The red bar indicates peptide A. The orange bar indicates peptide B. Scale bar: 100 μm . *** $P < 0.001$ vs control. ### $P < 0.001$ vs liraglutide and peptide A groups.

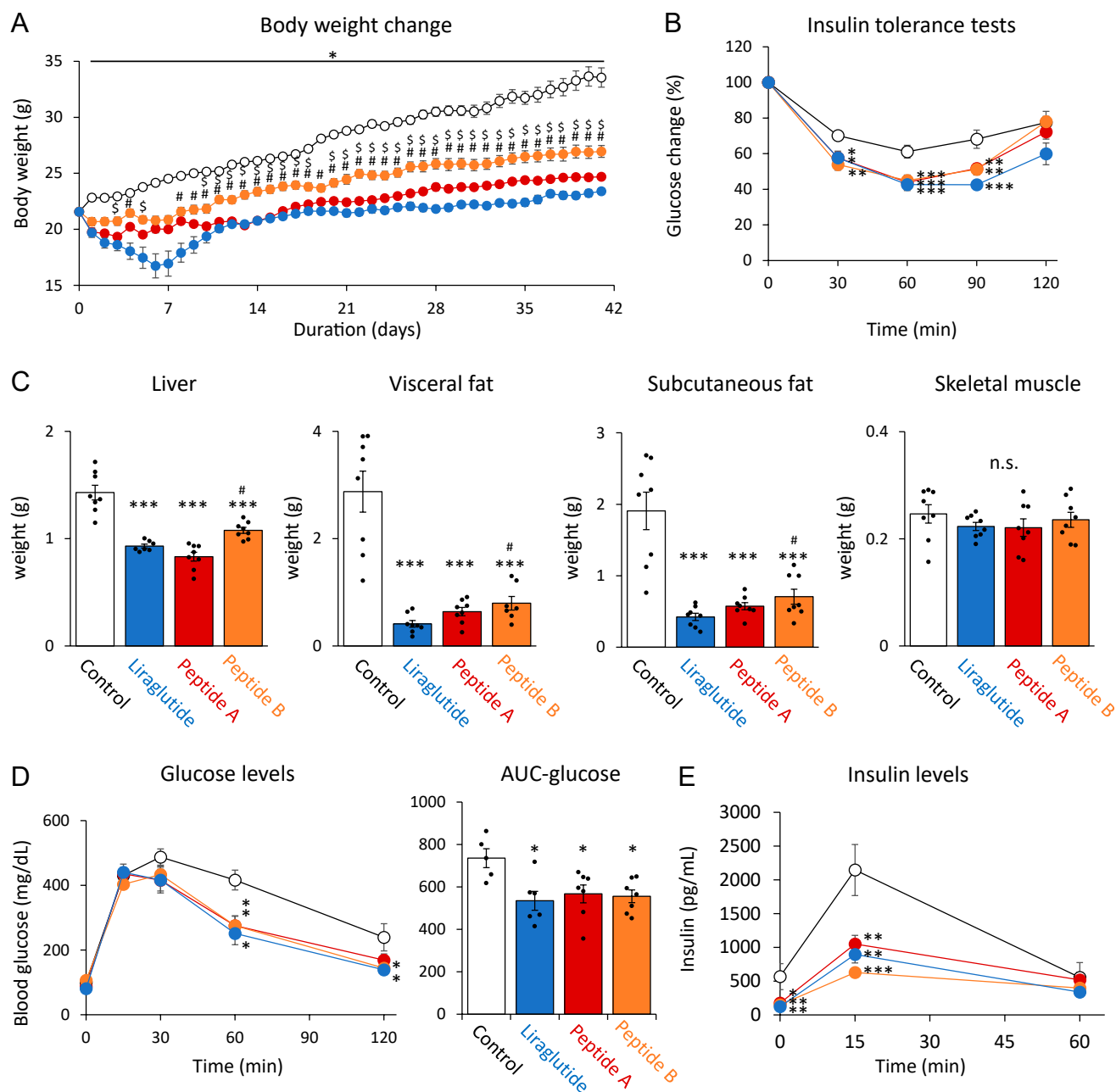
Regarding the effects of lipidated peptides *in vivo*, peptide A was found to activate satiety-promoting neurons in the ARC and thereby decrease food intake and body weight as effectively as liraglutide, while peptide B activated fewer neurons in the ARC than liraglutide and did not as strongly reduce food intake and body weight. In addition, the suppression of food intake after two-day administration of the peptides was transient, and liraglutide and peptide A elicited a rebound effect on food intake four days post-injection. A previous study reported that a single subcutaneous administration of liraglutide significantly reduced the amount of food intake for one day, which returned to close to baseline within four days (Le *et al.* 2023). Because the lipidated peptides were administered intraperitoneally in that study, the anorectic effect might be expected to last only a short time and be eliminated rapidly to result in a rebound effect. On the other hand, in the present study, peptides A and B both enhanced insulin secretion from pancreatic β -cells and inhibited gastric emptying as effectively as liraglutide. Peptide A has one hydrophilic miniPEG linker, while peptide B has three of them. Since the BBB consists mainly of lipids, hydrophilic substances cannot readily pass the barrier (Abbott *et al.* 2010), suggesting that peptide B might produce less postprandial satiation signaling than peptide A or liraglutide.

During the first seven days after administration of the peptides in HFD-fed mice, the body weight of the liraglutide group tended to be low compared to that of the peptide A group, further suggesting that structural differences between liraglutide and peptide A influence the anorectic effect via the central nervous system. Furthermore, the structural difference between semaglutide and liraglutide has been reported to be

involved in their differing distribution in the brain (Gabery *et al.* 2020). It is therefore necessary to elucidate the distribution of liraglutide and peptide A in the brain in future studies.

It is currently not possible to precisely assess the half-life of lipidated peptides in the blood because GLP-1 peptides bind to albumin in plasma. We therefore measured immunoreactive plasma active GLP-1 levels using the active GLP-1 ELISA kit. Active GLP-1 levels of the peptide B group returned to baseline at 24 h, while active GLP-1 levels of the peptide A group remained higher than those at baseline. These results suggest that peptide B may have a shorter half-life in plasma than peptide A. In addition, the miniPEG linker is hydrophilic and reduces affinity to albumin, which might affect measurement in plasma (Jin *et al.* 2015, Lee & Larson 2016). The difference in miniPEG linkers between peptides A and B might be involved in cross-reaction with the active GLP-1 antibodies in the ELISA kit. We therefore evaluated food intake upon administration of each peptide at both twice a day (250 $\mu\text{g/kg}$ body weight) and once a day (500 $\mu\text{g/kg}$ body weight). Liraglutide, peptide A and peptide B were found to have a suppressive effect on food intake. While peptide B suppressed appetite under both methods of administration, twice a day had a stronger appetite-suppressing effect than once a day. In addition, the appetite-suppressing effect of peptide B was found to be weaker than that of peptide A under both administration methods, suggesting that the difference in appetite reduction between peptides A and B might depend on both the half-life of plasma active GLP-1 levels and c-Fos activation in the ARC.

Lipidation of peptides has been applied to antidiabetic agents such as GLP-1 RAs and insulin

**Figure 7**

Phenotype of HFD-fed mice during administration of lipidated GLP-1 peptides. PBS, liraglutide, peptide A or peptide B was intraperitoneally administered (250 μ g/kg body weight, twice a day) to HFD-fed wild-type mice. (A) Body weight changes ($n = 8$). (B) ITTs after six weeks of HFD ($n = 7-8$). (C) Weight of liver, visceral fat, subcutaneous fat and skeletal muscle in the mice after 10 weeks of HFD ($n = 7-8$). (D) Glucose and (E) insulin levels during OGTT ($n = 5-7$). The white bars and circles indicate control. The blue bars and circles indicate liraglutide. The red bars and circles indicate peptide A. The orange bars and circles indicate peptide B. AUC indicates area under the curve. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs liraglutide group. $^{\$}P < 0.05$ vs peptide A. n.s., not significant. A full colour version of this figure available at <https://doi.org/10.1530/JOE-24-0026>.

(Damholt *et al.* 2006, Baggio & Drucker 2007, Heise *et al.* 2012, Marbury *et al.* 2017). We developed effective GLP-1 peptides by lipidation to tryptophan, which is more readily acylated selectively. Our present findings indicate that tryptophan-selective lipidated GLP-1

peptides have comparable effects on GLP-1R to conventional GLP-1 peptides regarding glucose tolerance. We also found that differences in the number of miniPEGs added to the GLP-1 peptide elicit differing effects on appetite and body weight.

While dual GIP/GLP-1 receptor agonists and triple GIP/GLP-1/glucagon receptor agonists are of interest for treatment of diabetes and obesity, our findings demonstrate the novel potential of single GLP-1 receptor agonists. It has been reported that semaglutide reduces body weight more effectively than dulaglutide, suggesting variable degrees of anorectic effect among GLP-1 receptor agonists (Pratley *et al.* 2018). Our method of tryptophan-selective lipidation has the potential to enable clinicians to adjust transmission of GLP-1 peptides into the brain to alter anorectic effects according to the weight loss requirement of the individual patient.

In conclusion, our findings demonstrate that tryptophan-selective lipidated GLP-1 peptides increase cAMP production and insulin release as effectively as human GLP-1(7–37) and liraglutide *in vitro*. Moreover, peptides A and B are shown to lower blood glucose levels, increase insulin levels and suppress gastrointestinal motility as effectively as liraglutide *in vivo*. While the increased number of miniPEG linkers attached to these novel peptides affected activation of satiety-promoting neurons in the ARC and subsequent suppression of food intake, both of them mitigated obesity and insulin resistance under HFD feeding conditions. Thus, our procedure for tryptophan-selective lipidation with readily applied synthetic operations may contribute to drug development to extend the half-life of GLP-1 while also providing new avenues to the control of body weight in people with T2D.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-24-0026>.

Declaration of interest

NH received scholarship grants from Mitsubishi Tanabe Pharma Co., Ltd, Ono Pharmaceutical Co. Ltd, Nippon Boehringer Ingelheim Co. Ltd and Sanofi K.K. NI received joint research grants from Daiichi Sankyo Co., Ltd, Terumo Co., Ltd and Drawbridge, Inc. NI received speaker honoraria from Kowa Pharmaceutical Co., Ltd, MSD, Astellas Pharma Inc., Novo Nordisk Pharma Ltd, Ono Pharmaceutical Co., Ltd, Nippon Boehringer Ingelheim Co., Ltd, Takeda Pharmaceutical Co., Ltd and Mitsubishi Tanabe Pharma Co., Ltd. NI received scholarship grants from Kissei Pharmaceutical Co., Ltd, Sanofi, Daiichi Sankyo Co., Ltd, Mitsubishi Tanabe Pharma Co., Ltd, Takeda Pharmaceutical Co., Ltd, Japan Tobacco Inc., Kyowa Kirin Co., Ltd, Sumitomo Dainippon Pharma Co., Ltd, Astellas Pharma Inc., MSD, Eli Lilly Japan, Ono Pharmaceutical Co. Ltd, Sanwa Kagaku Kenkyusho Co. Ltd, Nippon Boehringer Ingelheim Co., Ltd, Novo Nordisk Pharma Ltd, Novartis Pharma K.K., Teijin Pharma Ltd and LifeScan Japan Inc. All other authors have nothing to disclose.

Funding

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Japan Society for the Promotion of Science (JSPS) [Grant Nos. 22K08668, 21J13647 and 20H03731], the Ministry of Health, Labour and Welfare, the Ministry of Agriculture, Forestry and Fisheries, Japan Diabetes Foundation and Japan Association for Diabetes Education and Care.

Author contribution statement

XL planned the study and researched data. NH planned the study, researched the data, contributed to discussion and wrote, reviewed and edited the manuscript. TY researched the data and wrote and edited the manuscript. EI-O, DK, MD, YS and SY researched the data. AO contributed to discussion and edited the manuscript. NI planned the study, contributed to discussion and edited the manuscript. All authors approved the final version of the manuscript.

Acknowledgments

We thank Dr Junji Fujikura for technical suggestions to construct human GLP-1R pCMV-6c vector.

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