Trypsin-Treated β-Lactoglobulin Improves Glucose Tolerance in C57BL/6 Mice by Enhancing AMPK Activation and Glucose Uptake in Hepatocytes

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It was reported that trypsin-treated β-lactoglobulin (β-LG) had a glucose-lowering effect in the oral glucose tolerance test (OGTT) in mice and a dipeptidyl peptidase-4 (DPP-4) inhibition activity in vitro. However, whether trypsin-treated β-LG improves glucose tolerance by inhibiting DPP-4 in vivo has not yet been examined, and the mechanism of the glucose-lowering effect of trypsin-treated β-LG is thus unclear. Here we investigated the detailed mechanism underlying the glucose tolerance effect of trypsin-treated β-LG. The oral administration of trypsin-treated β-LG significantly decreased the blood glucose concentrations in both the OGTT and an intraperitoneal glucose tolerance test (IPGTT). However, trypsin-treated β-LG did not increase the insulin secretion after glucose loading. Trypsin-treated β-LG potently increased the level of phosphorylated AMP-activated protein kinase (AMPK) in human hepatocellular carcinoma (HepG2) cells and in mouse hepatocytes. Moreover, trypsin-treated β-LG significantly enhanced glucose uptake into the HepG2 cells. These results indicate that trypsin-treated β-LG decreases blood glucose levels after glucose loading by upregulating AMPK activation and glucose uptake in the liver, which could contribute to the reduction of postprandial hyperglycemia.

Key words β-lactoglobulin; glucose tolerance test; AMP-activated protein kinase; glucose uptake; mouse

The metabolic disorder diabetes mellitus is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It was estimated that worldwide in 2013, 382 million people had diabetes, and this number is expected to rise to 592 million by 2035. To prevent diabetes and its complications, it is important to control the blood glucose level as close as possible to normal.

Many types of drugs have been used to treat individuals with diabetes. For example, in several clinical studies, the antihyperglycemic drug sitagliptin has shown potential for decreasing blood glucose concentrations by inhibiting dipeptidyl peptidase-4 (DPP-4). DPP-4 is an enzyme that is responsible for the degradation of incretins such as glucagon-like peptide 1 (GLP-1). DPP-4 inhibition prevents the inactivation of GLP-1, which increases the levels of active GLP-1. This increases insulin secretion, thereby lowering glucose levels. The diabetes medication metformin has a glucose-lowering effect by upregulating glucose uptake and suppressing gluconeogenesis via the activation of the enzyme AMP-activated protein kinase (AMPK). AMPK plays a key role in the regulation of energy metabolism, which is activated by the phosphorylation of its Thr172 residue. It was reported that the activation of AMPK induces an upregulation of glucose uptake by glucose transporter-2 (GLUT2) and GLUT4 and a suppression of hepatic glucose production. However, it was shown that the continuous use of the above-described drugs for the prevention of diabetes induces side effects. Sitagliptin’s side effects include nausea, common cold-like symptoms and pancreatitis, and metformin’s side effects include nausea, vomiting and diarrhea.

Milk products are known to decrease the risk of diabetes mellitus and helped reduce the risk of diabetes and cardiovascular disease. Whey protein, which accounts for 20% of whole milk protein, has also been shown to reduce postprandial hyperglycemia in diabetic patients, and whey protein consumption was proposed as a novel approach for enhancing glucose-lowering strategies in diabetes. In addition to the activity of intact whey protein, peptides from whey protein exhibit further physiological functions. It was demonstrated that trypsin-treated β-lactoglobulin (β-LG), the major protein component of milk whey protein, had a glucose-lowering effect in an oral glucose tolerance test (OGTT) in mice and DPP-4 inhibition activity in vitro. It has not been determined whether trypsin-treated β-LG improves glucose tolerance by inhibiting DPP-4 in vivo. The mechanism of the glucose-lowering effect of trypsin-treated β-LG is thus unclear.

In the present study, we investigated the effect of trypsin-treated β-LG on glucose tolerance by performing an OGTT and an intraperitoneal glucose tolerance test (IPGTT) in mice. To investigate the detailed mechanism of the glucose-lowering effect of trypsin-treated β-LG, we further evaluated the effects of trypsin-treated β-LG on insulin secretion, AMPK activation, and glucose uptake in vivo and in vitro.

MATERIALS AND METHODS

Preparation of Trypsin-Treated β-LG β-LG (Sigma-Aldrich, St. Louis, MO, U.S.A.) dissolved in 100 mM phosphate buffer (pH 8.0) at a concentration of 1 g/20 mL was digested by adding 10 mg of trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h at 37°C. Digestion was stopped by heating for 5 min at 90°C. The reaction mixture was lyophilized.

Reagents Metformin was purchased from Dainippon

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Sumitomo Pharma (Osaka, Japan). Sitagliptin was purchased from MSD (Tokyo). Primary antibodies against AMPKα and p-AMPKα (Thr172) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Glucose was obtained from Wako Pure Chemical Industries, Ltd.

**Animal Experiments** Six-week-old male C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan) and housed in plastic cages under controlled temperature (22±1°C), humidity (55±15%), and 12-h light/dark cycle for the week prior to the commencement of the experiments. All animal experiments were performed in accord with the Meiji Co., Ltd. Guidelines for the Care and Use of Laboratory Animals.

**OGTT and IPGTT** Mice were fasted for 18 h before the experiment. Trypsin-treated β-LG was dissolved in distilled water and orally administered to the mice at a dose of 1 g/kg body weight at 30 min before the glucose tolerance test. Metformin or sitagliptin dissolved in distilled water was orally administered at a dose of 100 or 3 mg/kg body weight 60 min before the glucose tolerance test. The control mice were orally administered distilled water 30 min before the glucose tolerance test. The administration volume was 10 mL/kg body weight. Glucose was orally or intraperitoneally administered at a dose of 5 or 2 g/kg body weight. The blood glucose concentration was measured with BREEZE® 2 blood glucose meter (Bayer Yakuhin, Osaka, Japan) from a tail vein before and at 30, 60, 90, 120 min after glucose loading. The area under the curve (AUC) of the blood glucose concentrations during the glucose tolerance test was calculated.

**Measurement of Insulin Secretion** Mice were fasted for 18 h before the experiment. Trypsin-treated β-LG was dissolved in distilled water and orally administered at a dose of 1 g/kg body weight at 30 min before the glucose loading. Sitagliptin dissolved in distilled water was orally administered 60 min before the glucose tolerance test at a dose of 3 mg/kg body weight. The control mice were orally administered distilled water 30 min before the glucose loading. The administration volume was 10 mL/kg body weight. Glucose was administered orally at a dose of 5 g/kg body weight. At 20 min after the glucose loading, the mice were anesthetized with isoflurane and blood was collected by cardiac puncture. The blood was centrifuged for 15 min at 12000 rpm. Plasma insulin concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

**Preparation of Liver Samples** Mice were fasted for 18 h before the preparation of liver samples. Trypsin-treated β-LG dissolved in distilled water was orally administered at a dose of 1 g/kg body weight. The control mice were orally administered distilled water. The administration volume was 10 mL/kg body weight. At 30 min after the administration, the mice were sacrificed by cardiac puncture under isoflurane anesthesia. Their livers were collected and homogenized by sonication in lysis buffer and then centrifuged for 15 min at 15000 rpm. The supernatants were collected as liver extracts and frozen at −80°C. The protein concentration in each sample was determined using Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blot Analysis** Proteins in liver extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.) for Western blotting. The membrane was incubated at 4°C overnight with primary antibodies against AMPK-α or p-AMPK-α. After incubation, the membrane was incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody. The blots were visualized using enhanced chemiluminescence substrate (Wako Pure Chemical Industries, Ltd.) and imaged using a ChemiDoc XRS+ imaging system (Bio-Rad).

**Cell Culture** Human hepatocellular carcinoma (HepG2) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Cells were maintained in a 5% CO₂ incubator at 37°C. During subculture, cells were detached by trypsinization when they reached 80% confluency. The well-grown cells were harvested and seeded in plates for experiments.

**Cell-Based Enzyme Immunoassay** HepG2 cells were seeded in collagen-coated 96-well plates at a density of 3.5×10⁴ cells/well and incubated for 24 h. The medium was then replaced with glucose- and serum-free DMEM (control), glucose- and serum-free DMEM containing trypsin-treated β-LG (10 mg/mL) or glucose- and serum-free DMEM containing metformin (2 mM). After incubation for 3 h, the levels of AMPK phosphorylation were determined in the cells using an AMPK Phosphorylation Assay Kit (BioAssay Systems, Hayward, CA, U.S.A.).

**Measurement of Glucose Uptake** HepG2 cells were seeded in collagen-coated 96-well plates at a density of 3.5×10⁴ cells/well and incubated for 24 h. The medium was then replaced with glucose- and serum-free DMEM containing the fluorescent glucose analog 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)aminol]-D-glucose (2-NBDG, 10 µM) (control) or glucose- and serum-free DMEM containing trypsin-treated β-LG (10 mg/mL) and 2-NBDG (10 µM). After incubation for 2 h, the levels of glucose uptake were determined in the cells with the use of a Glucose Uptake Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, U.S.A.).

**Statistical Analysis** All data are expressed as mean±standard error of the mean (S.E.M.). Student’s t-test was used for the comparisons of pairs of groups. Dunnett’s test was used for the comparisons of multiple numbers of groups. Differences with p-values <0.05 (*) or p<0.01 (**) were considered significant.

**RESULTS**

**Effect of Trypsin-Treated β-LG on Blood Glucose Concentrations Shown by OGTTs in Mice** In the OGTTs, trypsin-treated β-LG significantly decreased the blood glucose concentrations at 30 and 60 min after the oral glucose administration (285.6±13.5 and 229.4±13.3 mg/dL) compared to the control values (365.4±22.4 and 322.2±31.8 mg/dL) (Fig. 1A), and lowered the AUC (21869±789 mg/dL.min) compared to the control (27951±2119 mg/dL.min) (Fig. 1B). Metformin and sitagliptin, the positive controls, also decreased the blood glucose levels at 30 and 60 min after the oral glucose administration (metformin; 222.0±8.6 and 219.9±8.0 mg/dL, sitagliptin; 262.5±16.6 and 190.7±10.1 mg/dL) and AUCs (metformin; 19758±623 mg/dL.min, sitagliptin; 19601±876 mg/dL.min) compared to the controls.

**Effect of Trypsin-Treated β-LG on Blood Glucose Con-
In the IPGTTs, trypsin-treated β-LG significantly decreased the blood glucose concentrations at 30 and 60 min after the intraperitoneal glucose administration (357.5 ± 15.2 and 262.1 ± 20.1 mg/dL) compared to the control (424.3 ± 13.0 and 330.1 ± 14.4 mg/dL) (Fig. 1C), and it lowered AUCs (25913 ± 1406 mg/dL min) compared to the control (31032 ± 1102 mg/dL min) (Fig. 1D). Metformin and sitagliptin also decreased the blood glucose concentrations at 30 and 60 min after the intraperitoneal glucose administration (metformin; 355.6 ± 14.4 and 251.7 ± 10.5 mg/dL, sitagliptin; 366.4 ± 13.3 and 251.2 ± 17.2 mg/dL) compared to the control, and also lowered the AUCs (metformin; 24864 ± 830 mg/dL min, sitagliptin; 26343 ± 1247 mg/dL min) compared to the control.

Effect of Trypsin-Treated β-LG on AMPK Phosphorylation in Vitro and in Vivo

The results of the levels of AMPK phosphorylation in vitro are shown in Fig. 3. Trypsin-treated β-LG significantly increased the level of phosphorylated AMPK in the HepG2 cells compared to the control, and metformin also increased AMPK phosphorylation. The results of the levels of AMPK phosphorylation in vivo are shown in Fig. 4. Trypsin-treated β-LG potently enhanced the level of phosphorylated AMPK in mouse hepatocytes compared to the control at 30 min after administration.

Effect of Trypsin-Treated β-LG on Glucose Uptake in Vitro

Trypsin-treated β-LG significantly increased the glucose uptake into the HepG2 cells compared to the control (Fig. 5).

DISCUSSION

It was reported that trypsin-treated β-LG decreased blood glucose concentrations in an OGTT (10 g/kg body weight) in mice. In accordance with that report, our present findings confirmed that trypsin-treated β-LG significantly decreased the levels of blood glucose in an OGTT (5 g/kg body weight).
Our results also showed that trypsin-treated β-LG had a glucose-lowering effect in the IPGTT. Higuchi et al. reported that the oral administration of meat hydrolysate (2 g/kg body weight) had no effect on the levels of blood glucose in an IPGTT, which suggested that not all proteins or peptides had a glucose-lowering effect. We speculate that trypsin-treated β-LG has a characteristic effect on the blood glucose levels in glucose tolerance tests. In an IPGTT, glucose is absorbed from the peritoneal cavity, and thus, the blood glucose levels are not affected by the gastric emptying and the glucose absorption from the intestine. Our above-mentioned IPGTT finding therefore indicates that the gastric emptying and the glucose absorption from the intestine were not associated with the main mechanism of the glucose-lowering effect of trypsin-treated β-LG.

DPP-4 inhibition prevents the inactivation of GLP-1, which increases the level of active GLP-1. This increases insulin secretion, thereby lowering the glucose level. Sitagliptin, a DPP-4 inhibitor drug, decreased the levels of blood glucose in OGTT and IPGTT in the present study. It has been reported that trypsin-treated β-LG had DPP-4 inhibition activity in vivo. It has thus been speculated that the glucose-lowering effect of trypsin-treated β-LG is caused by the inhibition of DPP-4.

In this study, we investigated whether trypsin-treated β-LG increased insulin secretion after glucose loading by exerting DPP-4 inhibitory effect. In an OGTT, the plasma insulin concentration sharply increases at approximately 15–30 min after glucose loading, and then decreases immediately until
60 min after glucose loading. In an OGTT in the present study, trypsin-treated β-LG decreased the blood glucose levels at 30 min after glucose loading. We evaluated insulin concentrations at 20 min after glucose loading because it was appropriate to investigate whether the glucose-lowering effect of trypsin-treated β-LG is caused by the increase of insulin secretion via an inhibition of DPP-4. Our results demonstrated that trypsin-treated β-LG did not increase insulin secretion, whereas sitagliptin significantly increased it. This suggests that the DPP-4 inhibitory activity of trypsin-treated β-LG in vitro was not reflected in insulin secretion in vivo, and that DPP-4 inhibition was not the main mechanism of the glucose-lowering effect of trypsin-treated β-LG. Although the reason why trypsin-treated β-LG did not increase insulin secretion in vivo is not clear, it might be because peptides that have DPP-4 inhibitory activity in trypsin-treated β-LG are inactivated by luminal digestion. Although it has been demonstrated that peptides from β-LG such as histidine (His)-alanine (Ala)-glutamic acid (Glu)-glycine (Gly)-threonine (Thr)-phenylalanine (Phe) and isoleucine (Ile)-proline (Pro)-Ala-valine (Val)-Phe have potent DPP-4 inhibitory activity in vitro, it is not yet known whether these peptides are actually absorbed without digestion in vivo. Further analyses regarding this issue are needed.

Another possibility is that the intensity of DPP-4 inhibitory activity is not sufficient to increase insulin secretion. It was reported that whyte protein administration increased insulin secretion by inhibiting DPP-4. That study demonstrated that the luminal digestion of whey protein generated small fragments (di- and tripeptides) that are substrates for DPP-4 and act as competitive inhibitors, resulting in increased insulin secretion. In that study, mice were administered approximately four times as much whey protein as was used in our present study. These data suggest that a high-dose administration of trypsin-treated β-LG might increase insulin secretion by augmenting the DPP-4 inhibitory effect.

AMPK plays a key role in the regulation of energy metabolism, which is activated by the phosphorylation of its Thr172 residue. Here we observed that the diabetes mellitus drug metformin, which activates AMPK in hepatocytes and muscles, actually decreased the levels of blood glucose in the OGTT and IPGTT. We then determined the effect of trypsin-treated β-LG on AMPK activation. The results showed that the 3-h treatment of trypsin-treated β-LG potently increased the levels of phosphorylated AMPK in HepG2 cells, and AMPK phosphorylation was also enhanced in mouse hepatocytes at 30 min after the administration of trypsin-treated β-LG. These results indicated that trypsin-treated β-LG enhanced the AMPK activity in the hepatocytes. It was reported that the activation of AMPK induces a suppression of hepatic glucose production. A glucose-lowering effect of trypsin-treated β-LG could be attributed to its ability to activate AMPK.

We next examined the effect of trypsin-treated β-LG on glucose uptake, and the results demonstrated that trypsin-treated β-LG significantly elevated the glucose uptake into the HepG2 cells, indicating that trypsin-treated β-LG suppressed the glucose levels by upregulating glucose uptake in hepatocytes, which could contribute to the reduction of postprandial hyperglycemia. It was reported that the activation of AMPK results in an upregulation of the glucose uptake by GLUT2, which is expressed mainly in the liver. Trypsin-treated β-LG may upregulate the glucose uptake by GLUT2 via AMPK activation. The phosphoinositide 3-kinase (PI3K)/Akt pathway is also known to regulate the glucose metabolism in the liver, but we observed that trypsin-treated β-LG had no effect on Akt phosphorylation (data not shown). The PI3K/Akt pathway may not be related to the glucose-lowering effect of trypsin-treated β-LG. It is known that β-LG constitutes more than half of milk whey protein, which accounts for 20% of whole milk protein, and several studies showed that whey protein or whey peptide consumption had no side effects. This indicates that trypsin-treated β-LG could be safe to consume, unlike drugs such as metformin, which has side effects include nausea, vomiting and diarrhea. Trypsin-treated β-LG may thus provide a valuable contribution to the reduction of postprandial hyperglycemia.

Bovine milk whey protein hydrolysate was reported to increase the AMPK activity in rat skeletal muscles, and we therefore speculated that trypsin-treated β-LG, the major whey protein, might have an upregulating effect on AMPK activity in skeletal muscles. Here we evaluated AMPK activity in hepatocytes, and tissues including muscles were not tested. Further studies are required to elucidate the site of action of trypsin-treated β-LG.

Trypsin is known to cleave peptide chains at the carboxyl side of the amino acids lysine or arginine, and the amino acid sequence of β-LG is clear. Peptides in trypsin-treated β-LG are apparent. In this study, the orally administered trypsin-treated β-LG may be little digested by peptic in the stomach because the gastric emptying of trypsin-treated β-LG is considered to be rapid in overnight fasting conditions. On the other hand, trypsin-treated β-LG might be digested by enzymes such as chymotrypsin in the intestine, and various additional peptides may be produced from trypsin-treated β-LG when it is absorbed from the intestine. Investigations of the types of absorbed peptides and evaluations of the effects of the peptides on AMPK should be performed to further clarify the effect of trypsin-treated β-LG.

In conclusion, our study demonstrated that trypsin-treated β-LG improved glucose tolerance in C57BL/6 mice by enhancing AMPK activation and glucose uptake in hepatocytes. These results suggest that the oral intake of trypsin-treated β-LG may attenuate postprandial hyperglycemia and reduce the risk of diabetes.

Conflict of Interest All authors are employees of Meiji Co., Ltd.

REFERENCES

1) American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care, 37 (Suppl. 1), S81–S90 (2014).
2) Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2030. Diabetes Res. Clin. Pract., 103, 137–149 (2014).
3) Raz I, Hanefeld M, Xu L, Caria C, Williams-Herman D, Khatami H. Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy in patients with type 2 diabetes mellitus. Diabetologia, 49, 2564–2571 (2006).
4) Hermansen K, Kjienes M, Luo E, Fanurik D, Khatahi H, Stein P. Efficacy and safety of the dipeptidyl peptidase-4 inhibitor, sitagliptin, in patients with type 2 diabetes mellitus inadequately...
controlled on glimepiride alone or on glimepiride and metformin. Diabetes Obes. Metab., 9, 733–745 (2007).

5) Goldstein BJ, Feinglos MN, Lunceford JK, Johnson J, Williams-Herman DE. Effect of initial combination therapy with sitagliptin, a dipeptidyl-peptidase-4 inhibitor, and metformin on glycemic control in patients with type 2 diabetes. Diabetes Care, 30, 1979–1987 (2007).

6) Thornber NA, Gallwitz B. Mechanism of action of inhibitors of dipeptidyl-peptidase-4 (DPP-4). Best Pract. Res. Clin. Endocrinol. Metab., 23, 479–486 (2009).

7) Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljungqvist O, Efendic S, Moller DE, Thorell A, Goodyear LJ. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes, 51, 2074–2081 (2002).

8) Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventire J, Doebber T, Fuji N, Misi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest., 108, 1167–1174 (2001).

9) Stumvoll M, Nurjan N, Perrello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N. Engl. J. Med., 333, 550–554 (1995).

10) Walker J, Jupin HB, Diaz H, Salehi P, Churchill T, Madsen KL. 5-Aminimidazole-4-carboxamide riboside (AICAR) enhances GLUT2-dependent jejunal glucose transport: A possible role for AMPK. Biochem. J., 385, 485–491 (2005).

11) Russell RR 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am. J. Physiol. Heart Circ. Physiol., 277, H643–H649 (1999).

12) Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandra, 2002–2009.

13) Aschner P, Kipnes MS, Lunceford JK, Sanchez M, Mickel C, Williams-Herman DE. Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with type 2 diabetes. Diabetes, 29, 2632–2637 (2009).

14) Olansky L. Do incretin-based therapies cause acute pancreatitis? J. Diabetes Sci. Technol., 4, 228–229 (2010).

15) Bouchoucha M, Uzzan B, Cohen R. Metformin and digestive disorders. Diabetes Metab., 37, 90–96 (2011).

16) Pereira MA, Jacobs DR Jr, Van Horn L, Slattery ML, Kartashov AI, Ludwig DS. Dairy consumption obesity, and the insulin resistance syndrome in young adults: the CARDIA study. JAMA, 287, 2081–2089 (2002).

17) Choi HK, Willett WC, Stampfer MJ, Rimm E, Hu FB. Dairy consumption and risk of type 2 diabetes mellitus in men: a prospective study. Arch. Intern. Med., 165, 997–1003 (2005).

18) Jakubowicz D, Froy O, Ahrén B, Boaz M, Landau Z, Bar-Dayan Y, Ganz T, Barnea M, Wainstein J, Incretin, insulinotropic and glucose-lowering effects of whey protein pre-load in type 2 diabetes: a randomised clinical trial. Diabetologia, 57, 1807–1811 (2014).

19) Uchida M, Ohshiba Y, Mogami O. Novel dipeptidyl peptidase-4-inhibiting peptide derived from β-lactoglobulin. J. Pharmacol. Sci., 117, 63–66 (2011).

20) Higuchi N, Hira T, Yamada N, Hara H. Oral administration of corn zein hydrolysate stimulates GLP-1 and GIP secretion and improves glucose tolerance in male normal rats and Goto–Kakizaki rats. Endocrinology, 154, 3089–3098 (2013).

21) Silveira SI, Martinez-Maqueda D, Recio I, Hernandez-Ledesma B. Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. Food Chem., 141, 1072–1077 (2013).

22) Gunnarsson PT, Winzell MS, Deacon CF, Larsen MO, Jelic K, Carr RD, Ahren B. Glucose-induced incretin hormone release and inactivation are differentially modulated by oral fat and protein in mice. Endocrinology, 147, 3173–3180 (2006).

23) Karim S, Adams DH, Lalar PF. Hepatic expression and cellular distribution of the glucose transporter family. World J. Gastroenterol., 18, 6771–6781 (2012).

24) Taniguchi CM, Kondo T, Sajan M, Luo J, Bronson R, Asano T, Fares R, Cantley LC, Kahn CR. Divergent regulation of hepatic glucose and lipid metabolism by phosphoinositide 3-kinase via Akt and PKCα/C. Cell Metab., 3, 343–353 (2006).

25) Bortolotti M, Maiolo E, Corazza M, Van Dike E, Schneider P, Boss A, Carrel G, Giusti V, Le K-A, Chong DG. Effects of a whey protein supplementation on intrahepatocellular lipids in obese female patients. Clin. Nutr., 30, 494–498 (2011).

26) Chalé A, Cloutier GJ, Hau C, Phillips EM, Dallal GE, Fielding RA. Efficacy of whey protein supplementation on resistance exercise—induced changes in lean mass, muscle strength, and physical function in mobility-limited older adults. J. Gerontol. A Biol. Sci. Med. Sci., 67, 269–274 (2012).

27) Kerksick CM, Rasmussen CJ, Lancaster SL, Magu B, Smith P, Melton C, Greenwood M, Almada AL, Earnest CP, Kreider RB. The effects of protein and amino acid supplementation on performance and training adaptations during ten weeks of resistance training. J. Strength Cond. Res., 20, 643–653 (2006).

28) Pins JJ, Keenan JM. Effects of whey peptides on cardiovascular disease risk factors. J. Clin. Hypertens., 8, 775–782 (2006).

29) Moura CS, Lollo PCB, Morato PN, Risso EM, Amaya-Farfan J. Bioactivity of food peptides: biological response of rats to bovine milk whey peptides following acute exercise. Food Nutr. Res., 61, 1290740 (2017).

30) Ichinoseki-Sekei N, Kakigi R, Miura S, Naito H. Whey peptide ingestion suppresses body fat accumulation in senescence-accelerated mouse prone 6 (SAMP6). Eur. J. Nutr., 54, 551–556 (2015).

31) Morato PN, Lollo F, Moura C, Batista T, Carneiro E, Amaya-Farfan J. A dipeptide and an amino acid present in whey protein hydrolysate increase translocation of GLUT4 to the plasma membrane in Wistar rats. Food Chem., 139, 853–859 (2013).