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

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Received May 30, 2017; accepted August 28, 2017

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 mice 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 reported 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 heart disease, and they are recommended for their beneficial effects on blood glucose levels. However, the mechanism of the glucose-lowering effect of milk proteins is not fully understood. 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. We investigated the detailed mechanism of the glucose-lowering effect of trypsin-treated β-LG, and 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 100mM phosphate buffer (pH 8.0) at a concentration of 1g/20mL was digested by adding 10mg of trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24h at 37°C. Digestion was stopped by heating for 5min 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 18h 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 18h 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 was collected and frozen at −80°C. 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 18h 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 2h 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 XR5+ 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 3h, 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]amino]-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 20 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 OGTTSs in Mice In the OGTTSs, 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-
Effect of Trypsin-Treated β-Lactoglobulin (β-LG) on Blood Glucose Concentrations by Oral Glucose Tolerance Test (OGTT) and Intraperitoneal Glucose Tolerance Test (IPGTT) in Mice

A: For the OGTT, mice were fasted for 18 h. Trypsin-treated β-LG (1 g/kg body weight) was orally administered at 30 min before the OGTT. Metformin (100 mg/kg body weight) or sitagliptin (3 mg/kg body weight) was orally administered 60 min before the OGTT. Glucose (5 g/kg body weight) was orally administered and the blood glucose concentration was measured from a tail vein.

B: The AUCs of blood glucose concentrations during OGTT.

C: For the IPGTT, mice were fasted for 18 h. Trypsin-treated β-LG (1 g/kg body weight) was orally administered at 30 min before the IPGTT. Metformin (100 mg/kg body weight) or sitagliptin (3 mg/kg body weight) was orally administered 60 min before the IPGTT. Glucose (2 g/kg body weight) was intraperitoneally administered and the blood glucose concentration was measured from a tail vein.

D: The AUCs of blood glucose concentrations during the IPGTT. Values are mean±S.E.M. (n=10). *p<0.05, **p<0.01 compared to the control group.

Effect of Trypsin-Treated β-LG on Insulin Secretion after Glucose Loading in Mice

Trypsin-treated β-LG did not increase the plasma insulin concentrations after oral glucose administration (1.18±0.21 ng/mL) compared to the control (1.31±0.18 ng/mL), whereas sitagliptin significantly increased the plasma insulin concentrations (2.82±0.74 ng/mL) (Fig. 2).

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.\(^{19}\) 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)
in mice. 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 vitro. 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
have potent DPP-4 inhibitory activity (Phe) and isoleucine (Ile)-proline (Pro)-Ala-valine (Val)-Phe.

Tartaric acid (Glu)-glycine (Gly)-threonine (Thr)-phenylalanine (Phe) peptides from β-luminal digestion. Although it has been demonstrated that β-LG are inactivated by β-inhibitory activity in trypsin-treated β-LG. 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 whey 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 including 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 muscle, 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 amionic 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 pepsin 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