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α-Lactalbumin Hydrolysate Stimulates Glucagon-Like Peptide-2 Secretion and Small Intestinal Growth in Suckling Rats[1,2]

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Abstract

We investigated whether bovine milk constituents influenced glucagon-like peptide (GLP)-2 secretion and intestinal growth in suckling rats. Male Sprague-Dawley rats (14 d old) received i.g. infusions of a milk protein fraction, a lactose solution, or the cream fraction of milk. The serum concentration of GLP-2, but not GLP-1, markedly increased in rats administered milk protein compared with those given the lactose solution or the cream fraction from 60 to 120 min after administration. In another experiment, both casein (CN) and whey protein isolate stimulated GLP-2 secretion at 120 min after administration, but soy protein and ovalbumin did not. Stimulation of GLP-2 secretion by several milk proteins was similar, including α-CN, α-lactalbumin (α-La), and β-lactoglobulin, in a separate experiment. A hydrolysate of α-La obtained by incubation with protease A extracted from *Aspergillus oryzae* (LaHPA) caused almost twice the GLP-2 release due to intact α-La and other α-La hydrolysates. Free amino acid concentrations and molecular size distributions did not differ among α-La hydrolysates, including LaHPA. In rat pups reared with milk formulae containing α-La or LaHPA, LaHPA significantly promoted small intestinal elongation and increased the number of crypt epithelial cells compared with a formula containing intact α-La. LaHPA administration also increased the maltase:lactase activity ratio, a marker of maturation of the intestinal mucosa. In conclusion, milk proteins stimulate GLP-2 secretion and contribute to growth and maturation of the small intestine in suckling rats. J. Nutr. 139: 1–6, 2009.

Introduction

The gastrointestinal tract of neonates is exposed to food, bacteria, and environmental antigens starting immediately after birth and is thus forced to adapt rapidly to the extrauterine environment. Moreover, growth factors found in milk are reported to have biological effects on the small intestine in neonates (1). Additionally, the intestinal epithelial cells of neonates express a variety of receptors for growth factors and hormones (1), suggesting that such factors in maternal milk may have a direct influence on the gastrointestinal tissues of neonates. In neonates, certain factors or nutrients obtained from milk stimulate enteroendocrine cells to secrete various intrinsic growth factors and/or hormones, including insulin-like growth factor, neurotensin, peptide YY, gastrin, cholecystokinin, and glucagon-like peptide (GLP)[5]-2 (1).

GLP-2 is a highly conserved 33-amino acid peptide that is produced by posttranslational processing of proglucagon, which also contains glicentin, oxyntomodulin, and GLP-1, in enteroendocrine L-cells found primarily in the distal small intestine in response to luminal nutrients, especially carbohydrates and lipids (2,3). GLP-2 promotes the proliferation of and suppresses apoptosis in intestinal epithelial cells, resulting in increased crypt-villus height and small bowel mass (4,5). GLP-2 is also involved in many gastrointestinal tract functions related to the absorption of nutrients, e.g. by promoting enlargement of the absorptive area and upregulating nutrient transporter expression (4,5). Moreover, it promotes intestinal barrier function, increases blood flow, and decreases intestinal motility (4–6).

These effects of GLP-2 on the gastrointestinal tract are thought to be mediated via a specific receptor localized on enteric neurons in rats or on enteroendocrine cells in humans (7). GLP-2 receptor expression and circulating GLP-2 concentration are transiently enhanced in neonatal rats (8) and the response to GLP-2 is temporarily upregulated after birth in pigs (9). Thus, GLP-2 is thought to be an important hormone for intestinal growth and maturation in neonates (10,11).

In this study, we investigated GLP-2 secretion in response to i.g. infusion of milk constituents or various food proteins in suckling rats. There are reports that peptone can stimulate the secretion of GLP-1 (12); thus, we chose one milk protein and

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[2] Supplemental Tables 1–3 are available with the online posting of this paper at jn.nutrition.org.
[5] Abbreviations used: α-La, α-lactalbumin; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; CN, casein; DPP-IV, dipeptidyl peptidase-IV; GLP, glucagon-like peptide; LaHP, α-lactalbumin pronase hydrolysate; LaHPA, α-lactalbumin protease A hydrolysate; LaHPF, α-lactalbumin pancreatin F hydrolysate; Mn, number-average molecular weight; MW, molecular weight; OVA, ovalbumin; SPI, soy protein isolate; WPI, whey protein isolate.
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prepared hydrolysates to examine the stimulatory effect on GLP-2 secretion and subsequent intestinal growth.

Materials and Methods

Rats

All animal experiments were performed in accordance with the Japanese standards relating to the care and management of experimental animals (notification no.6, March 27, 1980, of the Prime Minister's Office, Japan). Male Sprague-Dawley rats (7 d old; Charles River Laboratories Japan) with surrogate mother rats were used for the GLP-2 secretion experiments (Expt. 1). The surrogate dams were housed individually with 10 suckling rats in cages under temperature-controlled (23 ± 2°C) conditions and a 12-h-light/12-h-dark cycle. The suckling rats were kept with their surrogate dams until d 14.

Pregnant Sprague-Dawley rats (9 wk old) were obtained from the same breeder. They were allowed free access to a commercial diet[6] (F-2; Nosan) and tap water. The litters were reared by their mothers until d 7 after delivery and then were used for the rearing experiment from postnatal d 7 to d 14 (Expt. 2).

Expt. 1: Contribution of milk components and various proteins to GLP-2 secretion

GLP-1 and GLP-2 secretion. For the GLP secretion experiments, male rats (14 d old) were deprived of food by separating them from their surrogate dams for 16 h. The rats were administered 150 mg of a milk protein concentrate solution (Morinaga Milk Industry), 150 mg lactose solution (Nacalai Tesque), or 150 mg of the cream fraction obtained from homogenized cow milk (Morinaga Milk Industry) by gavage. The cream fraction had the following composition: lipids (455 mg/g), lactose (26 mg/g), proteins (16 mg/g), and minerals (3 mg/g). The energy value (2.5 kJ) was similar for the milk protein concentrate solution, lactose solution, and cream fraction. Usually, 14-d-old rats consume ~8 mL/d of maternal milk (13) and rat milk contains 10.5% protein, 3.7% carbohydrates, and 10.9% lipids (14). Thus, 14–d-old rats were calculated to have an energy intake of ~51.6 kJ/d. We administered 150 mg of each milk product with an energy content of 2.5 kJ, equivalent to one-twentieth of the normal daily energy intake at this age. The rats were decapitated under diethylether anesthesia at 15, 30, 60, or 120 min after administration of a test substance and blood was collected from the inferior vena cava. Blood samples were centrifuged at 2000 × *g*; 10 min at 4°C and the serum was stored at −35°C until analyzed. In a preliminary experiment, we monitored changes in the serum GLP-2 concentration over 240 min after administration of milk components and found that an increase in the serum GLP-2 concentration induced by nutrient was highest at 120 min postadministration.

To assess GLP-2 secretion induced by various food proteins, rats (14 d old) were deprived of maternal milk for 16 h, as described above, and were administered 150 mg of one of the following by gavage: casein (CN; Morinaga Milk Industry), whey protein isolate (WPI; Morinaga Milk Industry), ovalbumin (OVA; Sigma-Aldrich), soy protein isolate (SPI; Fuji Oil), bovine α-CN, bovine β-CN, bovine α-lactalbumin (α-La), bovine β-lactoglobulin, bovine serum albumin (BSA) (Sigma-Aldrich), each α-La hydrolysate (see below), or water (control group). When CN was reconstituted, the pH was adjusted to 7.0 with 1 mol/L NaOH. The rats were killed as above at 120 min after administration and blood samples were collected and processed as described previously.

Serum GLP-1 and GLP-2 assays. The serum GLP-1 and GLP-2 concentrations were measured using a rat GLP-1 EIA kit (YK160) and rat GLP-2 EIA kit (YK140) (Yanaihara Institute) according to the manufacturer's protocols.

Preparation of α-La hydrolysates. α-La (Davisco Foods International) was dissolved in distilled water at a concentration of 100 g/L. By the addition of 1 mol/L NaOH, the pH was adjusted to 7.0 for digestion with protease A from *Aspergillus oryzae* (Amano Enzyme) and to 8.0 for digestion with pancreatin F from porcine pancreas (Amano Enzyme) or with Pronase from *Streptomyces griseus* (Kaken Pharmaceutical). Each protease was dissolved in distilled water (100 g/L) and the pH of the protease solutions was also adjusted. The α-La and protease solutions were centrifuged at 14,000 × *g*; 20 min at 10°C to remove insolubles, passed through a 0.80-μm filter, and then sterilized by filtration (0.22 μm) before being mixed. The α-La solution was then mixed separately with each protease solution; the final concentration of protease in the reaction mixture was 30 mg/g substrate. Hydrolysis was performed at 50°C for 18 h according to the manufacturer's instructions and was terminated by heating at 90°C for 15 min. The resulting α-La protease A hydrolysate (LaHPA), α-La pronase hydrolysate (LaHP), and α-La pancreatin F hydrolysate (LaHPF) solutions were freeze-dried.

Free amino acid concentration of α-La hydrolysates. Free amino acids in α-La and its hydrolysates were measured using an amino acid analyzer L-8500 (Hitachi). α-La and its hydrolysates were dissolved in ultrapure distilled water (1.5 mg/L) and were deproteinized with 3 mmol/L sulfosalicylic acid. The sample solutions were sonicated for 5 min and filtrated through a 0.2-μm filter (Millex-LG; Millipore). Analysis was performed using a lithium citrate buffer system. Amino acid concentrations were calculated from individual peak areas and external amino acid mixture standard solutions (Wako Pure Chemical Industries) using the EZChrome Elite software (version 3. 1. 7.J; GL Sciences).

Molecular size distributions of α-La hydrolysates. Molecular size distributions of α-La and its hydrolysates were measured by high performance gel permeation chromatography using a HPLC system (Shimadzu) with a poly-hydroxyethyl-aspartamide gel column (4.6 × 200 mm; Poly LC). Samples were prepared (1 mg/L protein or hydrolysates in 50 mmol/L formic acid, 20 mmol/L NaCl solution); 20 μL was injected onto the column, and elution was isocratic, in the same solution at 30°C with a flow rate of 0.5 mL/min. Wavelength of the spectrophotometric detection was at 215 nm. The following proteins, peptides, and amino acids were used for calibration: IgG [molecular weight (MW) 160,000; Sigma-Aldrich], lactoperoxidase (MW 93,000; Sigma-Aldrich), OVA (MW 43,000; Sigma-Aldrich), chymotrypsinogen A (MW 25,000; Pharmacia), ribonuclease A (MW 13,700; Pharmacia), insulin (MW 5740; Wako Pure Chemical Industries), basitrasin (MW 1427; Sigma-Aldrich), oxytocin (MW 1007; Bachem Americas), enkephalinamide (MW 588; Bachem Americas), thyrotropin -releasing hormone (MW 362; BioProducts), L-methionine (MW 149; Kyowa Hakko Bio), and L-glutamine (MW 146; Kyowa Hakko Bio). A calibration curve was made from the MW of the markers and their respective elution times using calculation software (GPC; Shimadzu). The total area of the chromatograms was integrated and separated into 10 molecular mass ranges (<200, 200−500, 501−1000, 1001−1200, 1201−2000, 2001−3500, 3501−5000, 5001−10,000, 10,001−20,000, and >20,000 Da), expressed as a percentage of the total area. Number-average MW (Mn) and weight-average MW were calculated using same software. Mn was calculated by $\Sigma N_i M_i / \Sigma N_i$, and Mw was calculated by $\Sigma N_i M_i^2 / \Sigma N_i M_i$, where N_i is the number of molecules of MW M_i.

Expt. 2: Rearing of rat pups using milk formulae containing α-La or LaHPA

Preparation of milk formulae. The milk formulae for rearing rats were prepared (13) according to the report by Keen et al. (14), with slight modifications (**Supplemental Table 1**). The CN:whey protein ratio of each formula was 1:20 and α-La or LaHPA was used as the whey protein source. In this experiment, we assessed whether the intestinal maturation was influenced by proteins with the same amino acid concentration having different abilities to stimulate GLP-2 secretion.

Rearing of rat pups. In 7-d-old rat pups, an i.g. cannula was implanted according to the method of Hall (15). Briefly, a piece of piano wire covered with Silastic tubing (0.3 mm i.d.) was advanced perorally into the esophagus of a rat pup on ice. When the coated wire reached the pup's stomach, the Silastic tubing was withdrawn and the wire was

[6] The F-2 diet (Nosan Corporation) contains the following nutrients per kg: water, 70 g; protein, 208 g; fat, 45 g; carbohydrate, 620 g.

advanced to pierce the stomach wall and abdominal skin. A gastric cannula was then pulled out from the stomach by attaching it to the other end of the wire. The pups were reared in styrol cups on a temperature-controlled water bath set at 39°C. Wood shavings were placed at the bottom of each cup as bedding. The i.g. cannula was joined to a tube (Intramedic polystyrene tubing, PE-50; Beckton Dickinson), which was then connected to a syringe mounted on an infusion pump (PHD 2000 programmable; Harvard Apparatus). The syringe containing milk formula and the pump were kept in a refrigerator (4°C) to prevent growth of bacteria in the milk. The rat pups were given milk formula via intermittent gastric infusion (25 min every 2 h) under computer control. The daily volume (mL) of formula for each pup was calculated as the age (in days) multiplied by 0.5 (e.g. at 7 d old, the volume of milk was 3.5 mL). To promote urination and defecation, the anogenital region was gently stimulated with a swab twice a day. Rearing of rat pups was performed from d 7 to 14 of life. On d 7 of the rearing period (d 14 of life), all pups were decapitated while under diethylether anesthesia 2 h after the last infusion of milk. At 1 h before death, each pup received a subcutaneous injection of 5-bromo-2′-deoxyuridine (BrdU) solution (15 mg/kg of BrdU and 1.5 mg/kg of 5-fluoro-2′-deoxyuridine in saline) for immunohistochemical analysis (16). The entire small intestine and colon were removed for measurement of the weight and length. Then, the small intestine was divided into halves. A segment (2 cm) of the ileum located 1 cm from the ileocecal valve was removed for histochemical examination and was fixed with 100 mL/L formalin in PBS. The mucosa of the remaining ileum was gently scraped off with a glass slide, added to 10 mL of ice-cold PBS (pH 7.4), and stored at −35°C for biochemical analysis.

Disaccharidase assays. Maltase and lactase activity were assayed according to the method of Dahlqvist (17) with slight modifications. Briefly, aliquots of mucosal homogenates were incubated at 37°C for 30 min in the presence of either maltose or lactose as the substrate and glucose production was measured. The protein concentration of each reaction mixture was measured with a protein assay (Bio-Rad Laboratories) using BSA as the standard. Then enzyme activity was calculated as μmol substrate hydrolyzed/(g protein · min). We also calculated the maltase:lactase activity ratio, a biomarker used to evaluate maturation of the small intestine (18,19).

Immunohistochemistry. Frozen sections were prepared and immunostaining for BrdU was performed as previously reported (16). The sections were treated with mouse anti-BrdU antibody (NA-20; Calbiochem, EMD Chemicals) and biotinylated rabbit anti-mouse IgG+A+M (H+L) (Zymed Laboratories) as the primary and secondary antibodies, respectively. Then the sections were immersed in horseradish peroxidase-conjugated streptavidin (Zymed Laboratories) to enhance the signals and 3,3′-diaminobenzidine was used to stain reaction products. Epithelial cells were scored from the bottom of each crypt to the position of cell number 20 under a microscope and 50 well-organized crypt sections were examined at each site of the intestine (20). The number of BrdU-incorporating epithelial cells was counted and the ratio of positive cells: total number of epithelial cells in each half-crypt section was determined.

Statistical analyses. Values are expressed as means ± SEM. In time course experiments for GLP-1 and GLP-2 secretion from 0 to 120 min after administration, the significant difference from time 0 was determined using the Dunnett's test and the significant differences between groups at each time point were determined using the Tukey-Kramer honestly significant difference test for multiple comparisons. In other experiments, the significance of differences was determined using the Tukey-Kramer honestly significant difference test for multiple comparisons and Student's *t* test for comparison between 2 groups (JMP software; SAS Institute). A *P*-value <0.05 was considered significant.

Results

Expt. 1

Milk components and GLP-1 and GLP-2 secretion. We detected an increase in GLP-1 secretion at 15 min and 60 min only after administration of the lactose solution (**Fig. 1***A*). However, all of the components tested stimulated GLP-2 secretion increase in suckling rats compared with time 0 (Fig. 1*B*). The serum GLP-2 concentrations at 60 and 120 min were significantly higher after milk protein concentrate was administered compared with the lactose solution or the cream fraction, although an early significant increase compared with time 0 was also detected when rats were given lactose or cream (Fig. 1*B*). GLP-2 secretion was significantly increased relative to time 0 at 15–60 min after administration of the lactose solution and at 15–120 min after administration of the cream fraction.

Food proteins and GLP-2 secretion. Proteins isolated from cow milk (CN and WPI) promoted significantly more GLP-2 secretion than OVA or SPI (**Fig. 2***A*). All of the milk proteins significantly enhanced GLP-2 release at 120 min after administration compared with the control (water administration) group (Fig. 2*B*), but serum GLP-2 concentrations were significantly higher in rats administered α-CN, β-lactoglobulin, and α-La than in rats given BSA. BSA is also a bovine protein, but it did not affect GLP-2 secretion compared with the control group. Among α-La hydrolysates, LaHPA was more effective than α-La, LaHPF, and LaHP at stimulating GLP-2 secretion (Fig. 2*C*). The serum GLP-2 concentration at 120 min after LaHPA administration was almost twice that after dosing with intact α-La and other hydrolysates.

Characteristics of α-La hydrolysates. Free amino acid concentrations and the molecular size distributions of α-La and its hydrolysates were analyzed (**Supplemental Tables 2** and **3**). The degree of hydrolysis was similar among the hydrolysates obtained with the different enzyme treatments, because the

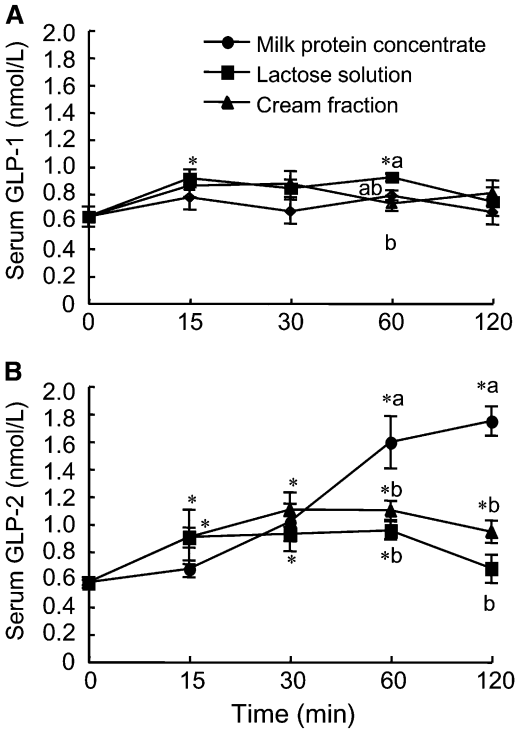

FIGURE 1 Changes in serum GLP-1 (*A*) and GLP-2 (*B*) concentrations in neonatal rats in response to gastric infusion of milk protein concentrate, a lactose solution, or the cream fraction. Values are means ± SEM, $n = 4$. *Different from time 0, $P < 0.05$. Labeled means at a time without a common letter differ, $P < 0.05$.

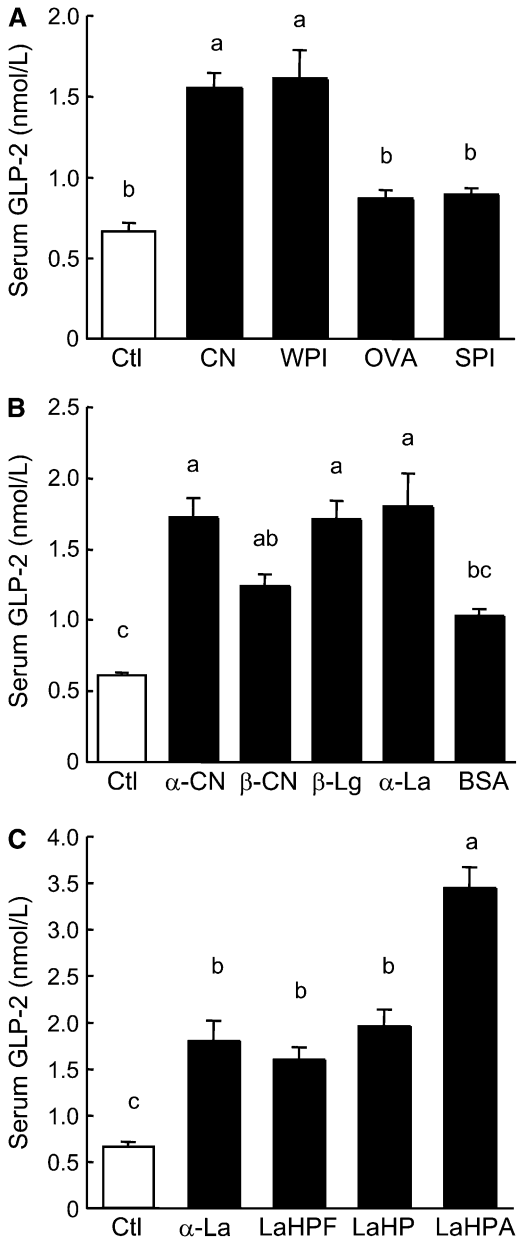

FIGURE 2 Serum GLP-2 concentration at 120 min after i.g. administration of various food proteins (*A*), milk proteins (*B*), and α-La hydrolysates (*C*) in neonatal rats. Values are means ± SEM, n = 5. Means without a common letter differ, $P < 0.05$.

free amino acid concentrations and the Mn of these hydrolysates were similar.

LaHPA and GLP-1 and GLP-2 secretion. GLP-2 secretion was stimulated by administering LaHPA to suckling rats (**Fig. 3**). There was a significant increase in GLP-2 from 30 to 120 min after administration compared with time 0. The concentration vs. time curve for LaHPA was similar to that obtained with the milk protein fraction (Fig. 1*B*). In contrast, there was no stimulation of GLP-1 secretion after administration of LaHPA.

Expt. 2

Morphological and biochemical features of the small intestine. Rearing of suckling rats with formula was performed from d 7 to 14 of life. The small intestine was significantly longer in pups reared on LaHPA than those given α-La (**Table 1**), whereas length of the colon did not differ. Intestinal mucosal lactase activity in LaHPA-reared rats was significantly lower than that in α-La-reared rats. In contrast, maltase activity tended to be greater (P = 0.078) in the small bowel mucosa of LaHPA-reared rats. The maltase:lactase ratio of the small bowel mucosa was significantly higher in LaHPA-reared rats than in α-La–reared rats (Table 1).

We also evaluated small bowel epithelial changes by BrdU immunohistochemistry. Morphology did not differ between pups reared on α-La and those reared on LaHPA. The number of epithelial cells per half-crypt section was significantly higher in the LaHPA-reared rats than in the α-La–reared rats (Table 1), although the groups did not differ in mitotic figures or the number of BrdU-incorporating epithelial cells (results not shown).

Discussion

GLP-2 is a hormone involved in intestinal epithelial proliferation and subsequent intestinal growth. Oral administration of nutrients has been reported to stimulate GLP-2 secretion by intestinal enteroendocrine L-cells (4,5). Moreover, it has been reported that exogenous GLP-2 enhances intestinal maturation (4,5,21), GLP-2R expression and circulating GLP-2 are enhanced in the neonatal period (8), and the response to GLP-2 is temporarily upregulated after birth (9). Thus, certain milk components may support the growth and maturation of the gastrointestinal tract via GLP-2 secretion in neonates before ingestion of various foodstuffs. The aim of this study was to identify the most effective milk component for stimulating intrinsic GLP-2 secretion in suckling rats.

The stimulus for the secretion of GLP is thought to be primarily nutrient intake and to involve luminal stimulation of L-cells as well as indirect enteroendocrine and neural mechanisms (3). Few studies have reported on GLP-2 secretion being affected by nutrient intake. GLP-2 release is stimulated by administration of carbohydrates and lipids but not by a protein-rich diet in human volunteers (2), administration of SCFA in rats (22), or ingestion of dietary fiber in streptozotocin-induced

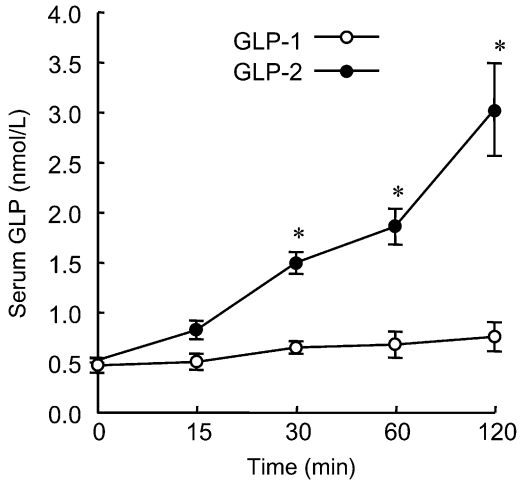

FIGURE 3 Changes in serum GLP-1 and GLP-2 concentrations in neonatal rats in response to gastric infusion of LaHPA. Values are means ± SEM, n = 4–5. *Different from time 0, $P < 0.05$.

TABLE 1 Final body weight, intestinal length, maltase:lactase activity ratio, serum GLP-2 concentration, and small bowel crypt epithelial cell number in rats administered milk formula containing α-La or LaHPA for 7 d[1]

	α-La	LaHPA
Final body weight, *g*	20.3 ± 1.4	20.0 ± 0.4
Small intestinal length, *cm*	36.6 ± 2.7	45.0 ± 1.7*
Colon length, *cm*	3.9 ± 0.3	4.3 ± 0.3
Maltase activity, *μmol/(g protein·min)*	210.1 ± 43.7	381.1 ± 64.8
Lactase activity, *μmol/(g protein·min)*	54.9 ± 5.9	30.8 ± 4.2*
Maltase:lactase activity ratio	3.7 ± 0.5	13.3 ± 2.4*
Serum GLP-2, *nmol/L*	0.59 ± 0.14	1.16 ± 0.09*
Epithelial cells, *n/half crypt section*	13.4 ± 0.7	17.5 ± 1.1*

[1] Values are means ± SEM, $n = 4–5$. *Different from α-La, $P < 0.05$ (Student's *t* test).

diabetic rats (23). To the best of our knowledge, the present study is the first report showing that food proteins induce GLP-2 secretion in rats.

In contrast, many studies have reported that nutrients influence GLP-1 secretion. It has been reported that oleic acid can stimulate GLP-1 secretion in fetal rat intestinal culture (24) as well as in adult rats (25). In humans, oleic acid has been reported to stimulate GLP-1 and GIP secretion in healthy adults (26). Olive oil and butter can also stimulate GLP-1 and GIP secretion (27). These findings suggest that the stimulatory mechanism of L- and K-cells is common between humans and rats.

In our results, there was a significant, but weak, stimulation of GLP-1 secretion in response to lactose. Neither milk protein nor the cream fraction induced GLP-1 secretion, although the cream fraction contains relatively high levels of oleic acid (28). Interestingly, milk protein stimulated GLP-2 secretion in suckling rats. These results suggest that regulation of GLP secretion is different between neonates and adults. Xiao et al. (2) reported that cream stimulated GLP-2 secretion, but whey protein did not, in healthy human volunteers. However, other reports have stated that whey protein and milk protein did stimulate GLP-1 secretion (26). These reports suggest that GLP-1 and GLP-2 secretion are not always parallel and that regulation of the secretions depends on developmental stage.

We tried to determine which protein was the most effective in induction of GLP-2 secretion in suckling rats. Milk proteins were obviously more effective than OVA or SPI when GLP-2 secretion was assessed at 120 min postadministration (Fig. 2*A*). The GLP-2 secretory effect was comparable among the various milk proteins tested (Fig. 2*B*). Among the bovine proteins used in this study, BSA did not stimulate GLP-2 secretion. These results suggest the importance of milk proteins in the neonatal period. Cordier-Bussat et al. (12) reported that peptone can stimulate the secretion of GLP-1; thus, we chose α-La and prepared 3 hydrolysates (LaHPF, LaHP, and LaHPA) using 3 enzymes to examine whether the stimulatory effects on GLP-2 secretion are influenced by hydrolysis. As a result, LaHPA, which is one of the α-La hydrolysates, was the most effective in stimulating GLP-2 secretion. Stoll et al. (29) found that piglets fed an elemental formula (free amino acids, glucose, lipid) had ~70% higher GLP-2 levels than piglets fed a cow milk-based formula (protein, lactose, lipid). Their results suggested that free amino acids or extensively hydrolyzed peptides could enhance GLP-2 secretion more than undigested protein. Thus, we next analyzed concentrations of free amino acids and degree of hydrolysis in the hydrolysates. As a result, there was no notable difference in free amino acid concentrations or molecular size distributions (Supplemental Tables 2 and 3). The pattern of the change in the GLP-2 concentrations in LaHPA-administered rats was similar to that in rats administered the milk protein fraction (Fig. 1*B* and Fig. 3). Nevertheless, the GLP-2 secretory effect of LaHPA was almost twice that of intact α-La or the other hydrolysates at 120 min after administration (Fig. 2*C*), indicating that the existence of peptide fragments stimulate GLP-2 secretion and/or inhibit dipeptidyl peptidase-IV (DPP-IV) in the α-La hydrolysate by protease A (from *Aspergillus oryzae*). In fact, Gunnarsson et al. (30) showed that whey protein inhibited DPP-IV activity in the proximal small intestine. The other 2 enzymes used for digestion studies were from a microorganism and porcine pancreas. The animal protease did not seem to have any advantage in producing suitable peptides from α-La to induce GLP-2 release.

We studied whether promotion of GLP-2 secretion by LaHPA contributed to intestinal growth in suckling rats. To investigate the difference in GLP-2 secretory effect without other influences (e.g. amino acid composition), we used α-La, which has the same amino acid content as LaHPA, as a control. Our rearing study revealed that LaHPA significantly promoted elongation of the small intestine with an increase in the number of crypt epithelial cells (Table 1). LaHPA also increased the maltase:lactase ratio, an index of mucosal maturation (18), in the small intestine. Another important aspect of intestinal growth is crypt fission (31). The crypt fission rate is 7 times higher in neonatal rats than in adult rats, but epithelial mitosis starts to increase from d 21 (32). Therefore, an increase in serum GLP-2 would thus be expected to trigger crypt fission in the ileum during the suckling period, contributing to expand mucosal surface and subsequent increase in the intestinal length.

We also examined whether an increase in intrinsic GLP-2 was required for intestinal growth using a neutralization study with anti-GLP-2 in weanling rats. This experiment demonstrated that intrinsic GLP-2 was involved in elongation of the small intestine in weanling rats (data not shown). This study confirmed that endogenous GLP-2 supported intestinal growth in the weanling period. The inhibitory effect of anti-GLP-2 on BrdU incorporation was primarily observed in the distal ileum (data not shown), where L-cells are heavily localized and the concentration of active GLP-2 is much higher than in the jejunum. Usually, DPP-IV rapidly inactivates intact GLP-2 after its release (33) and the plasma half-life of active GLP-2 is estimated to be ~7 min due to rapid clearance by DPP-IV. Thus, the effect of intrinsic GLP-2 on intestinal growth is probably limited to around the ileum.

GLP-2 promotes the intestinal mucosal barrier and reduces intestinal mucosal permeability (6). Arvola et al. (34) reported that whey protein hydrolysate reduced permeability to almost the same extent as breast-feeding in neonatal rats. The mechanism of this barrier-promoting effect by whey protein hydrolysate might be partially due to increased GLP-2 secretion. Milk protein-stimulated GLP-2 secretion is considered to support epithelial barrier function and prevent extravasation of luminal substances into the lamina propria, resulting in the suppression of inflammation.

In conclusion, we demonstrated the promotion of GLP-2 release by proteins from cow milk, which supported growth of the small intestine in suckling rats. These findings suggest that milk proteins enhancing the secretion of endogenous GLP-2 are involved in supporting normal intestinal growth and maturation.

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