Large molecule protein feeding during the suckling period is required for the development of pancreatic digestive functions in rats

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Kinouchi T, Koyama S, Harada E, Yajima T. Large molecule protein feeding during the suckling period is required for the development of pancreatic digestive functions in rats. Am J Physiol Regul Integr Comp Physiol 303: R1268–R1276, 2012. First published October 24, 2012; doi:10.1152/ajpregu.00064.2012.—We examined if large molecule protein feeding during the suckling period is prerequisite for the proper development of pancreatic digestive functions. Most amino acids in breast milk exist as the constituent of large proteins and not as oligopeptides or free amino acids. Accumulating evidence indicates the nutritional importance of large protein feeding for suckling infants; however, evidence on the physiological significance remains small. We thus artificially reared rat pups on a standard rat formula with milk protein or a formula with milk protein hydrolysate from 7 to 21 days of age, and thereafter, fed a standard solid diet until 42 days of age. Pancreas weight and the stock of pancreatic digestive enzymes in the hydrolysate-fed rats were significantly lower than those in the protein-fed rats during and also after the suckling period. Plasma insulin, a stimulator of amylase synthesis, was also significantly low in the hydrolysate-fed rats compared with the protein-fed rats. At 28 days of age, we evaluated the pancreatic secretory ability in response to dietary protein and cholecystokinin (CCK) by means of pancreatic duct cannulation. Pancreatic secretion stimulated by dietary protein in the hydrolysate-fed rats was significantly weaker than that in the protein-fed rats. No significant difference was observed in the increasing rate of pancreatic enzyme secretion in response to CCK between the two groups. These results suggest that the presence of large proteins in breast milk is significant for the development of pancreatic digestive functions and the outcomes could remain even later on in life.

Most amino acids in breast milk exist as the constituent of large molecule proteins. In human milk, for instance, about 95% of all amino acids constitute α-lactalbumin (14 kDa) or larger proteins (3). Infants are able to adequately digest such large proteins and absorb sufficient amino acid nutrition from the digest. On the other hand, it is often thought that protein hydrolysates might be nutritionally equivalent to the original large proteins for infants. However, several reports have suggested that for suckling infants, it is important that amino acids exist mainly as large proteins in breast milk.

Accumulating evidence indicates that large proteins may be superior to protein hydrolysates or free amino acids as a dietary source of amino acid nutrition for sucking infants. Some groups suggested that the utilization of nitrogen from standard formula might be higher than that from hydrolysate formula in infants (25, 32, 33). In adults, it has been shown that body nitrogen accumulation from “slow” proteins, amino acids of which are slowly absorbed, is larger than that from “fast” protein (6). This might account for the lower efficiency of nitrogen accumulation seen in hydrolysate formula-fed infants, because the gastrointestinal transit time of hydrolysate formula in infants may be shorter than that of standard formula (27). Interestingly, it is also suggested that some nutrients such as fat, phosphorus, and calcium could be efficiently absorbed from milk formula based on large proteins in infants compared with protein hydrolysate formula (33).

From a physiological point of view, dietary proteins may be similarly significant for infants. For instance, milk-borne large proteins per se, which require digestion by pancreatic enzymes for intestinal absorption, might be involved in the developmental processes of pancreatic digestive functions in infants. In adults, dietary protein can act as a critical factor to stimulate the secretion and synthesis of pancreatic digestive enzymes at least in rats and perhaps in humans as well (13, 14, 34). Although there have been few studies on the physiological importance of large protein intake in infancy, there is a report that insufficient pancreatic digestive functions were observed in rats fed an amino acid diet with milk from dams during the weaning period (28). These results arouse interest in a possible role of large dietary proteins taken during the suckling-weaning period in the development of pancreatic digestive functions.

In recent years, various kinds of antigen-reduced milk protein hydrolysate formulas are often used not only for infants diagnosed as allergic but also for healthy infants in the expectation of allergy prevention. Although some researchers have pointed out concerns about the nutritional adequacy of protein hydrolysate infant formulas and their long-term effects (2, 41, 46), there is still little evidence especially on the physiological significance of large protein feeding in infancy. Recently, there is growing evidence that dietary experience in infancy can critically affect some physiological functions not only during the feeding period but also later on in life.

In the present study, we examined the impact of large protein feeding during the suckling period on the development of pancreatic digestive functions during and after the suckling period, using a rat artificial rearing technique.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan) and housed in separate cages under conditions of controlled temperature (25 ± 2°C), humidity (55 ± 2% relative humidity), and light (lights on 07:00–19:00). For the assessment of the stimulatory effects of milk proteins and whey protein hydrolysate on pancreatic enzyme secretion, female rats weighing between 250 and 300 g were used. For artificial rearing studies, we purchased 11 pregnant rats. The day of birth was referred to as day 0. The litter sizes...
were adjusted to 10 pups on day 1 to maintain a standard nutritive status. All rats were given free access to a standard chow diet (CRF-1; Japan Clea, Tokyo, Japan) and water. The experimental protocols were approved in advance by the Committee for Research on Experimental Animals of our institute and was conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals (NRC, 1996).

**Milk formula for rats.** Two kinds of milk formulas for rat pups (Prot and Hydr) were prepared from different protein sources: acid-precipitated bovine casein and whey protein isolated from bovine milk (Prot) and whey protein hydrolysate (Hydr), following a previously described procedure (19). The whey protein hydrolysate was made by digesting whey protein isolated from bovine milk with a bacterial enzyme that randomly digests peptide bonds in protein and is often used for the production of food materials including protein for infant milk formula. The mean molecular weight (~900) and the molecular weight distributions of the hydrolysate (Table 1) were obtained by means of HPLC with a TSKgel G2000SWx1 column (TOSOH, Tokyo, Japan). We collected rat milk from 7 to 13 days after birth and measured the protein concentration, following a previously described method (20), and the results showed that protein concentration of rat milk during the period was 7.9 ± 0.7%. Thus, in the present study, the protein content of the milk formulas was set at 7.5% (Table 2), and the compositions of fat and carbohydrate and energy of both milk formulas were also set likewise (Table 2). Both formulas contain minerals, micronutrients, and vitamins close to those of rat milk (19). The osmolality (mosmol/kg H2O) of the milk formulas was 291 and 480, respectively. The prepared milk was divided into 50-ml sterilized bottles and then stored at 4°C until used.

**Artificial rearing.** The rat is immature at birth, and pancreatic digestive enzymes in the small intestine begin to increase during the second half of the second postnatal week (17), whereas, in humans, pancreatic enzymes in the small intestine begin to increase at around 1 mo of age (44). We then started the feeding study at 7 days of age when the developmental status of the pancreatic digestive functions is likely close to those in human newborns. On day 7, 80 pups, male and female, were randomly divided into two artificial rearing groups (Prot and Hydr, n = 30 and 30) and a dam-fed (DF) group (n = 20) of equal mean body weight. Rat pups for artificial rearing were implanted with a pancreatic cannula made of polyethylene tubing at 7 days of age, and the pups were fed either of the milk formulas or fed by a dam (10 pups in each group) and weaned following the same procedure.

**Experimental protocols.** To evaluate the stimulatory effects of protein materials on pancreatic secretion, the adult female rats were anesthetized with isoflurane (Forane, Dainippon Pharmaceutical, Osaka, Japan) using a gas anesthesia apparatus after an overnight fast and kept warm on thermal pads throughout the experiment. The abdomen of the rats was cut, and a pancreatic cannula, which is a silicone tube tipped with a short metal tube, was inserted into the pancreatic duct and fixed so that all the secreted bile-pancreatic juice could be collected through the cannula. Another cannula was inserted into the jejunum close to the papilla to return the bypassed pancreatic juice while the pancreatic juice was not being collected. Rats were kept for one and a half hours after the cannulation. Two samples of pancreatic juice secreted for 5 min were collected with a 10-min interval to measure the basal secretion. Then 2 ml of protein solution (including 100 mg of casein, whey protein, or the whey protein hydrolysate) were administered into the stomach through a silicone tube fixed in advance. Osmolality and pH of the solutions were adjusted to 310 mosmol/kg H2O with NaCl and 7.4 with NaOH, respectively. Five-minute pancreatic juice collections after 10-min intervals were started just after the administration, and interval-collection cycles were continued for 75 min. After the pancreatic juice collection, the rats were euthanized by draining the blood with a syringe from the abdominal aorta. As for the artificially rearing study, some artificially reared rats and dam-fed rats were randomly selected at 14, 21, 28, and 42 days of age, regarding equality of original litter variation. Rats at 14, 21, or 42 days of age were euthanized by draining the blood from the abdominal aorta under anesthesia with ether within 30 min after the last feeding of the milk formula, separation from the dam, or removal from the standard chow diet in the morning (0900–1100). The pancreas, heart, liver, kidney, and spleen were removed and weighed. Animals at 28 days of age were used for the evaluation of pancreatic secretory functions. A pancreatic cannula was fixed by the method described above. To evaluate pancreatic secretory activity in response to dietary protein, soybean trypsin inhibitor (SBTI; Sigma, St. Louis, MO) was used. First, two samples of the pancreatic juice secreted for 5 min were collected at a 10-min interval for to measure the basal secretion. Then the rats were injected with 1 ml of SBTI solution (20 mg/ml saline) into the duodenum using the fixed silicone tube, and 10-min interval-5 min collection cycles were continued for 75 min. After the pancreatic juice collection, the rats were euthanized by draining the blood from the abdominal aorta, and the pancreas, the heart, the liver, the kidney, and the spleen were removed and weighed. For the evaluation of pancreatic secretory ability to CCK, rats at 28 days of age were used and a pancreatic cannula was fixed by the same method. The pancreatic juice was collected for 10 min to measure the basal secretion, and 50 μl of CCK (sulfated CCK-octapeptide, Peptide Institute, Osaka, Japan) solution (50 ng/ml saline) was injected into the femoral vein. The pancreatic juice was collected for 10 min immediately after the administration. The animals were then euthanized by draining the blood from the abdominal aorta. The blood sample was immediately transferred into a heparin tube. The plasma was separated and stored at

<table>
<thead>
<tr>
<th>Component</th>
<th>Prot</th>
<th>Hydr</th>
</tr>
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<tbody>
<tr>
<td>Casein, g/dl</td>
<td>4.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Whey protein, g/dl</td>
<td>2.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Whey protein hydrolysate, g/dl</td>
<td>0.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Fat, g/dl</td>
<td>11.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Lactose, g/dl</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Energy, kcal/dl</td>
<td>136.6</td>
<td>136.6</td>
</tr>
</tbody>
</table>

Prot, protein; Hydr, hydrolysate.
−40°C until assayed. The pancreas was homogenized with ice-cold PBS and centrifuged. The supernatant was removed and frozen at −40°C until assayed.

Assays. The protein concentration was determined using a Coomassie Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. Amylase activity was determined with a kit Amylase B-Test Wako (Wako, Osaka, Japan) based on a method using carboxymethyl amylase as substrate. Amylase activity was shown as units of the Caraway method. Trypsin activity was determined with benzoyl-arginine p-nitroanilide (Sigma) as a substrate, after activation with enterokinase (Sigma). One unit of trypsin activity was defined as 1 μmol p-nitroaniline liberated per minute at 37°C. Total plasma concentrations of insulin were determined by rat insulin [125I] radioimmunoassay system (Amersham, Buckinghamshire, UK).

Statistical analysis. Comparisons between the formula-fed groups were carried out by Student’s t-test. In the evaluation of area under the curve in the experiments on the stimulatory effects of protein materials and pancreatic secretory ability to SBTI, one-sample t-test was used to compare the values against zero. In the evaluation of the effects of protein sources fed during the suckling period on pancreatic responsive ability to CCK, two-way repeated measures ANOVA with post hoc analysis by t-test was performed with Bonferroni’s adjustment. Significance was set at P < 0.05.

RESULTS

Stimulatory effects of protein hydrolysate on pancreatic enzyme secretion. We first evaluated the stimulatory effect of the whey protein hydrolysate used for the Hydr formula on digestive enzyme secretion from the pancreas using adult rats. As shown in Fig. 1, intragastric administration of whey protein solution or casein solution significantly increased pancreatic enzyme secretion. In contrast, the hydrolysate hardly had any effect on pancreatic secretion. These results suggest that the hydrolysate does not have substantial capability to enhance pancreatic enzyme secretion in contrast to whey protein or casein at least in adult rats. Also, these results indicate that the physiological characteristics of the hydrolysate are evidently different from those of the original whey protein even though it still has the same amino acid profile as the original protein.

We next assessed the stimulatory effects of some commercial infant formulas on the pancreas using the same model. The increases in pancreatic amylase secretion 15 min after administration of the formulas were 469 ± 116% (standard formula, n = 6), 186 ± 44% (whey protein hydrolysate formula, Mw: <3,500, P < 0.05, vs. standard formula, n = 6), 230 ± 53% (casein hydrolysate and whey protein hydrolysate formula, mol wt: 96% <3,500, P < 0.05, vs. standard formula, n = 5), and 112 ± 8% (elemental formula, P < 0.05, vs. standard formula, n = 5). The areas under the curves until 45 min were 99.5 ± 34.3%·h (P < 0.05, vs. 0%-h), 43.3 ± 18.5%·h, 3.5 ± 20.8%·h, and −2.8 ± 2.7%·h, respectively.

Growth and development of the pancreas. The weight of the pancreas in the Hydr group was significantly less than that in the Prot group at 14 days of age during the suckling period and also at 28 and 42 days of age after weaning (Fig. 3). The pancreatic weight in the Prot group was equivalent to that in the DF group at each time point throughout the experimental period. The difference at 42 days of age, 3 wk after the experimental formula-feeding period, was even larger compared with that at 28 days of age or younger and the ratio was maintained.

Next, we compared the concentrations of digestive enzymes in the pancreas in the Prot group with those in the Hydr group. As shown in Fig. 4A, amylase concentration to soluble protein ratio in the pancreas tissue in the Prot group increased constantly...
until 28 days of age, and the level was unchanged from 28 to 42 days of age. Likewise, in the Hydr group, pancreatic amylase concentration increased until 28 days of age; however, the concentrations were lower than those in the Prot group, and the difference expanded until 28 days of age. After 28 days of age, the amylase concentration was unchanged in the Hydr group also, and the difference between the Hydr group and the Prot group at 42 days of age was still as large as that at 28 days.

The ratios of the two groups were constant throughout the experimental period, even 3 wk after weaning from the experimental formulas. Amylase concentration in the Prot group was close to that in the DF group at each time point. The amount of amylase in the whole pancreas in the Hydr group was significantly lower than that in the Prot group as well [7.06 ± 1.00 (DF), 6.25 ± 1.22 (Prot), and 2.68 ± 0.45 (Hydr) kU/pancreas at 28 days, 13.01 ± 1.36 (DF), 14.72 ± 2.10 (Prot), and 5.67 ± 0.81 (Hydr) kU/pancreas at 42 days].

On the other hand, trypsinogen concentrations in the pancreas did not change in both groups, and there was no difference between the groups during the suckling period (Fig. 4B). After the weaning, trypsinogen concentrations increased of age. The ratios of the two groups were constant throughout the experimental period, even 3 wk after weaning from the experimental formulas. Amylase concentration in the Prot group was close to that in the DF group at each time point. The amount of amylase in the whole pancreas in the Hydr group was significantly lower than that in the Prot group as well [7.06 ± 1.00 (DF), 6.25 ± 1.22 (Prot), and 2.68 ± 0.45 (Hydr) kU/pancreas at 28 days, 13.01 ± 1.36 (DF), 14.72 ± 2.10 (Prot), and 5.67 ± 0.81 (Hydr) kU/pancreas at 42 days].

The body weights of rats used for sample collection at 14, 21, 28, and 42 days of age are shown in Fig. 2. Dam fed (DF) rats were also weaned onto the same chow diet at 21 days of age. Rats at 14 and 21 days of age were weighed within 30 min after the removal of the diets in the morning. Rats at 28 and 42 days of age were weighed after an overnight fast. Numbers of the animals used for the experiments (Figs. 2–7) are showed above each column. Values are presented as means ± SE.

Fig. 2. Body weights of rats used for sample collection at 14, 21, 28, and 42 days of age. Rats were artificially raised on protein (Prot) or hydrolysate (Hydr) from 7 to 21 days of age, and then weaned onto a standard chow diet. Dam fed (DF) rats were also weaned onto the same chow diet at 21 days of age. Rats at 14 and 21 days of age were weighed within 30 min after the removal of the diets in the morning. Rats at 28 and 42 days of age were weighed after an overnight fast. Numbers of the animals used for the experiments (Figs. 2–7) are showed above each column. Values are presented as means ± SE.

Fig. 3. Pancreas weights of rats artificially raised on Prot or Hydr and DF rats at 14, 21, 28, and 42 days of age. Values are presented as means ± SE. *Significantly different from the Prot group at each age (P < 0.05).

Fig. 4. Concentrations of amylase (A) and trypsinogen (B) to soluble protein in the pancreas of rats raised on Prot or Hydr and DF rats. The tissue samples were taken at 14, 21, 28, and 42 days of age. Values are presented as means ± SE. *Significantly different from the Prot group at each age (P < 0.05).
slightly in both groups; however, no significant difference in trypsinogen concentration between the Prot group and the Hydr group was observed at 28 and 42 days of age. We further compared the total amounts of pancreatic trypsinogen in the two groups at 28 and 42 days of age because pancreas weights in the Hydr group were significantly less than those in the Prot group. As shown in Fig. 5, the amount of trypsinogen in the whole pancreas in the Hydr group was remarkably lower than that in the Prot group at 1 wk and also 3 wk after the weaning. The ratio of the two groups was unchanged (1:0.54 at 28 days, 1:0.63 at 42 days), and the difference between the two groups increased from 28 to 42 days of age. The values of trypsinogen in the Prot group were close to those in the DF group at each time point.

Plasma insulin levels. Insulin is the main stimulator of pancreatic amylase synthesis. The lower amount of amylase observed in the Hydr group was possibly due to the low plasma concentration of insulin. We, therefore, measured plasma concentration of insulin. As shown in Fig. 6, the plasma concentrations of insulin in the Hydr group were significantly lower than those in the Prot group at 14 and 21 days of age. The plasma insulin concentrations in the Prot group were equivalent to those in the DF group.

Ability to increase pancreatic enzyme secretion in response to dietary protein. We evaluated the capability to increase pancreatic secretion of digestive enzymes in response to dietary protein in rats raised on experimental formulas. For this purpose, SBTI, which is a sort of soybean protein and often used as an experimental stimulator of pancreatic enzyme secretion, was administered intraduodenally to the rats 1 wk after the weaning, and the secreted pancreatic juice was collected. Digestive enzyme secretion from the pancreas increased notably after the SBTI administration in the Prot group as well as the DF group (Fig. 7). On the contrary, the response of pancreatic enzyme secretion after the SBTI administration in the Hydr group was significantly weaker than that in the Prot group, and the ratios of the average increases in protein, amylase, and trypsinogen over 60 min after the administration between the Prot group and the Hydr group were 1:0.24, 1:0.30, and 1:0.27, respectively.

Responsive ability of the exocrine pancreas to circulating CCK. Finally, we assessed the ability of the pancreas to increase digestive enzyme secretion in response to circulating CCK in rats raised on Prot or Hydr. As shown in Table 3, absolute amounts of basal protein, amylase, and trypsinogen secretion in the Hydr group were significantly lower than those in the Prot group. Then CCK administration significantly increased the secretion of protein, amylase, and trypsinogen in both formula-fed groups. The absolute amounts of secreted enzymes in the Hydr group were still significantly low, even after CCK administration, compared with the Prot group. However, no significant interaction (formula × CCK) was observed, although the increased rates in the Hydr group were lower than those of the Prot and DF groups.

DISCUSSION

We evaluated the impact of large molecule protein feeding in infancy on pancreatic digestive functions in the developmental stage by means of our rat artificial rearing system and a protein hydrolysate formula. The results demonstrated that large protein feeding during the suckling period is a prerequisite for the proper development of pancreatic digestive functions, and the outcomes can persist long term after weaning.

Our present results showed a new functional aspect of dietary protein in terms of the physiological significance of large protein feeding for infants. From the nutritional point of view, although most human infants fed on protein hydrolysate formula show good body weight gain (36), the large protein in milk formula may enhance the availability of some coexisting nutrients in human infants.
infants (25, 32, 33). On the other hand, the physiological significance of the form of dietary protein for infants has received little attention. Dietary protein is known to stimulate pancreatic enzyme secretion (13), and prolonged intake of a protein-rich diet increases pancreas weight and digestive enzymes in the pancreas (14, 34). In this connection, insufficiency of pancreatic digestive functions was reportedly observed in 9-wk-old rats fed an amino acid diet with milk from dams for about 1 wk around the weaning period (28). The results implied a possibility that appropriate intake of large proteins during the suckling-weaning period might be a prerequisite for the proper development of pancreatic digestive functions. In this study, we have for the first time clearly demonstrated the physiological role of large dietary proteins during the suckling period for the development of pancreatic digestive functions.

The present study suggested that pancreatic digestive functions cannot develop appropriately without repeated stimulation by dietary proteins during the suckling period. Dietary proteins rich in specific peptide bonds that are hydrolyzed by trypsin are supposed to be responsible for triggering pancreatic enzyme secretion, growth of the pancreas, and the synthesis of proteases in the pancreas mainly via CCK release from the small intestine to the circulation (13, 14, 34, 35). The hydrolysate we used in this study includes relatively large peptides (mol wt: 1,500–3,500) (Table 1). In addition, based on the calculation using molecular weight distribution, the total number of peptide bonds and the number of specific peptide bonds for trypsin digestion in the hydrolysate should be about 70% of those in the original whey protein. This can be supported by the fact that the intraduodenal administration of the hydrolysate increased pancreatic enzyme secretion as potently as the whey protein did (data not shown). Nevertheless, the peptide bonds of protein hydrolysates are all naked, and moreover, peptides have a larger number of COOH and NH₄ terminals compared with native proteins. Therefore, protein hydrolysates can be

Table 3. Changes in pancreatic secretion after CCK administration in the protein group and the hydrolysate group

<table>
<thead>
<tr>
<th>Secretion</th>
<th>Group</th>
<th>Basal, mg/10 min</th>
<th>Stimulated, mg/10 min</th>
<th>Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>DF</td>
<td>55.3 ± 5.0</td>
<td>119.8 ± 13.6</td>
<td>216 ± 19</td>
</tr>
<tr>
<td></td>
<td>Prot</td>
<td>46.6 ± 2.9</td>
<td>95.9 ± 3.4</td>
<td>210 ± 10</td>
</tr>
<tr>
<td></td>
<td>Hydr</td>
<td>37.0 ± 1.8*</td>
<td>65.7 ± 4.7*</td>
<td>181 ± 16</td>
</tr>
<tr>
<td>Amylase</td>
<td>DF</td>
<td>17.8 ± 2.5</td>
<td>58.9 ± 11.2</td>
<td>344 ± 53</td>
</tr>
<tr>
<td></td>
<td>Prot</td>
<td>19.6 ± 2.3</td>
<td>57.0 ± 4.4</td>
<td>314 ± 32</td>
</tr>
<tr>
<td></td>
<td>Hydr</td>
<td>12.5 ± 0.9*</td>
<td>34.3 ± 3.6*</td>
<td>277 ± 26</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>DF</td>
<td>23.9 ± 3.5</td>
<td>98.2 ± 16.1</td>
<td>425 ± 73</td>
</tr>
<tr>
<td></td>
<td>Prot</td>
<td>19.2 ± 2.8</td>
<td>72.4 ± 6.4</td>
<td>422 ± 49</td>
</tr>
<tr>
<td></td>
<td>Hydr</td>
<td>11.6 ± 1.0*</td>
<td>40.3 ± 5.6*</td>
<td>359 ± 47</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Rats fed on the protein (Prot) formula (n = 9) or the hydrolysate (Hydr) formula (n = 8) and Dam-fed (DF) rats (n = 10) were used at 28 days of age. Pancreatic juice was collected for 10 min to measure the basal secretion. Next, 50 µl of cholecystokinin (CCK) solution (50 ng/ml saline) was injected into the femoral vein. Pancreatic juice was collected for 10 min immediately after the administration. Secreted amounts were analyzed by ANOVA repeated measures (formula × CCK). Protein secretion: formula, P < 0.05; CCK, P < 0.05; interaction (formula × CCK), not significant. Amylase secretion: formula, P < 0.05; CCK, P < 0.05; interaction (formula × CCK), not significant. Trypsinogen secretion: formula, P < 0.05; CCK, P < 0.05; interaction (formula × CCK), not significant. *Significantly different from the Prot group (P < 0.05).

Fig. 7. Pancreatic enzyme secretory response to dietary proteins in rats raised on Prot or Hydr and DF rats at 28 days of age. Rats were intraduodenally administered 1 ml of soybean trypsin inhibitor (20 mg/ml saline) solution under anesthesia, and the bile-pancreatic juice was collected via a bile-pancreatic cannula. The amount of protein (A), amylase (B) and trypsinogen (C) in the bile-pancreatic juice was measured. Values are presented as means ± SE. *Significantly different from the Prot group at each time point (P < 0.05).
quickly digested by gastric pepsin and various pancreatic enzymes and rapidly lose their trypsin substrate sites and CCK-releasing ability during the gastrointestinal transit compared with the original native proteins. It is most likely that the forms, sizes, and digestibility of dietary proteins can largely affect the potency of their stimulatory effects on CCK release and the exocrine pancreas (12). The practical stimulating effect of dietary protein hydrolysates on the exocrine pancreas does not simply depend on the number of peptide bonds or trypsin substrate sites. On the other hand, pancreatic enzyme secretion is barely stimulated by free amino acids in the intestinal tract in rats, which we used in the present study, whereas in humans and dogs, the stimulatory effect of free amino acids is relatively potent (40, 43). However, pancreatic enzyme secretion appears to be stimulated more strongly by large proteins than by free amino acids in humans (9, 23). Moreover, prolonged intake of an elemental diet without large proteins was reported to significantly decrease pancreatic exocrine secretion in dogs (34). Dietary protein, which cannot be absorbed without adequate digestion, may be a prerequisite for developing infants including human infants.

Recently, the impact of nutrition in infancy on health in later life is being increasingly emphasized (10, 39). In this study, we followed the outcomes of digestive functions modified by the lack of large protein feeding after weaning and found significant differences between the Hydr group and the Prot group even at 3 wk after weaning onto a standard chow diet. It is noteworthy that the differences were increasing, but the ratios remained constant until at least 42 days of age. We also found that pancreatic secretory response was considerably weak in the Hydr-fed rats at 1 wk after weaning. These results suggest that the physiological effects of dietary protein in infancy on digestive functions could persist long term after weaning. Thus studies about long-term outcomes of the lower digestive functions may be necessary. In addition, it is worthwhile to further elucidate whether or not complete catch up of the digestive functions can eventually take place and also when and how long the critical window is.

CCK is well known as the most important regulatory factor of the exocrine pancreas. Our present results strongly suggest that CCK can readily regulate the exocrine pancreas and induce its development in response to large dietary proteins during the suckling period. It has been shown that pancreatic trypsin secretion was significantly decreased by reduced protein intake for 1 mo after birth in human infants (45), suggesting that CCK-mediated stimuli by dietary proteins can promote the development of the pancreas in human infants also. Interestingly, it is more likely that the responsive ability of pancreatic exocrine cells to circulating CCK is still adequate in the Hydr group because no difference was observed between the experimental groups regarding the increased rates of pancreatic secretion in response to CCK injection. In contrast, the results of the SBTI administration study suggest that intestinal CCK endocrine cells cannot develop appropriately without stimuli by large dietary proteins during the suckling period. In 4-day-old human infants, increase in plasma CCK concentrations after feeding was shown to be biphasic, which is different from that seen in 9-mo-old infants or adults (37, 42). These observations might suggest that CCK endocrine cells still are in the developmental stage after birth in human infants also, and dietary factors can affect the developmental process, which may cause the long-persisting lower pancreatic digestive functions.

We observed a low plasma concentration of insulin that might be responsible for the low amylase concentration in the pancreas in the Hydr group. Interestingly, we observed a larger difference in pancreatic amylase concentration between the two groups than in trypsinogen concentration. The observation might suggest that stimulation of insulin to amylase synthesis is more potent than that of CCK to the synthesis of soluble protein and trypsinogen in the pancreas during this period. On the other hand, low plasma insulin concentrations or low pancreatic amylase content are found in animals that experienced malnutrition in infancy (15, 29). Interestingly, several reports showed CCK is an important factor for the maturation of pancreatic β-cells (16), and also CCK is one of the cofactors to stimulate insulin release from β-cells (1). These facts might suggest that proper intake of large proteins is also important for the development of the endocrine pancreas, and the functional value of hydrolysate formula may be similar to that of a low-protein diet. In this study, we couldn’t obtain measurements regarding pancreatic β-cell functions after weaning because weaned rats were used to exclusively evaluate the functions of the exocrine pancreas, thus the long-term impact of protein intake in infancy on the endocrine pancreas and metabolic capacity should be assessed in the future.

In recent years, hydrolysate formulas for healthy infants who may be at risk of allergy are commercially available. Although ESPACI/ESPHAN recommended the use of hydrolysate formulas only to bottle-fed infants with a documented hereditary risk of atopy (a family history of allergic disease) with a clear indication (18), such formulas are quite popular in some countries. From an allergologic point of view, it is in fact still difficult to accurately estimate the potential risk of allergy in infants. Moreover, the practical efficacy of hydrolysate formulas for allergy prevention has still not been proven (4, 11, 31). On the contrary, a few infants can be sensitized to the residual allergenicity of such hydrolysate formulas without any apparent symptoms and experience severe anaphylactic shock when fed on milk allergen (7, 8). In this context, low pancreatic digestive ability that is likely due to hydrolysate formulas may rather be a disadvantage in terms of the degradation of allergenic dietary proteins. On the other hand, the nutritional and physiological values of hydrolysate formulas have received little attention because hydrolysate formula-fed infants usually show proper growth without any notable effect in appearance. However, our current study suggests that growth may not always be a proper index to evaluate hydrolysate formulas, and the results from the study with some commercial infant formulas suggest that hydrolysate formulas may not be equivalent to standard formulas. Evidence showing the physiological importance of large protein feeding in infancy is required hereafter for proper designing and proper choice of infant milk formula (5).

**Perspectives and Significance**

Our present study demonstrated that it is physiologically important that the greater part of amino acids in breast milk constitute large proteins. Some recent studies may also suggest the physiological importance of large protein intake in infancy. First, proteinase-activated receptor 2 on the small intestinal...
epithelium can control some intestinal functions in response to the degradation status of dietary protein in the intestinal tract, apart from CCK-mediation (22). Second, insulin may play important roles in the maturation process of major energy-metabolizing tissues during the suckling-weaning period (29, 21), which might relate to modified metabolic rates such as fatty acid oxidation in CCK-knockout mice (24). Third, modified taste preferences were observed in 4–5/10-yr-old children that were fed on hydrolysate formula in infancy (26, 38). The physiological significance of proteins in infant nutrition should be further elucidated from various and long-term viewpoints and be considered more profoundly.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: T.K. and T.Y. conception and design of research; T.K. and S.K. performed experiments; T.K. and S.K. analyzed data; T.K., E.H., and T.Y. edited and revised manuscript; T.K.

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