Bile-Pancreatic Juice-Independent Increases in Pancreatic Proteases and Intestinal Cholecystokinin by Dietary Protein in Rats (44220)

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Abstract. **Luminal bile-pancreatic juice (BPJ) is involved in the induction of pancreatic proteases in rats fed a high-protein diet. Recently, we have demonstrated that a BPJ-independent mechanism is responsible for enhancement of pancreatic secretion after feeding of a dietary protein in chronic BPJ-diverted rats. The aim of the present study was to explore the existence of a BPJ-independent mechanism during adaptation of the exocrine pancreas to dietary protein. Rats, whose BPJ was diverted into the ileum through a common bile-pancreatic duct catheter for 5 days (PBD rat), were fed a fat-free diet containing 25% or 60% casein for 3 days. Messenger RNA levels for pancreatic enzymes, cholecystokinin, and secretin in the jejunal mucosa were evaluated by northern blotting method. Pancreatic trypsin and chymotrypsin activities and mRNA levels of their zymogens were higher in PBD rats than in rats whose diverted BPJ was returned into the duodenum (PBD returned rat). In the PBD groups, pancreatic protease activities were further increased by 3-day feeding of a high-protein diet without changes in mRNA levels of these proteases. Cholecystokinin mRNA was increased after feeding of a high-protein diet in the PBD rats. These results indicate that pancreatic proteases are induced by feeding a high-protein diet by a mechanism independent of luminal BPJ, which is associated with an increase in intestinal cholecystokinin mRNA level.** [P.S.E.B.M. 1998, Vol 217]

The exocrine pancreas is capable of adapting to dietary composition (1), and feeding a high-protein diet increases pancreatic protease activities. Proteins or amino acids in foods may signal information to the mucosal surface of the small intestine, which is transmitted to the pancreas through hormonal and nervous systems. Another factor involved in the pancreatic adaptation is luminal protease activity in bile-pancreatic juice (BPJ), decreasing in protease activity in the proximal small intestinal lumen increasing pancreatic secretion, and pancreatic protease synthesis.

Pancreatic protease induction, and pancreatic hypertrophy and hyperplasia by dietary protein, is analogous to that induced by administration of trypsin inhibitors or by BPJ diversion. The changes in the pancreas resulting from trypsin inhibitor or BPJ diversion are due to the masking of or a deficit of trypsin activity in the upper small intestine (2, 3), and depend on cholecystokinin (CCK) secretion in rats (4, 5). This enteric hormone is also responsible for the pancreatic adaptation on feeding a high-protein diet in rats (6, 7).

Dietary protein may mask the activities of luminal pancreatic proteases in a manner similar to trypsin inhibitors, and the pancreatic changes seen in normal rats are considered to be due to this masking effect. However, certain compounds, such as amino acids in the intestinal lumen may interact directly with the mucosal surface of the small intestine and induce pancreatic secretion in humans (8) and dogs (9). In addition, we demonstrated previously that dietary protein stimulates pancreatic enzyme secretion by a mechanism independent of BPJ in chronic BPJ-diverted rats (10).

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The aim of the present study was to examine the mechanism of the exocrine pancreatic adaptation to dietary protein, by determining whether the exocrine pancreas adapts to a high-protein diet in rats whose BPJ is excluded from the proximal small intestine chronically. BPJ-diverted rats lack the pancreatic stimulatory mechanism associated with the masking of luminal trypsin activity. To characterize pancreatic adaptation to a high-protein diet in BPJ-diverted rats, we studied the relationship between pancreatic enzyme mRNA levels and pancreatic enzyme activities. Also, to evaluate the role of the small intestine in pancreatic adaptation, we examined changes in intestinal CCK mRNA and secretin mRNA while feeding a high-protein diet to chronic diverted rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan), weighing about 250 g, were divided into three groups after a 24-hr fast. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight; Abbott Laboratories, North Chicago, IL), and had operations to implant cannulas into the common bile-pancreatic duct to divert BPJ. The BPJ was diverted into the ileum (two groups) or the duodenum (one group) through intestinal cannulas as described in a previous report (11). Briefly, a polyethylene catheter (SP 28; i.d. 0.4 mm, o.d. 0.8 mm; Natsume Seisakusyo, Tokyo, Japan) was inserted into the common bile-pancreatic duct, and a silicone catheter (i.d. 0.5 mm, o.d. 1.0 mm; Dow Corning Co., Kanagawa, Japan) for returning BPJ to the lumen was placed through a fistula at 45 cm distal from the ligament of Treitz [Pancreatico-biliary diverted (PBD) rat] or 1 cm proximal to the ampulla of Vater (PBD-returned rat). These catheters were led subcutaneously behind the neck and connected to each other to confirm the flow of BPJ.

After the operation, rats were fed a semi-purified, sucrose-based, fat-free diet containing casein [25% casein diet, Table I (12–15)] for 5 days. Rats in one PBD group had their diet changed to a 60% casein diet (Table I) for 3 days. Rats from another PBD group and the PBD-returned group were fed a 25% casein diet for a further 3 days. Rats in all groups had free access to their test diets. Rats were killed in the postabsorptive state (no dietary chyme in the stomach and the upper small intestine) to minimize influences of enhanced enzyme secretion by diet on the pancreatic enzyme activities. In a previous experiment, we confirmed that trypsin and chymotrypsin activities were not detected in the proximal small intestine in PBD rats (Data not shown).

Table I. Composition of Diets

	25% Casein diet (g/kg diet)	60% Casein diet (g/kg diet)
Intact casein[a]	250	600
Corn oil[b]	50	50
Mineral mixture[c]	40	40
Vitamin mixture[d]	10	10
Granulated vitamin E[e]	1.0	1.0
Choline bitartrate	4.0	4.0
Sucrose	to make 1 kg	

[a] Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).
[b] Retinyl palmitate (7.66 μmol/kg diet) and ergocalciferol (0.0504 μmol/kg diet) were added to corn oil.
[c] The mineral mixture is prepared based on the AIN-76 Workshop held in 1989 (12). It provided (mg/kg diet); Ca, 4491; P, 2997; K, 3746; Mg, 375; Fe, 100; I, 0.32; Mn, 10.0; Zn, 34.7; Cu, 6.00; Na, 4279; Cl, 6542; Se, 1.05; Mo, 1.00: Cr, 0.50; B, 0.50; V, 0.25; Sn, 2.00; As, 1.00; Si, 20.0; Ni, 1.00; F, 2.72; Co, 0.20.
[d] The vitamin mixture was prepared in accordance with the AIN-76 mixture (13) except that menadione and L-ascorbic acid were added to make 5.81 μmol/kg (14) and 284 μmol/kg (15) diet, respectively.
[e] Vitamin E granule (Juvela, Eisai Co., Tokyo) supplied 423 μmol all-rac-α-tocopheryl acetate/kg diet.

A segment of pancreatic tissue (100 mg) of the dorsal area and the 10-cm jejunum segment immediately distal from the ligament of Treitz were removed for extraction of RNA after the collection of 4 ml of portal blood (into a syringe containing aprotinin and heparin) from the rats under pentobarbital anesthesia. The mucosa was lightly scraped from the jejunal segment using a glass slide. The pancreatic tissue and mucosa were immediately homogenized in Isogen (RNA extraction mixture, Nippon Gene, Tokyo, Japan) using a Polytron homogenizer (KINEMATICA, Amlehnhalde, Switzerland). Quantification of extracted RNA was achieved spectrophotometrically (absorbance at 260 nm). Animals were bled *via* the aorta, and

Table II. Hypertrophy in the Pancreas in Rats with Bile-Pancreatic Juice (BPJ) Diversion into the Ileum (PBD) in Comparison with Rats whose BPJ was Returned into the Duodenum (PBD returned), and Additional Increase in Protein/DNA by Feeding a High Protein Diet in the PBD Rats

Group	Weight (wet g)	DNA	RNA (mg/g dry pancreas)	Protein	Protein/DNA
PBD					
25% casein diet	1.59 ± 0.092[a]	51.6 ± 4.51[b]	107 ± 8.37[a]	761 ± 23.4	15.5 ± 1.15[b]
60% casein diet	1.69 ± 0.058[a]	43.4 ± 2.99[b]	105 ± 5.19[a]	826 ± 31.7	19.6 ± 1.32[a]
PBD returned					
25% casein diet	1.11 ± 0.147[b]	75.9 ± 7.40[a]	63.5 ± 5.73[b]	734 ± 30.9	11.5 ± 1.83[c]
P value	0.0015	0.0005	0.0002	0.0902	0.0024

Notes. Values are means ± SEM (nine rats for PBD groups, eight rats for PBD returned group).
[a,b,c] Values not sharing a common superscript letter differ significantly ($P < 0.05$).

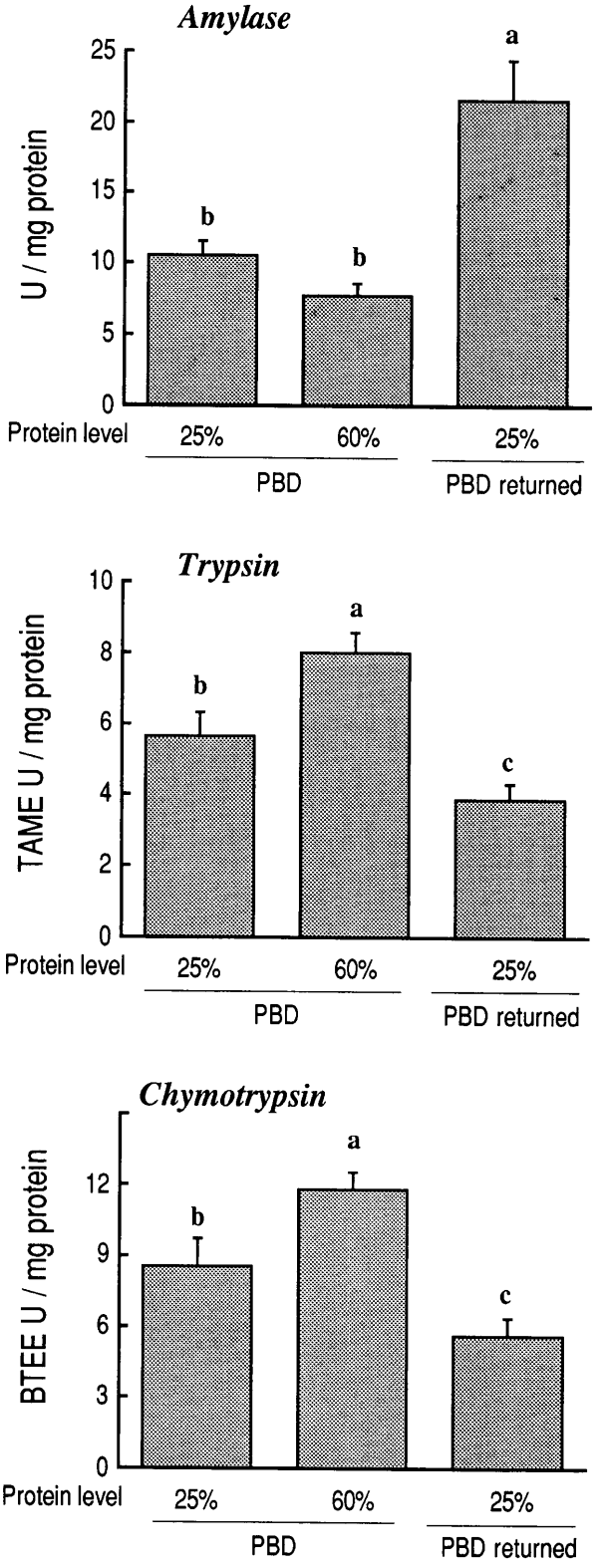


Figure 1. Specific activities of trypsin and chymotrypsin were increased and that of amylase was decreased in chronic bile-pancreatic juice diversion rats (PBD) compared with rats whose BPJ was returned to the duodenum (PBD returned), and activities of the proteases were further increased after 3 days feeding a 60% casein diet. Values were mean ± SE (nine rats for PBD groups and eight rats for PBD returned group). *P* values estimated by one-way analysis of variance were < 0.0001 for amylase, 0.0002 for trypsin and 0.0004 for chymotrypsin. Mean values not sharing a letter are significantly different between diet groups ($P < 0.05$).

the residual pancreatic tissue was removed and frozen in liquid nitrogen.

The study was approved by the Hokkaido University Animal Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Analyses. Enzyme mRNAs in the total pancreatic RNA and CCK, secretin and β-actin mRNAs in polyadenylated [poly(A)$^+$] RNA from the upper (10 cm) jejunal mucosa were quantified by northern blotting method using digoxigenin (DIG)-labeled cDNA hybridization (16, 17). Poly(A)$^+$ RNA was extracted from total RNA of the jejunal mucosa using oligo(dT) latex polymer (Oligotex-dT30 super, Takara Suzo Co., Ltd., Tokyo, Japan). Extracted total or poly(A)$^+$ RNA were electrophoresed on 1% agarose gels, and RNA was transferred from agarose gels to nylon membrane (Hybond-N, Amersham, Oakville, Ontario). The northern blot was hybridized with DIG-labeled trypsinogen I or II, chymotrypsinogen B, amylase, CCK, secretin, or β-actin cDNA, and DIG-labeled hybridized probes, and visualized using DIG-Luminescent-Detection Kit (Boehringer Mannheim, Mannheim, Germany). The intensity of each mRNA band was quantified by exposing the blots to x-ray film and subsequent scanning densitometry (Flying-Spot Scanner, Shimadzu, Kyoto, Japan).

The cDNAs for trypsinogen I or II, chymotrypsinogen B, and amylase were the reverse-transcriptase polymerase chain reaction (RT-PCR) products from total RNA of the pancreas; and for β-actin they were the RT-PCR products from total RNA of the jejunal mucosa. CCK and secretin cDNAs were prepared by RT-PCR from poly(A)$^+$ RNA of the jejunal mucosa. Trypsinogen I and II, CCK and secretin probes were labeled with DIG-PCR (17) from the RT-PCR products using Taq polymerase (Gene Taq, Nippon Gene, Tokyo, Japan), specific primers, and DIG-DNA labeling mixture (Boehringer Mannheim). Other probes were labeled with DIG-DNA labeling kit (Boehringer Mannheim). Primers for cholecystokinin were as described previously (18). Secretin cDNA was prepared using sense primer (position 24–45) and antisense primer (position 428–450) for rat secretin (19). Pancreatic enzymes and β-actin cDNAs were prepared using sense primer (position 46–68) and antisense primer (position 461–486) for rat trypsinogen I (20); sense primer (position 57–76) and antisense primer (position 213–232) for rat trypsinogen II (20); sense primer (position 378–401) and antisense primer (position 906–929) for rat chymotrypsinogen B (21); sense primer (position 700–723) and antisense primer (position 1146–1169) for rat amylase (22); and sense primer (position 31–51) and antisense primer (position 1000–1020) for rat β-actin (23).

Pancreatic ribosomal RNA, blotted on to membranes, was stained with methylene blue solution (0.04%) and quantified by densitometry.

Trypsinogen and chymotrypsinogen in freeze-dried pancreas were activated by enterokinase (Sigma Chemical Co., St. Louis, MO) at 30°C for 20 min in 15 m*M* Tris

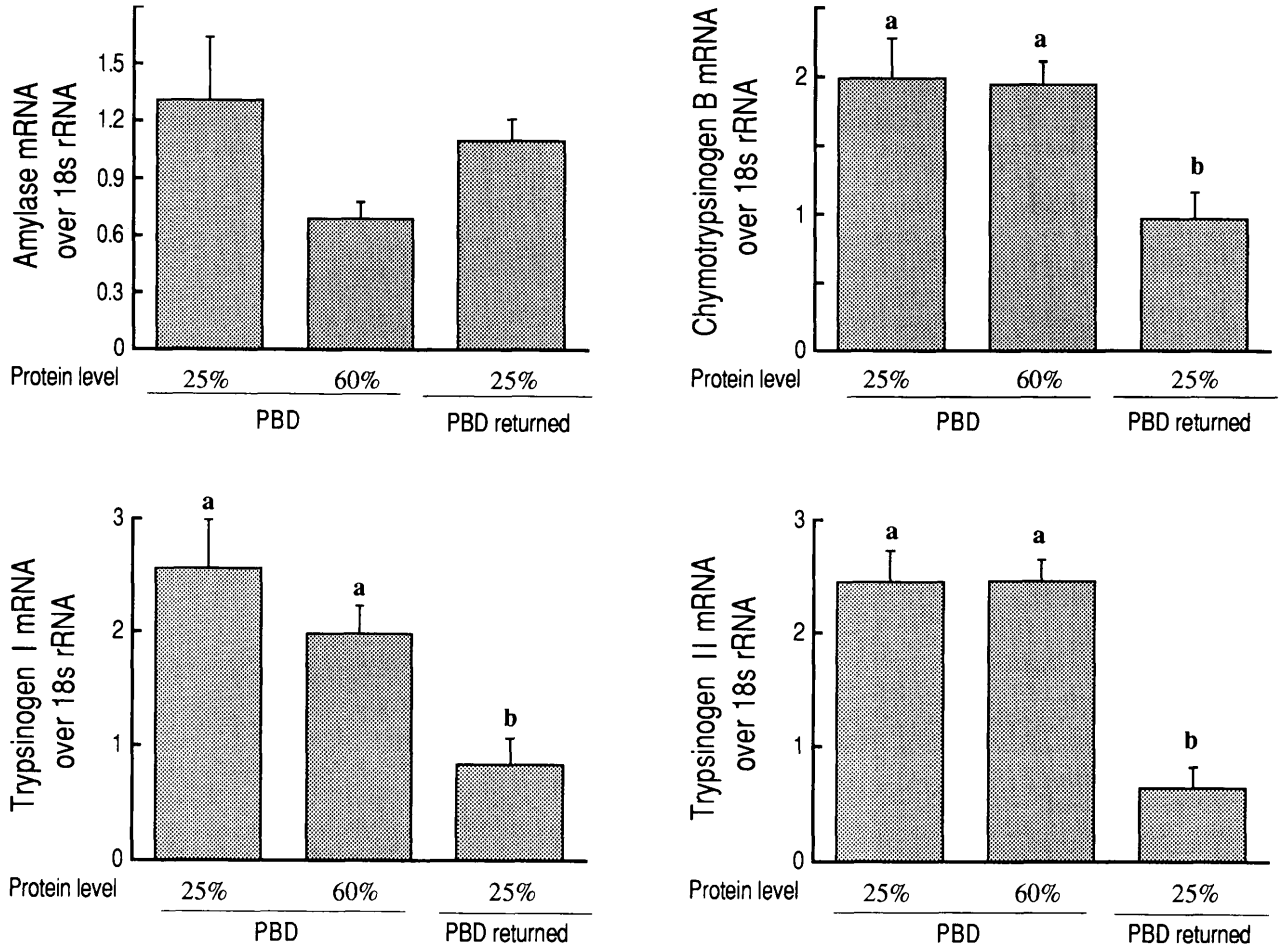


Figure 2. Pancreatic trypsinogen I and II, chymotrypsinogen mRNA were increased in chronic bile-pancreatic juice diversion rats (PBD) compared with rats whose BPJ was returned to the duodenum (PBD returned), and these protease mRNA levels were not further increased after 3 days feeding a 60% casein diet. Values were mean ± SE (nine rats for PBD groups and eight rats for PBD returned group). *P* values estimated by one-way analysis of variance were 0.1568 for amylase, 0.0064 for chymotrypsinogen B mRNA, 0.0023 for trypsinogen I mRNA, < 0.0001 for trypsinogen II mRNA. Mean values not sharing a letter are significantly different between diet groups ($P < 0.05$).

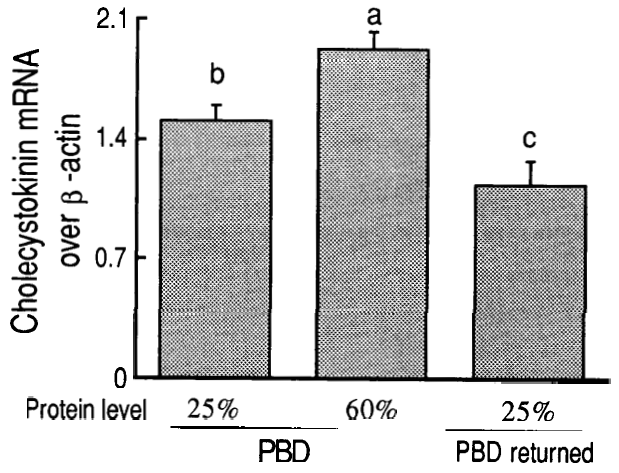
buffer (pH 8.1). Trypsin and chymotrypsin activities were estimated photometrically using synthetic substrates, *N*α-*p*-toluenesulfonyl-L-arginine methyl ester (TAME) (24) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) (25), respectively. Amylase activity in the pancreas was measured with procion yellow starch (26). Protein was measured by a modified version of Lowry's method (27, 28). DNA content was measured by the method of Brunk *et al.* (29) using 4′,6-diamidino-2-phenylindole. The concentration of total RNA was determined colorimetrically by the orcinol method (30) following extraction as described by Fleck and Munro (31).

Plasma cholecystokinin concentration was evaluated following the bioassay procedure described by Liddle *et al.* (32). Portal plasma (2 ml) was passed through a Sep-Pak C18 cartridge, freeze-dried, and incubated with dispersed pancreatic acini prepared from a fasted rat (33, 34). The amylase released into the medium was quantified, and the cholecystokinin concentration in the plasma was derived from a CCK-8 (Peptide Institute Inc., Osaka, Japan) standard curve.

Calculation. The activities of the pancreatic enzymes were expressed as U/mg protein (specific activity) in the residual pancreas (more than 90% of whole pancreas based on wet weight). One unit of trypsin and chymotrypsin was defined as the activity that hydrolyses 1 μmol of substrate/min at 30°C. Procion yellow starch, the substrate for amylase assay, was calibrated by purified α-amylase from porcine pancreas (Type 1A, Sigma Chemical Co.) at 37°C. Cholecystokinin mRNA and secretin mRNA levels were expressed as a ratio of β-actin mRNA. Pancreatic enzyme mRNA was expressed as in a ratio of 18s ribosomal RNA as β-actin mRNA was not detected by the β-actin probe used in intestinal analyses. The data were analyzed by analysis of variance, and the significant difference between groups was determined by Duncan's multiple range test ($P < 0.05$, SAS version 6.07, SAS Institute Inc., Cary, NC).

Results

There was no statistical difference between the groups in body weight gain (3.7 ± 0.8 g/day, $n = 26$, $P = 0.4740$)



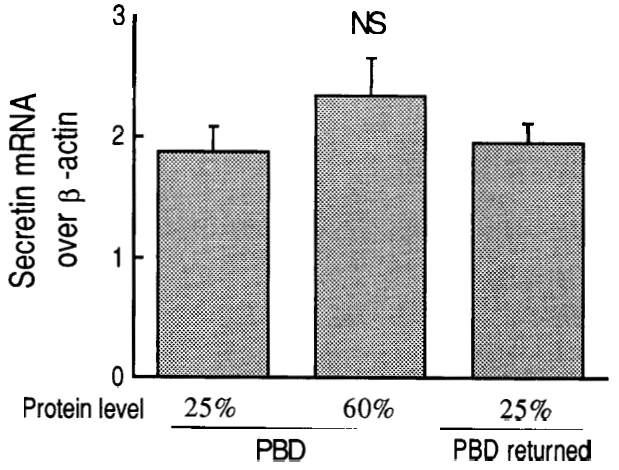


Figure 3. Jejunal cholecystokinin mRNA level was increased in chronic bile-pancreatic juice diversion rats (PBD) compared with rats whose BPJ was returned to the duodenum (PBD returned), and that was further increased after 3 days feeding a 60% casein diet, and secretin mRNA was not significantly changed. Values were mean ± SE (nine rats for PBD groups and eight rats for PBD returned group). *P* values estimated by one-way analysis of variance were 0.0003 for CCK mRNA, 0.3385 for secretin mRNA. Mean values not sharing a letter are significantly different between diet groups ($P < 0.05$).

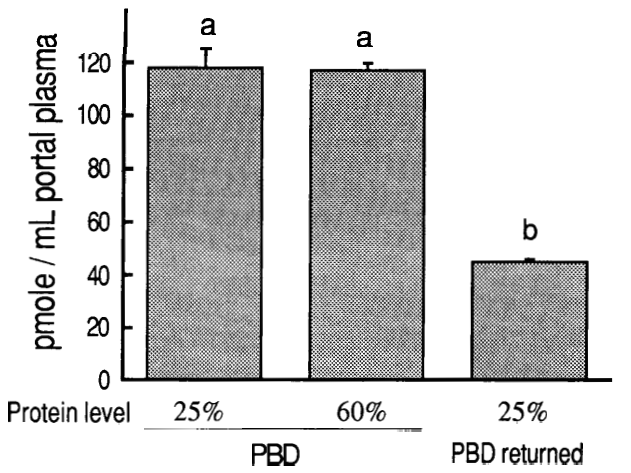


Figure 4. Plasma concentration of CCK was much higher in chronic bile-pancreatic juice diverted rats (PBD) compared with that in PBD returned rats, and the concentration was not increased after 3 days feeding a 60% casein diet. Values were mean ± SE (nine rats for PBD groups and eight rats for PBD returned group). *P* values estimated by one-way analysis of variance was < 0.0001. Mean values not sharing a letter are significantly different between diet groups ($P < 0.05$).

and food intake (16.1 ± 0.87 g/day, $n = 26$, $P = 0.5521$) during the 3-day test-diet period.

As shown in Table II, pancreatic wet weight (whole pancreas) and RNA concentration (residual pancreas) were higher, and DNA concentration was lower in the PBD groups than those in the PBD-returned group. No additional effect of 60% casein diet was observed in these parameters in the PBD group. Protein/DNA, a measurement of hypertrophy, in the PBD groups was higher than that in the PBD returned group, and within the PBD animals was greater in rats receiving the 60% casein diet. Changes in protein concentration tended to be similar to changes in protein/DNA.

As shown in Figure 1, activities of trypsin and chymotrypsin per mg protein significantly increased with a BPJ diversion (25% PBD vs 25% PBD returned), and was further increased by feeding a high-protein diet (60% PBD vs 25% PBD). Amylase specific activity was decreased by a BPJ diversion.

Pancreatic protease mRNA levels, as shown in Figure 2, were significantly higher in rats of the PBD groups compared to the PBD-returned group. In the PBD groups, there were no additional increases in the protease mRNA levels with feeding of 60% casein. Changes in pancreatic amylase mRNA level were not significant.

Changes in CCK mRNA level (Fig. 3) were similar to those in pancreatic protease-specific activities. The CCK mRNA level was significantly increased by a chronic BPJ diversion and was further increased after feeding a 60% casein diet in the PBD rats. Variations in secretin mRNA and CCK mRNA were analogous but were not significant. The portal CCK concentration (Fig. 4) was 3-fold higher in the PBD groups than in the PBD-returned group but remained further unaffected by feeding the 60% protein diet in the PBD rats.

In Figure 5, representative northern blots of pancreatic RNA hybridized with DIG-labeled cDNA for trypsinogen I and II, chymotrypsinogen B, amylase, and rRNA on blots stained by methylene blue (panel A), and of poly(A)$^+$RNA of the jejunal mucosa with cDNA for CCK, secretin, and β-actin (panel B) are shown.

Discussion

The enzymatic activity of the pancreas is determined by both the rate of enzyme synthesis and secretion. In the chronic BPJ-diverted rats (PBD rats), pancreatic protease activities, expressed as trypsin and chymotrypsin, increased in response to feeding a high-protein diet (Fig. 1). These increases appear to be due to an enhancement in the synthesis rates as the rate of secretion in rats fed a high-protein

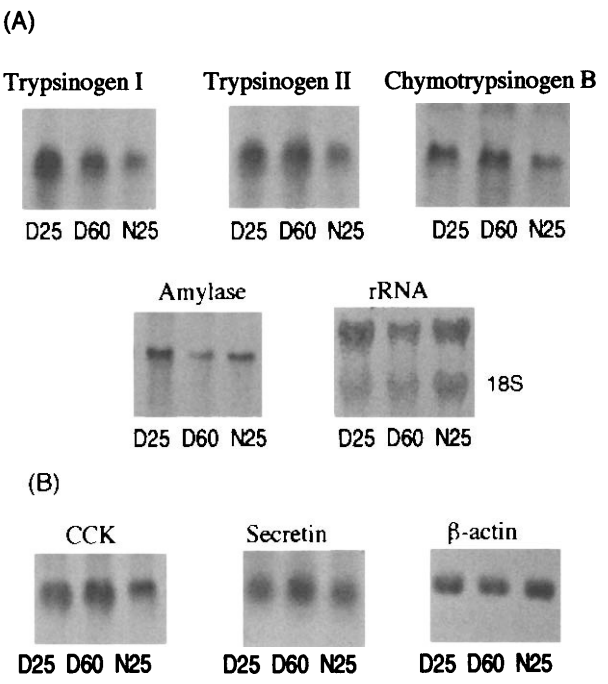


Figure 5. Representative northern blotting of amylase, trypsinogen I and II, chymotrypsinogen mRNA, and ribosomal RNA in the pancreas (A), and CCK, secretin and β-actin mRNA in the mucosa of the upper (10 cm) jejunal polyadenylated RNA (B) in rats of the PBD groups fed a 25% casein diet (D25) and a 60% casein diet (D60) and in rats of PBD returned group fed a 25% casein diet (N25). Hybridization was performed with digoxigenin-labeled cDNA. RNA separated by an electrophoresis (1% agarose gels) was transferred to nylon membrane and exposed to x-ray film.

diet is not lower than that in rats fed a 25% casein diet. These chronic BPJ-diverted rats lacked the regulatory mechanism associated with luminal BPJ, negative feedback regulation (35, 36), and the result indicates that pancreatic proteases are thus induced by a BPJ-independent stimulatory mechanism. However, this induction was not associated with an increase in mRNA level of the protease zymogen (Fig. 2), suggesting that a translational control is involved in pancreatic protease induction by dietary protein in the PBD rats.

There are many reports of pancreatic protease induction by feeding high-protein diets in normal rats. Such induction in normal rats is associated with increases in mRNA levels (37–39), whereas feeding a high-protein diet did not increase mRNA levels of pancreatic proteases in chronic BPJ-diverted rats. A possible explanation for this is as follows. In normal rats, dietary protein induces pancreatic proteases by a mechanism dependent on mRNA increases. The mechanism operates mainly by masking luminal proteases. However, in PBD rats dietary protein induces pancreatic proteases by a mechanism independent of mRNA levels in which dietary protein may interact directly with the mucosal surface or stimulate pancreatic acini after absorption.

As reported here, levels of CCK mRNA in the jejunal mucosa increased following chronic BPJ diversion. This is in agreement with similar changes observed following acute BPJ diversion (18). Furthermore, we showed that CCK mRNA increased after feeding a high-protein diet after a chronic BPJ diversion. This finding suggests that dietary protein affects CCK mRNA with a mechanism independent of luminal BPJ. The fact that plasma CCK concentration was maintained in a high level during a chronic BPJ diversion from the proximal small intestine also suggests that it is unlikely that a luminal BPJ-dependent mechanism is induced in the distal small intestine. If a BPJ-dependent mechanism were induced in the distal small intestine, plasma CCK levels would be reduced by high concentration of pancreatic protease in that part of the intestine. The mechanisms induced in the observed changes remain unclear but are hypothesized that dietary protein may be recognized by the mucosal surface of the jejunum, increasing intestinal CCK mRNA levels and increasing pancreatic proteases in the PBD rats. A BPJ-independent increase in CCK mRNA by dietary protein is further supported by the works of Beucher *et al.* (40) who reported that a glycomacropeptide, peptic hydrolysate of casein, stimulated CCK secretion in the vascularly-perfused duodenojejunum of rats.

In the present study, the concentration of plasma CCK was 3-fold higher in PBD rats than in PBD-returned rats (Fig. 4). The result is in agreement with other reports (41, 42). However, there was no further increase in the PBD group fed a high-protein diet despite the increase in CCK mRNA. This discrepancy may be due to the fact that CCK measurements were made during the postabsorptive state.

In conclusion, the study reported here demonstrates that the induction of CCK mRNA and pancreatic proteases can be achieved by dietary protein through a mechanism independent of BPJ.

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