Red kidney bean lectin is a potent cholecystokinin releasing stimulus in the rat inducing pancreatic growth

K-H Herzig, S Bardocz, G Grant, R Nustede, U R Fölsch, A Pusztai

Abstract

***Background*—Lectins are proteins capable of specific binding to carbohydrates without altering their covalent structure. As an essential part of plants they are ingested in our daily diet. By binding to glycosyl side chains of receptors lectins can mimic or inhibit the action of the ligand. Oral administration of phytohaemagglutinin (PHA) in rats dose dependently induces growth of the small intestine and the pancreas by an unknown mechanism.**

***Aims*—To investigate the mechanism of PHA induced intestinal and pancreatic growth.**

***Methods*—Thirty day old male rats were pairfed for 10 days with lactalbumin as a control diet or lactalbumin plus PHA or purified soybean trypsin inhibitor (STI) as a positive control (42 mg/rat/day) with or without 20 µg of the cholecystokinin A (CCK-A) antagonist MK 329. To investigate further the effect of PHA on CCK release intestinal mucosal cells were isolated from rats which were continuously perfused in a perfusion apparatus. CCK release into the medium was assayed.**

***Results*—PHA and STI significantly stimulated growth of the pancreas and the small intestine. MK 329 blocked this growth effect in the pancreas but not in the small intestine. In vivo, PHA significantly increased CCK plasma levels from 0.75 to 6.67 (SEM 2.23) compared with 2.3 (0.35) pM in the control group. In addition, in vitro PHA dose dependently stimulated CCK release with a maximal effect at 100 ng/ml.**

***Conclusion*—In vivo and in vitro PHA is a potent stimulus for CCK release in the rat, thereby inducing pancreatic growth, whereas intestinal growth is stimulated by a CCK independent mechanism.**

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Keywords: phytohaemagglutinin; cholecystokinin; cholecystokinin A receptor antagonist; pancreatic growth; intestinal growth

I. Department of Internal Medicine, Christian-Albrechts-Universität, Kiel, Germany
K-H Herzig
U R Fölsch

Department of Surgery, Georg-August-Universität, Göttingen, Germany
R Nustede

The Rowett Research Institute, Bucksburn, UK
S Bardocz
G Grant
A Pusztai

Correspondence to:
Dr K-H Herzig,
I. Department of Internal Medicine,
Christian-Albrechts-Universität,
Schittenhelmstraβe 12,
24105 Kiel, Germany.

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Lectins are proteins of a non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrates without altering their covalent structure.[1 2] They are usually detected by their capability to agglutinate erythrocytes, but can also aggregate other cells. First isolated by Stillmark in 1888 from castor beans,[3] the haemagglutinin was called lectin (from Latin *legere*: to choose) according to its ability to select its ligand.[4] Lectins are a ubiquitous plant constituent, but members of the Leguminosae and seeds are especially rich in these sugar binding proteins.[2] They are therefore a major part of our daily food intake. Nachbar and Oppenheim investigated the prevalence of lectins in American food.[5] Of 88 foods tested including common salad ingredients, fresh fruits, roasted nuts, and processed cereals, 29 were found to possess significant lectin-like activity. In addition, review of the literature uncovered 53 other edible plants with haemagglutination activity. Little is known about their biological function except as a protective against insects in seeds, fixation of nitrogen binding bacteria in Leguminosae, or as a storage protein.[6]

Some lectins are at least partially heat stable and most survive passage through the gastrointestinal tract intact. Their effect on the host depends on the carbohydrate specificity of the lectins; for example, by binding to the glycosyl side chains of receptors lectins can mimic or inhibit the action of the respective ligand. Phytohaemagglutinin (PHA) is a lectin present in raw kidney beans; it has strong mitogenic and haemagglutinating activity[7] and comprises five isolectin forms which are destroyed by cooking. Each isolectin contains four subunits in five possible forms (E for erythroagglutinating; L for lymphoagglutinating): E_4, E_3L, E_2L_2, EL_3, L_4.[8] In humans PHA caused severe outbreaks of gastroenteritis among the population of West Berlin in 1948 who received improperly cooked beans as a major nutritional source during the air lift.[9] Noah *et al* reported eight outbreaks of gastroenteritis associated with eating raw red kidney beans in Great Britain.[10] The mechanisms of these antinutritional effects of PHA are unknown. In the rat PHA strongly binds to the epithelial cells of the stomach, the brush border membrane of the small intestine, the caecum, and the colon.[11 12] In contrast to its antinutritional effects it is one of the most powerful growth factors for the rat alimentary tract.[13 14] Oral administration of PHA for 10 days induced dose dependent growth of the small intestine and the pancreas by an unknown mechanism.[15] Gastrointestinal peptides, especially cholecystokinin (CCK), have been shown to be involved in regulation of the

pancreas.[16] In this study therefore, we investigated the possibility that PHA acts by releasing CCK.

Materials and Methods

MATERIAL

Animals used were male Sprague-Dawley rats from Harlan-Winkelmann, Borchem, Germany or male Hooded-Lister rats of the Rowett strain. N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and bovine serum albumin (BSA) (fraction V) were purchased from E. Merck (Darmstadt, Germany), collagenase from Bayer Diagnostic (München, Germany), the calcium ionophore A23187 from Boehringer Mannheim, and Sephadex G-50 from Pharmacia. The highly potent peripheral CCK A-receptor antagonist MK 329 was a gift of Merck Sharp & Dohme.

PURIFICATION OF LECTINS

Ground kidney bean (100 g) was extracted by acetic acid and centrifuged; the supernatant was dialysed against eight changes of distilled water and freeze dried.[17] This preparation contained 35% PHA isolectins mostly of L-type PHA, 6–8% trypsin inhibitor, and 14–16% amylase inhibitor and uronic acid containing polysaccharides. The lectin preparation was then further purified by affinity chromatography on a fetuin-Sepharose-4B column.[18] Mixed E- and L-type PHA isolectins were eluted with a 0.05 M glycine-HCl buffer, pH 3.0, containing 0.5 M NaCl. The eluate was dialysed for desalting and after pH adjustment to 6.8 freeze dried. The material obtained from this preparation was used in the study. Soybean trypsin inhibitor was purified from defatted soybeans by affinity chromatography on anhydrotrypsin-Sepharose-4B.[19]

IN VIVO EXPERIMENTS

Rats (about 80 g) were divided into six groups and kept in metabolic cages throughout the experiment. Before and at the end of the experiment (ninth day) blood samples were drawn from the tail vein under local anaesthesia. Groups 1 and 2 were fed 6 g lactalbumin (control) diet per day for 10 days. The test groups were also given 6 g of this diet but for groups 3 and 4 the diet also included 42 mg PHA daily and for groups 5 and 6, 42 mg soybean trypsin inhibitor (STI), used as an internal positive control. Diets were made by including these factors into the diet at the expense of an equivalent amount of lactalbumin in the control diet. Rats in groups 2, 4, and 6 were also given 20 μg CCK-A receptor antagonist MK 329 mixed into the diet. In the morning of the last (tenth) day of the experiment, two hours after their last meal of 1.5 g of their respective diet, rats were killed by a halothane overdose, and the small intestine and pancreas were removed, weighed, and freeze dried.

Histology

Rats were killed two hours after the morning feed and small sections, 2 cm each, one 5–7 cm from the pylorus and a second 5–7 cm from the ileo-caecal valve, were taken, slit open, stuck to a piece of cardboard with the mucosa side up, and fixed in 4% buffered formalin. The fixed sections were embedded in paraffin wax, sectioned (3 μm), and stained with haematoxylin and eosin. Ten randomly selected crypts were examined under the microscope.[11 12]

Protein content was determined by a modified Lowry method.[20] DNA measurements were performed according to Løvtrup.[21]

CCK RADIOIMMUNOASSAY

Blood samples were immediately centrifuged (10 min at 2876 *g* and 4°C) and stored at –20°C. Plasma was extracted with absolute alcohol to remove unspecific plasma interference, centrifuged, and freeze dried. CCK was measured using a published method, modified by using the specific and sensitive antibody G160.[22 23] The antibody generated against CCK-33 recognises the sulphated tyrosine group in the C-terminal part of the peptide. It cross reacts with sulphated gastrin-17 at about 1.5% with less than 0.001% cross reactivity against non-sulphated gastrin or other gastrointestinal peptides. Final dilution of the antibody was 1/100 000. Sulphated CCK-8 in a concentration range of 0.39–50 pmol/l was used as a standard. Radioiodine labelled CCK-8 (2200 mCi/mmol) was purchased from New England Nuclear (NEN), Boston, Massachusetts, USA. The dried blood samples were dissolved immediately prior to determination in 0.02 M veronal buffer (pH 8.4 with 0.1% BSA). The recovery rate averaged 92%. Samples and standards were incubated with the diluted antibody for 72 hours before the labelled tracer was added for an additional 48 hours. The bound fraction was separated from the unbound fraction by charcoal. The sensitivity of the assay was 0.75 pmol/l, the intra-assay variance 5.8%, and the interassay variance 8.3%.

IN VITRO EXPERIMENTS

Dispersed intestinal mucosal cells were prepared as described by Bouras *et al*[24]: 20 cm of the proximal small bowel of male Sprague-Dawley rats, 2 cm distal to the pylorus, was removed, washed with saline, and incubated for 10 minutes in 15 ml of oxygenated calcium free KHB buffer containing 2.5 mM EDTA. After gentle shaking of the incubation flask the remaining tissue was removed and the cell suspension centrifuged for three minutes at 100 *g*. The pellet was washed twice with fresh KHB buffer with calcium and filtered through a 200 μm gauze. Two ml of the cell suspension was mixed with 2 ml of preswollen Sephadex G-50 medium resin and perfused in a perfusion apparatus at 1 ml/min.[25] After a 50 minute period perfusates collected for two five minute periods were taken as basal level followed by three five minute periods in which the stimulatory agent was added. The 15 minute stimulation period was followed by four additional five minute periods. CCK release into the medium was assayed using a pancreatic acini bioassay system as described previously.[26] Amylase release was measured using an autoanalyser

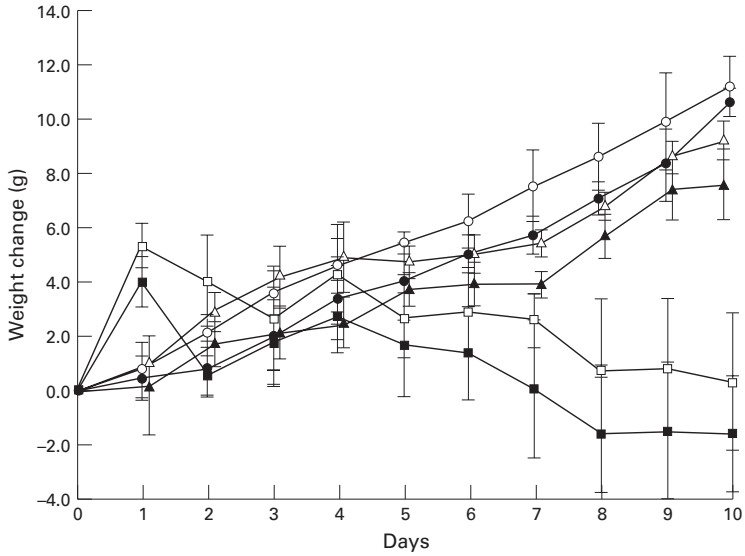

Figure 1: Growth of rats fed control (lactalbumin) diet (open circles), control diet + MK 329 (closed circles) or diets containing kidney bean lectin (PHA) (open squares), PHA + MK 329 (closed squares), STI (open triangles), or STI + MK 329 (closed triangles) for 10 days (initial weight 82 (1) g; daily food intake 6 g/rat).

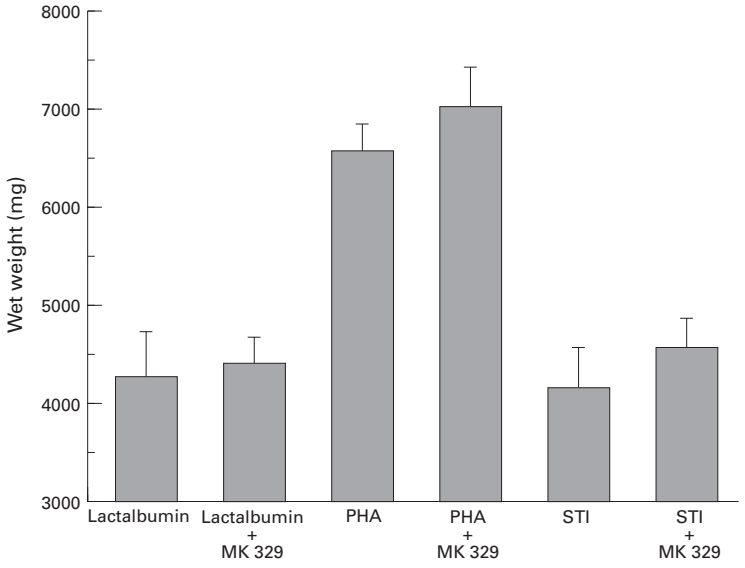

Figure 2: Effects of lactalbumin, PHA, and STI with or without MK 329 on growth of the small bowel (n=5; p<0.05).

(Eppendorf ACP 5040, Hamburg, Germany) according to the method of Kruse-Jarres *et al*.[27]

STATISTICS

Results are expressed as mean (SEM). Data were analysed using analysis of variance by the Minitab computer program (Scottish Agricultural Statistics Service, Edinburgh, UK) or Student's *t* test where appropriate. Statistical significance was set at $p<0.05$.

Results

IN VIVO STUDIES

Over the 10 day experimental period the PHA fed rats lost weight progressively although the rats were pair-fed (fig 1). At day 10 the weight difference in comparison with the lactalbumin control group was approximately 15%. The weight reduction in the PHA fed animals compared with the pairfed control animals suggests a reduced efficiency of nutrient utilisation. The peripheral CCK-A receptor antagonist MK 329 had no effect on weight development of the rats over the 10 day experiment, excluding the possibility of CCK induced satiety. PHA significantly increased the weight of the small bowel compared with the control and STI diet. MK 329 had no effect on PHA stimulated small bowel growth suggesting that PHA stimulates small bowel growth by a direct effect (fig 2). There was no evidence of lymphocytic infiltration or damage of the villi. PHA and STI significantly induced pancreatic hypertrophy (fig 3) with an increased protein content (fig 4) without changes in the DNA content (data not shown). The trophic effect of PHA and STI on the pancreas was significantly blocked by the CCK-A receptor antagonist by 72% and 69%, respectively, indicating a CCK dependent mechanism. CCK levels were significantly stimulated in PHA fed rats (fig 5). STI increased CCK levels but not significantly. In both groups the CCK-A receptor antagonist MK 329 further increased CCK plasma levels; it had no effect in the lactalbumin fed group.

IN VITRO STUDIES

To investigate further the mechanism by which PHA stimulates CCK secretion we perfused dispersed intestinal mucosal cells. In this perfusion system KCl (52 mM) and the calcium ionophore A23187 (1 μM) stimulated CCK release to 10 (1.27) and 8.9 (2.33) pM respectively. Both substances served as internal positive controls in our perfusion system. PHA dose dependently stimulated CCK release with a maximal effect at 100 ng/ml (fig 6). CCK release was unaffected by the presence of the neurotoxin tetrodotoxin, indicating that PHA directly stimulates CCK release with no neural involvement.

Discussion

Lectins are part of our daily diet, yet little is known about their effects in the intestinal tract. Their proteolytic resistance accounts for their physiological and nutritional effects. PHA binds to complex oligosaccharide structures of the small intestinal epithelial cells.[28] Diets containing raw kidney beans supplying 5–10% dietary protein caused severe damage to the epithelial cells of rats with vesiculation and shorting of the microvilli resulting ultimately in the death of the animals.[29 30] The mechanism of this PHA effect is unknown. PHA has been shown to induce bacterial overgrowth.[31] Germ free rats did not show this toxic effect of PHA suggesting that its toxicity depends on the presence of bacteria in the gut.[32]

For our experiments we used a daily intake of 42 mg of highly purified isolectins which were free of fibre or trypsin/amylase inhibitors present in the crude extract. There was no evidence of lymphocytic infiltration in the duodenum or pancreas or any damage to the villi during the course of the experiment; changes at the microvillous level could not be excluded. Rats challenged with a single bolus of 300 mg pulverised raw kidney beans, evaluated by elec-

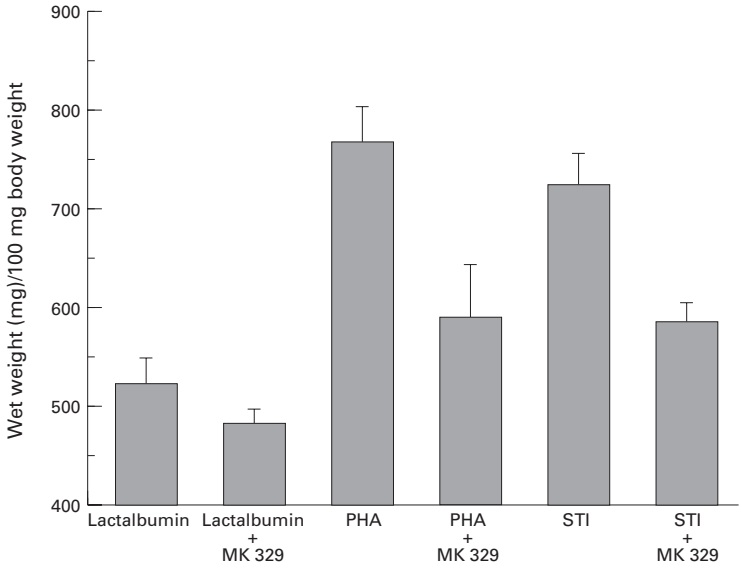

Figure 3: Effects of lactalbumin, PHA, and STI with or without MK 329 on growth of the pancreas (n=5; p<0.05).

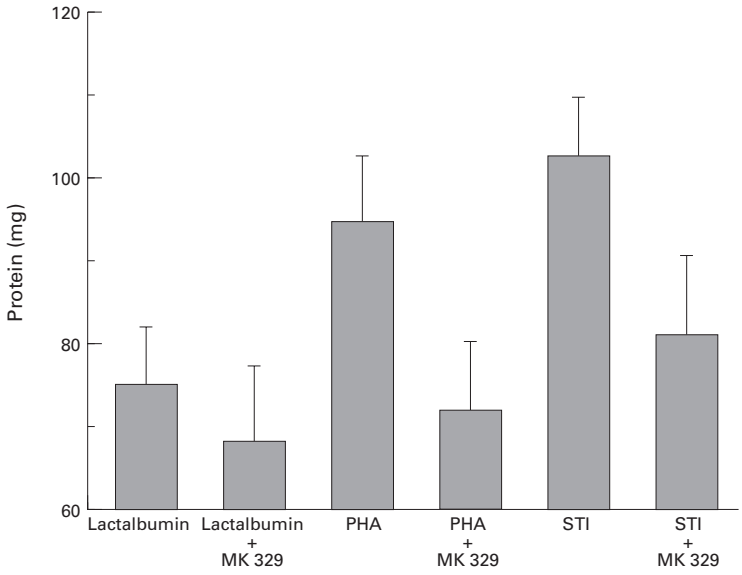

Figure 4: Effects of lactalbumin, PHA, and STI with or without MK 329 on protein content of the pancreas (n=5; p<0.05).

tron microscopy, showed a reduction in the entire villus length and vesiculation and shortening of the microvilli.[33] In contrast, using purified PHA, Bardocz *et al* found a noticeable increase in villus height after exposure to 42 mg purified PHA for three days.[34]

PHA significantly stimulated small bowel growth by a CCK independent mechanism and pancreatic hypertrophy by a CCK dependent mechanism as the CCK-A receptor antagonist MK 329 significantly inhibited PHA induced pancreatic but not intestinal growth. MK 329 (or L-364718)—a benzodiazepine derivative—is a highly potent peripheral CCK-A receptor antagonist which can be applied both intravenously or orally.[35] We obtained 72% inhibition of PHA stimulated pancreatic growth using 20 µg MK 329 in the diet; this is in good agreement with results reported by Schmidt *et al* who showed that MK 329 at a dose of 0.1 mg/kg given by gavage reduced the trophic effect of caerulein on pancreatic wet weight by 50–60% while 1 mg/kg MK 329 almost completely inhibited the caerulein induced trophic effect.[35]

In addition to the effect of PHA in vivo, we showed that PHA directly and dose dependently released CCK from isolated intestinal mucosal cells in vitro. In this system, CCK release has been shown to be induced by short term exposure to depolarising concentrations of KCl and the calcium ionophore A23187.[24] The mechanism of lectin induced CCK release from the CCK cells in the intestine is unknown. We hypothesise that lectins—by binding to the glycosyl side chains of voltage gated channels—might influence their activity.[36]

Banwell *et al* investigated the effect of PHA on small intestinal growth in control and germ free rats.[14] They showed a direct hyperplastic response of the small intestinal epithelial cells without influence of any bacteria. However, the intestinal flora augmented the growth effect of PHA on the small intestine which could be partially blocked in their study by oral antibiotics. There were no changes in tissue content and plasma levels of gastrin, enteroglucagon, glucagon, and peptide YY indicating that these peptides did not mediate the trophic response of the small intestine to PHA. Recently, Bardocz *et al*[34] reported that a single dose of PHA fed by gavage to rats transiently depressed plasma insulin levels. Feeding of a kidney bean diet for 10 days lowered the insulin content of the pancreas by an unknown mechanism. Insulin has however been shown to have a trophic effect on the pancreas[37] and the intestine.[38 39] Therefore, the observed stimulation of pancreatic and small intestinal growth are mediated by other mechanisms and mediators.

Polyamines play a major role in the growth of the gastrointestinal mucosa and the pancreas.[40–42] Polyamine accumulation has been demonstrated in PHA stimulated small bowel and pancreatic growth.[13 43] In both organs, activity of ornithine decarboxylase, the first enzyme of de novo synthesis and the rate limiting step, was slightly or not elevated at all during PHA treatment. Therefore the polyamines must be derived from the lumen for small bowel growth[40] or via the polyamine interconversion pathway and/or polyamine uptake from the circulation for pancreatic growth.[44] The bacterial microflora contribute to the supply of luminal polyamines; this explains the findings of previous studies, that in germ free animals or in animals treated with antibiotics, PHA had a smaller effect.

CCK is a potent stimulator of pancreatic growth,[16] but its significance as a regulator of small bowel growth is unclear. In dogs and rats on total parenteral nutrition CCK infusions prevented mucosal atrophy[45 46]; other studies however could not show a trophic effect of CCK on small bowel mucosa.[47–49] In our study the CCK-A receptor antagonist MK 329 did not inhibit PHA stimulated small bowel growth, while the antagonist blocked the effect

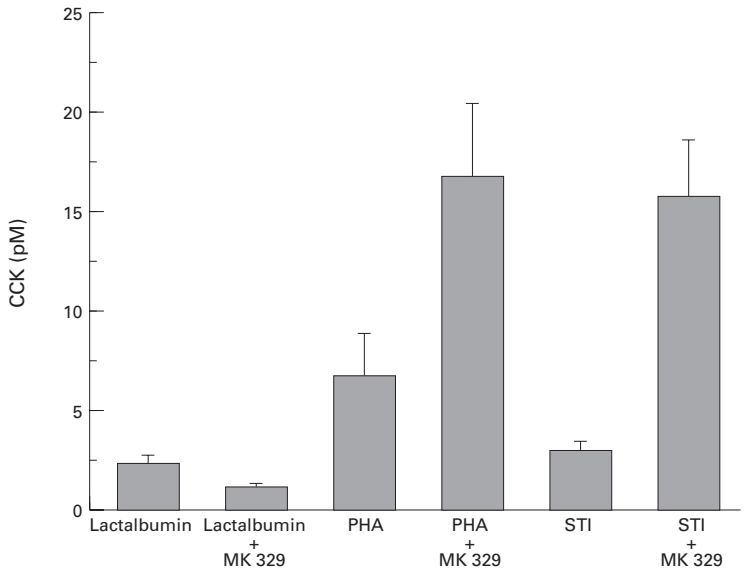

Figure 5: Effects of lactalbumin, PHA, and STI with or without MK 329 on CCK plasma levels (n=5; p<0.05).

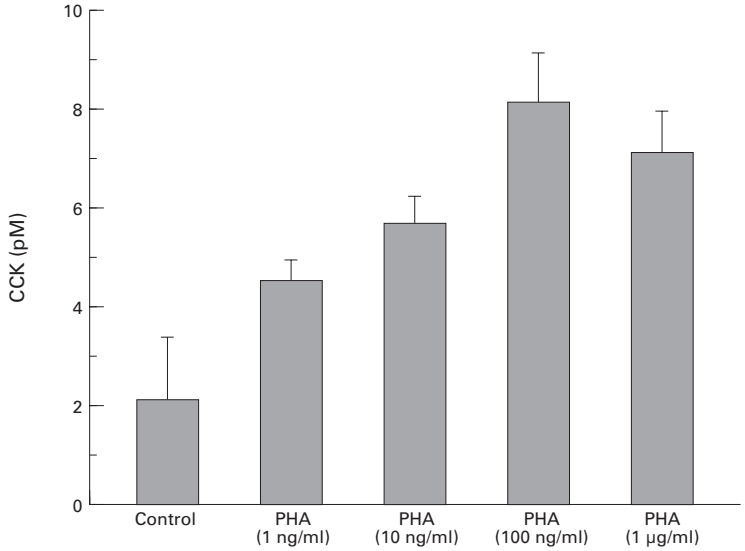

Figure 6: Dose dependent effect of PHA on CCK release from rat isolated intestinal mucosal cells (n=6–12; p<0.05).

of PHA on the growth of the pancreas. These findings suggest that PHA has a CCK independent growth promoting effect on the small bowel. Lectins are able, for example, to stimulate proliferation of colonic cells directly.[50 51]

The CCK-A receptor antagonist MK 329 further stimulated CCK plasma levels except with the control diet. Basal plasma CCK levels are not potentiated and only slightly elevated at higher concentration by the CCK-A receptor antagonist in humans.[52] In addition, loxiglumide, another potent CCK receptor antagonist, did not elevate basal plasma CCK levels.[53–55] Potentiation of CCK plasma levels by the CCK-A receptor antagonist only occurs in the stimulated state. Liddle *et al*[52] proposed that the antagonist causes a complete blockade of the pancreatic CCK-A receptors resulting in diminished pancreatic secretion and duodenal protease concentration which in turn triggers CCK release. Schmidt *et al*[56] suggested a negative short loop feedback control of CCK release by which CCK inhibits its own secretion or by the suppression of bile secretion in humans. Lactalbumin did not stimulate CCK release; therefore the CCK-A receptor antagonist did not further increase CCK plasma levels.

STI was used as an internal positive control in our study. It had no effect on small bowel growth but stimulated pancreatic proliferation. The stimulation was blocked by the CCK-A receptor antagonist MK 329 indicating a CCK dependent mechanism. We did not measure elevated CCK plasma levels by STI alone, only in combination with the CCK receptor antagonist. Green *et al* reported that CCK plasma levels were elevated on acute exposure to STI and then gradually declined during chronic exposure, depending on the protein content of the diet.[57] The reason we did not detect significantly elevated CCK levels induced by STI might be due to the timing of blood sampling, and the amount of STI and dietary protein used in this study.

In conclusion, our results indicate that PHA stimulates small bowel growth by a CCK independent mechanism. In contrast, PHA releases CCK from the duodenal mucosa, which in turn stimulates pancreatic growth.

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