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Potato protease inhibitors inhibit food intake and increase circulating cholecystokinin levels by a trypsin-dependent mechanism

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Abstract

Objective—To investigate the mechanisms underlying the satiety-promoting effects of a novel protease inhibitors concentrate derived from potato (PPIC).

Methods—Acute and prolonged effects of oral PPIC administration (100 mg/kg per day) on food intake, body weight, and gastric emptying were evaluated in healthy rats. Parameters of body weight, food intake, plasma glucose, insulin, and cholecystokinin (CCK) were measured. Duodenal proteolytic activity and CCK expression were determined in tissue extracts. Intestinal STC-1 cell culture model was used to investigate the direct effect of PPIC on CCK transcript level and secretion.

Results—Acute oral administration of PPIC reduced immediate food intake during the first two hours following the treatment, delayed gastric emptying, and decreased proteolytic activity in the duodenum. Repeated oral ingestion of PPIC reduced weight gain in male rats and significantly elevated the plasma CCK levels. Although duodenal mucosal CCK mRNA levels increased in response to PPIC administration, the concentrate failed to elevate CCK expression or release in STC-1 cells. The 14-day ascending dose range study (33 to 266 mg/kg PPIC per day) showed no adverse side effects associated with PPIC administration.

Conclusion—These findings provided evidence that PPIC is effective in reducing food intake and body weight gain in healthy rats when administered orally by increasing circulating CCK levels through a trypsin-dependent mechanism.

Keywords

satiety; body weight; cholecystokinin; protease inhibitors; potato

Introduction

Restriction of energy intake remains the most effective way to lose weight and improve glucose control in individuals with obesity or diabetes. As such, obesity is normally treated

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Conflict of interest

IR serves as a board member of Phytomdics Inc.

by diet and exercise, but attempts to sustain significant weight loss by lifestyle intervention often fail (1). Recent data suggest that the 2-year persistence rate with orlistat or sibutramine, the only FDA approved drug therapies for obesity, does not exceed 2% (2). Therefore, novel therapeutics to reduce food intake and body weight with minimal adverse reactions are highly desirable.

One way to improve dietary adherence rates in clinical practice may be to enhance satiety through the use of protease inhibitors. For a long time, plant protease inhibitors were considered as major anti-nutritional agents (3). Their presence in many seeds and tubers in high amounts has caused much speculation as to whether these inhibitors have any role in the control of proteolysis during development of plant tissues (4). The idea that protease inhibitors would interfere with animal digestive processes subsequently led to the discovery of a many serine protease inhibitors capable of protecting plants from mammalian digestive enzymes (5).

Potato tuber is the source of potato protease inhibitor II (PI2) active in eliciting a satiety response (6) and delayed gastric emptying in humans (7). While several methods to isolate and purify PI2 have been developed over the years on a laboratory scale, all of them are laborious and expensive (8–11). Potato protein recovery is also often complicated by interactions with non-protein components of potato tubers that lead to poor solubility and reduced biological activity of the protein fraction, thus hampering the potential therapeutic applications (12). Given the low yield and complexity of PI2 isolation, we thought it was important to modify the extraction procedure and test the satiation activity of a crude potato protease inhibitor concentrate (PPIC) that contains several thermostable protease inhibitors, including PI2. As PPIC showed potential satiety-promoting activity *in vivo*, the additional aim of this study was to examine the mechanism of reduction of food intake following PPIC administration. It was suggested that PI2 promotes satiety by increasing circulating levels of cholecystokinin (CCK) similar to soybean trypsin inhibitor (13), however neither the effects nor the mechanisms underlying these acute or prolonged effects of PI2 or PPIC administration have been investigated previously.

The gastrointestinal tract is rich in endocrine and neuronal cells that synthesize and secrete CCK (14). CCK is released in response to intraluminal stimuli associated with ingestion of a meal, and has been shown to reduce food intake and elicit behaviors associated with satiety in mammals (15). Similar to humans (16), CCK secretion is inhibited by trypsin and chymotrypsin activity in the proximal small intestine in rats (17). The presence of an intraluminally secreted, trypsin-sensitive CCK-releasing intestinal factor mediating this response has been suggested as a result (18), and the active luminal CCK-releasing factor LCRF was purified several years later (19). Therefore, this study was undertaken to test the hypothesis that orally administered PPIC reduces food intake by modulating intraluminal proteolytic activity and therefore interfering with protease-induced negative feed back loop that controls CCK secretion.

Materials and methods

Preparation of potato proteinase inhibitors concentrate (PPIC)

PPIC was prepared using an improved purification method based on previously reported extraction procedures (9, 12) and relevant information on abundance and distribution of protease inhibitors in potato juice (11). One kg of potato tubers was homogenized with 400 ml of the extractant buffer (sodium chloride: acetic acid: water 1:1:9, w:v:v) using a handheld commercial blender for 5 min at room temperature. The resulting mixture was centrifuged at 13,000g for 10 min and filtered through several layers of Whatman filter paper. The resulting liquid (450 ml) was transferred to an Erlenmeyer flask and heated to 70 °C on a hot plate while stirring. The solution was rapidly cooled to 30 °C and centrifuged at 13,000g for 10 min to pellet the visible precipitate of denatured proteins. Thermostable proteins from the clarified potato extract (400 ml) were precipitated with addition of 100 g of sodium chloride and centrifugation at 13,000g for 10 minutes to isolate the visible pellet, designated as the wet PPIC. At the final step, the wet PPIC was dissolved in 50 ml of water and loaded onto a gel filtration column (a total bed volume of 150 ml) packed with Bio-Gel P-6 DG matrix (Bio-Rad, Hercules, CA). The first three 20 ml fractions were discarded, while the next six fractions were pooled, lyophilized, and designated as PPIC used in animal and cell culture studies. Potato protease inhibitor II (PI2) was further isolated from PPIC concentrate according to the method of Pouvreau et al (11).

PPIC analysis

PPIC samples were analyzed on 12% polyacrylamide gel/ Coumassie Brilliant Blue G-250 staining for protein content and protease inhibitory activity was visualized using a slightly modified method (20). Briefly, nondenaturing PAGE gels were treated with trypsin or chymotrypsin and subsequently developed in the presence of *N*-Acetyl-phenylalanine-naphthylester and o-dianisidine tetrazotized for 10 min at 37 °C (21). After visualization, major protein bands were excised from the SDS-polyacrylamide gel, and MALDI-TOF protein spectra were recorded on Bruker Reflex III mass spectrometer following the service protocols of Mass Spectrometry Core Facility (University of Guelph, Canada).

Animals

Male Wistar rats (180–200 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats were housed individually under controlled temperature (24 ± 2 °C) and light (12 h light-dark cycle, lights on at 0200 h) with access to AIN-93 standard diet (Research Diets, New Brunwick, NJ) and tap water *ad libitum*. Following an adaptation period of 1 week, animals were introduced to daily 6 h food deprivation (beginning at 0800 h) and a daily gavage at 1400 h until it was determined that the process of gavaging did not significantly affect the daily food uptake (1 week). The 6 h fasting time prior to PPIC administration was introduced to eliminate variation in feeding patters among different animals by ensuring that all animals did not consume food immediately before the treatment. For all studies, animals were randomized into groups (n=6–8) with approximately equal mean body weight (within 2 g). Body weight and food consumed by each animal were determined daily by differential weighing after correcting for spillage on balances accurate to 0.01 g. All animal experimental procedures were carried out at the AAALAC-accredited Cook Animal Facility

in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and approved by Rutgers IACUC.

Acute administration of PPIC and PI2 to rats

Three groups of rats fasted for 6 h received a single *per os* dose of control solution (100 mg/kg casein), PPIC concentrate (100 mg/kg), or purified PI2 (100 mg/kg) in a total volume of 2 ml distilled water at the beginning of the feeding cycle (lights off, 1400 h). Food intake was measured at 1, 2, and 24 h postgavage and corrected for spillage. We included casein in the control treatment to account for possible effect of protein supplementation on food intake and satiety (22).

Effect of PPIC administration on gastric emptying and proteolytic activity

Gastric emptying was measured essentially as described by Shi el al (23). In short, the pre-warmed 40% peptone meal suspended in 1 ml of distilled water and supplemented with 1 mg/ml of phenol red (non absorbable dye marker) was given orally through a stainless steel tube immediately following the gavage of 100 mg/kg of PPIC (treatment group) or 100 mg/kg casein (control group) in 2 ml of distilled water. One hour after the treatment, all animals were sacrificed. Their stomachs were immediately ligated, opened, and gastric content was collected in graduated tubes. The phenol red concentration in each stomach was determined spectrophotometrically at 520 nm (Molecular Devices, Sunnyvale, CA). Phenol red concentration found in stomachs of animals sacrificed immediately after administration of the peptone meal (baseline group) served as a 100% reference point.

In the same set of experiments, the remaining proteolytic activity in the duodenum washes was recorded 1 hour following the treatment. Duodenum was ligated, opened, and duodenal content was collected in Eppendorf tubes with 0.5 ml of PBS buffer. Total serine protease activity in the samples was estimated as described elsewhere (20), using standard curve of known trypsin concentrations. Proteolytic activity found in duodenum of animals treated with casein (control group) served as a 100% reference point.

Repeated PPIC administration

Two groups of rats fasted for 6 h were provided with a single *per os* dose of control solution (100 mg/kg per day casein) or PPIC concentrate (100 mg/kg per day) in a total volume of 2 ml distilled water for 10 days. Food intake and body weight gain were recorded daily prior to the treatment. At the end of experiment, plasma CCK levels were measured immediately before and 15 min after the animals were dosed with PPIC in submundibular vein blood samples using indirect quantification of CCK-induced amylase release from isolated rat pancreatic acini (13). Blood was collected by cardiac puncture at the time of sacrifice into EDTA-coated tubes. Plasma glucose (colorimetric assay kit, Sigma, St. Louis, MO) and insulin (ELISA kit, Linco Research, St. Charles, MO) levels were measured.

PPIC toxicity studies

PPIC toxicity was investigated following daily oral gavage administration to healthy rats for 14 consecutive days by ITR Laboratories, Quebec, Canada (study no 7392). Twenty five male and 25 female rats were randomized among five groups of rats (n=10) and treated with

0, 33, 66, 133, and 266 mg/kg body weight of PPIC. In-life observations included mortality, cage-side clinical signs (ill health, behavioral changes), body weights and daily food consumption. Clinical pathology testing was performed on all animals at termination and included hematology (red blood cell count, white blood cell count, hematocrit, hemoglobin, mean corpuscular hemoglobin, cell morphology, platelet count), coagulation (activated partial thromboplastin time, prothrombin time), blood chemistry (total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, total bilirubin, creatinine, triglycerides, total cholesterol, urea, inorganic phosphorus, potassium, sodium, calcium, chloride) and urianalysis (volume, color and appearance, specific gravity, pH, bilirubin, protein, blood, sediment microscopy, glucose, urobilinogen, ketone). Gross pathology consisted of an external examination, including identification of all clinically recorded lesions, as well as a detailed internal examination including organ weights (adrenals, heart, kidney, liver, lungs, ovaries, pituitary, spleen, testes, thymus, and thyroid).

The mutagenic potential of PPIC concentrate was evaluated in Ames test using five histidine-dependent strains of *Salmonella* up to the maximum dose of 5 mg per plate either with or without metabolic activation by MDS Pharma Services, Lyon, France (study no AA41707).

Cell culture

The enteroendocrine STC-1 cell line that expresses CCK mRNA and secretes the biologically active form of the peptide, CCK-8 (24) was a kind gift from Dr. Douglas Hanahan, University of California, San Francisco). Cells were maintained in DMEM supplemented with 4.5% glucose and 10% fetal bovine serum at 37 °C in 5% CO_2, and passaged every 3–4 days. Routinely, 5×10^5 cells were plated on 24-well culture dishes and were used for studies 24 h after plating. Cell viability was estimated by the MTT assay (25). After dimethyl sulfoxide was added to solubilize formazan crystals, absorbance was read at 550 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Gene expression analysis

Total RNA was isolated from the scraped mucosa layer of duodenum samples rinsed with ice-cold PBS buffer or cell culture using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by absorption measurements at 260 and 280 nm using the NanoDrop system (NanoDrop Technologies Inc., Wilmington, DE). Total RNA (20 μg) were loaded and subjected to electrophoresis on a 1% denaturing agarose gel containing formaldehyde before capillary blotting onto a Hybond N+ nylon membrane (Amersham, Piscataway, NJ). Hybridizations with ^{32}P-labeled cDNA CCK and β-actin probes, respectively, were performed according to published procedures (26). Results were expressed as ratio of CCK to β-actin mRNA to normalize any differences in RNA loading and transfer.

For cDNA synthesis and quantitative PCR analysis, RNA was treated with DNAse I, reverse-transcribed to cDNA, and amplified essentially as described (27) using the following primers: rat CCK precursor (Genbank X01032), forward 5’-CAC GAC CCC TCG CCT CTA A-3’ and reverse 5’-GGC TGC ATT GCA CAC TCT GA-3’; and β-actin (Genbank NM_031144), forward 5’-GGG AAA TCG TGC GTG ACA TT-3’ and reverse 5’-GCG

GCA GTG GCC ATC TC-3'. Samples were subjected to a melting curve analysis to confirm the amplification specificity. The relative change in the target gene with respect to the endogenous control gene was determined using 2ΔΔCT method (28).

Statistics

Data are represented as mean ± s.e.m. Statistical analyses were performed using GraphPad Prism 4.0 (San Diego, CA) using Student's t-test or one-way ANOVA (as appropriate). Body weight was analyzed with one-way repeated-measures ANOVA with post hoc testing to localize significant differences at each time point. P-values of less than 0.05 were considered significant.

Results

PPIC treatment reduces food intake in rats

Potato proteinase inhibitors concentrate (PPIC) prepared from acidified potato tuber juice yielded 0.15–0.25 mg total protein per gram of potato tuber fresh weight (data not shown). PPIC contained essentially thermostable proteins in the molecular weight range of 5–160 kDa (Figure 1a) and showed a prominent inhibitory activity against trypsin *in vitro* (Figure 1b). Potato proteinase inhibitor II, a dimeric protein with two 10 kDa subunits (9), was identified in PPIC based on its molecular weight and anti-trypsin activity and isolated to 90% purity (Figure 1) to serve as reference treatment in subsequent studies. MALDI-TOF mass spectrometry identified three major proteins in the PPIC extract as potato protease inhibitor II (PI2), potato protease inhibitor I, and potato kunitz-type protease inhibitor.

A significant reduction in food intake was seen in the first (41.2%) and second (42.6%) hour following oral administration of 100 mg/kg PPIC to 6 h fasted rats in the early dark phase ($p<0.01$, Figure 2). A residual non-significant 6.9% decrease in food intake was also observed at the 24 h time point. To account for a possible effect of protein intake on satiety (22), 100 mg/kg casein solution served as a negative control that resulted in weak food intake reduction of 16.9% and 12.1% in the first and second hour only. Purified potato protease inhibitor II (PI2) was used as a positive control (6) to ensure that the observation system used was sensitive enough to detect significant changes in food intake. Oral administration of 100 mg/kg PI2 significantly reduced food intake in rats (25.2% and 17.4% in the first and second hour, respectively), albeit to a lesser extent than PPIC at the same concentration ($p<0.05$, Figure 2).

Effect on gastric emptying and proteolytic activity

The acute effect of oral PPIC administration on gastric emptying and proteolytic activity in the duodenum was also investigated. Approximately 53.8% of the peptone test meal supplemented with the inert dye left the stomach of control animals treated with 100 mg/kg casein within 1 hour of treatment. Under identical conditions, PPIC-treated animals exhibited a significant delay in gastric emptying by 20.1% over the control group ($p<0.05$, Figure 3a). Since one week of feeding with diets of different protein content was not sufficient to achieve a statistically significant deceleration of gastric emptying (23), the observed delay in gastric emptying can be attributed to the PPIC treatment alone.

An isolated duodenum system was used to assess the effect of PPIC on proteolytic activity in the gastrointestinal tract. A single dose of 100 mg/kg decreased trypsin-like activity in duodenum by 47.3% one hour after PPIC administration ($p<0.05$, Figure 3b).

Repeated PPIC administration

Following confirmation of the anorexigenic effect of oral administration of PPIC, the effects of repeated administration of PPIC were investigated. After 10 days of PPIC treatment (100 mg/kg per day), initial body weight of rats increased by 36.9%, while that of control rats receiving 100 mg/kg casein increased by 48.2%, suggesting that PPI administration was associated with 11.3% decrease in body weight gain ($p<0.05$, Figure 4a). At the same time, PPIC-treated animals significantly decreased cumulative food intake by 6.9% ($p<0.05$, Figure 4b).

Basal plasma CCK levels in control animals measured on the last day of the treatment were 0.96 pmol/l and were not significantly different from those of test animals prior to PPIC administration. Fifteen minutes after PPIC application, plasma CCK levels increased strongly and significantly higher as compared to control animals ($p<0.05$; Figure 5a). The postprandial mRNA levels (expressed as CCK mRNA to β-actin mRNA ratio) in the duodenal mucosa of PPIC-treated animals were elevated 2.4-fold compared to casein-treated controls (Figure 5b). Plasma glucose levels in control rats (6.4 ± 0.2 mM) were similar to those of rats treated with PPIC (6.6 ± 0.4 mM). Animals treated with PPIC exhibited a lower plasma insulin concentration (112 ± 26 pM) as compared to the control group (167 ± 19 pM; $p<0.05$). As a result, insulin to glucose ratio was lower in animals treated with PPIC.

PPIC toxicity

An ascending dose range study was performed to determine toxicity of PPIC following a daily oral gavage administration to the rat for 14 consecutive days, and to exclude the possibility that PPIC inhibits food intake and causes weight loss due to its toxicity to the animals of both sexes. Parameters evaluated in this study included monitoring of mortality, behavioral changes, clinical signs, body weight, food consumption, clinical pathology investigation and post-mortem examinations. There were no deaths during the course of the study. Reduced food intake up to 11.4% was observed in both male and female rats treated with PPIC at 133 and 266 mg/kg throughout the treatment period, but only the males showed a significantly reduced weight gain at the highest dose levels (Figure 6). There were no treatment-related effects on hematology parameters, clinical chemistry, or urinalysis. Apparent marginal elevations in mean activated partial thromboplastin times were noted in PPIC-treated groups, however these elevations were minor, did not achieve statistical significance, were not associated with any other changes, and did not exhibit a correlation to dose (not shown). No organ weight differences, macroscopic, or microscopic changes were noted at necropsy.

When tested up to the maximum dose level of 5 mg per plate using both the plate incorporation and pre-incubation methods, PPIC did not induce any biologically significant increases in the number of revertants in 5 *Salmonella* strains used, either with or without metabolic activation (data not shown).

Effect of PPIC on CCK expression *in vitro*

To answer the question whether PPIC increases CCK production directly or through a trypsin-dependent mechanism by preserving trypsin-sensitive CCK-releasing intestinal factor (18, 19), we examined changes in CCK expression and release in murine enteroendocrine STC-1 cell line. This cell line is derived from an intestinal tumor arising in double transgenic mice carrying the rat insulin II promoter cloned upstream to the coding region of the SV 40 T antigen and the polyoma small t antigen genes (26) and secrets the biologically active form of the peptide, CCK-8 (24). No significant difference in both CCK mRNA levels and CCK release was observed in PPIC-treated cells in the dose range 0.1 to 100 μg/ml (data not shown), suggesting that PPIC did not directly stimulate CCK expression and release *in vitro*. No cytotoxic effect of PPIC on STC-1 cells was noted at these concentrations.

Discussion

Potato proteinase inhibitor II belongs to the class of serine proteinase inhibitors that reduce food intake in humans when administered orally (6, 7). However, all prior studies utilized purified PI2, largely because of the previous claims that other potato tuber proteins have no beneficial affect on appetite suppression, or will have a detrimental effect (29). PI2 represents only 5% of all soluble proteins found in the potato juice (30), yet proteinase inhibitors represent 50% of the total amount of soluble protein present in the tuber (11). We therefore hypothesized that by generating PPIC concentrate that includes all heat stable potato proteinase inhibitors, we will develop a simple method to efficiently recover a fraction of protease inhibitors from potato tubers and considerably increase its biological activity to modulate food intake and weight gain.

PPIC reduced food intake during the first and second hour following oral administration, twice as efficiently as PI2 alone (Figure 2), as compared to negative control group of animals receiving an equivalent dose of casein to account for possible effect of total protein intake on satiety (22). This higher satiation activity may be due to the greater total anti-trypsin activity of PPIC concentrate (Figure 1b) or presence of additional classes of potato protease inhibitors (31). For example, a recent report showed that 70% of the protease inhibitors present in potato juice belong to the serine protease inhibitors from the Kunitz family which is different from PI2 family (32). Indeed, MALDI-TOF mass spectrometry identified three major proteins in the PPIC extract as potato protease inhibitor II (PI2), potato protease inhibitor I, and potato kunitz-type protease inhibitor. All of these proteins have been reported in potato tubers previously and have been known to possess strong serine protease inhibitory activity (11, 32).

Ten to fourteen days of oral intake of PPIC reduced body weight gain in healthy rats (Figure 4 and 6). This body weight gain adaptation seems to be time dependent since at least 6 days are required for PPIC to induce significant changes in body weight gain. The reduction can be partially explained by a decrease in cumulative food intake during the time of the experiment. According to previously published data (33), two week reduction of food intake by 20% in rats was associated with 35% reduction in body weight gain, suggesting that each point % reduction in food intake is expected to reduce body weight gain by approximately

1.75%. In our study, repeated PPIC administration for 2 weeks resulted in 6.9% reduction in food intake and 11.3% reduction in body weight gain. This is equivalent to 1.63% reduction in body weight gain per each point % reduction in food intake and is in close agreement with the previously published value. The data suggest that change in food intake alone is sufficient to explain the observed reduction in body weight gain associated with PPIC administration.

The observed reduction in food intake was associated with delayed gastric emptying and decreased proteolytic activity in the duodenum of PPIC-treated animals (Figure 3). The mechanism underlying this adaptive response could be mediated by changes in nutrient receptor density or release/sensitivity to hormonal mediators. For example, several previous studies indicated that food intake and gastric emptying are influenced by patterns of previous nutrient intake mediated by changes in CCK synthesis and release (23, 34). The two responses can be considered together because both involve activation of the vagal afferent pathway by CCK (17).

Stimulation of CCK by dietary nutrients is mediated through endogenous trypsin-sensitive CCK-releasing peptides (16–19). The potential of PPIC to reduce food intake by increasing CCK response will therefore depend on its capacity to mask trypsin in the small intestine. Indeed, the rapid increase in postprandial CCK levels after oral treatment with PPIC seems to reflect an increase in plasma CCK, as well as induction of CCK synthesis by gastrointestinal cells (Figure 5). These results are in agreement with those reported previously, where CCK release was associated with changes in CCK mRNA levels (35). To answer the question whether PPIC stimulates CCK release by acting directly on intestinal mucosa cells similar to protein hydrolysates (36), we employed an enteroendocrine STC-1 cell line that expresses CCK mRNA and secretes the biologically active form of the peptide, CCK-8 (24). No significant difference in both CCK mRNA levels and CCK release was observed in PPIC-treated STC-1 cells in the dose range 0.1 to 100 μg/ml, suggesting that PPIC did not directly stimulate CCK expression and release *in vitro*. This data suggests that PPIC enhances CCK response primarily by inhibition of trypsin-like proteolytic activity in the small intestine and not by direct stimulation of CCK-producing cells. An alternative explanation for enhanced CCK response associated with PPIC administration is increased stability of CCK without significant changes in CCK secretion, however the current data is insufficient to differentiate between these effects.

Metabolic syndrome and obesity are generally associated with an increased energy intake relative to calorie requirements and delayed satiation (37). Since changes in gastric motor and sensory functions in obesity may present useful targets to prevent and treat metabolic disorders (38), managing CCK-controlled gastric emptying and satiety (39) could be a good strategy for reducing the risks associated with metabolic pathologies. To ensure efficacy and safety of PPIC concentrate, we used healthy rats in these studies to exclude possible interactions with high fat diet used in diet induced obesity (DIO) animal models and to observe any putative toxic effects of PPIC without possible interactions with metabolic/behavioral changes associated with obesity/diabetes complications in DIO or genetic animal models. The 14-day ascending dose range study (33 to 266 mg/kg PPIC per day) showed no adverse side effects associated with PPIC treatment. Although males and females differ in

eating behavior (40), and there are reported gender differences in postprandial satiation and satiety responses (41), both genders responded identically to high doses of PPIC by decreasing food intake throughout the treatment period. However, only the males showed a significantly reduced weight gain at the highest dose levels (Figure 6). To our knowledge, there are no studies describing the differential effect of PI2 inhibitor or other protease inhibitors on satiety in males and females.

The gender differences observed in the study are similar to those observed when mice were treated orally with HIV protease inhibitor ritonavir. While ritonavir-treated male mice revealed whole-body lipoatrophy, female mice showed a small fat reduction that was restricted to the gonadal depot (42). Gender differences in the CCK response after food intake has also been noted (43) and might be responsible for the observed effects. Alternatively, a yet unknown interaction and/or activity within the proteins of PPIC could be responsible for differential reduction in weight gain. For example, PPIC treatment was associated with decreased plasma insulin levels without changes in plasma glucose. Insulin to glucose ratio, a measure of insulin resistance, was therefore also significantly lower in animals treated with PPIC. The significance of this observation remains to be investigated.

In conclusion, oral intake of PPIC reduces immediate food intake, delays gastric emptying, and decreases proteolytic activity in the duodenum of healthy rats. Repeated oral ingestion of PPIC reduces weight gain in male rats and significantly elevated the plasma CCK levels through a trypsin-dependent mechanism. PPIC appears to be safe and non-toxic in these studies. Therefore, increasing the intake of PPIC or PPIC-enriched foods can be a strategy for counteracting increased energy intake relative to calorie requirements and delayed satiation. Although to a lesser degree, obese animals remain sensitive to CCK-induced satiety (44), therefore the potential value and impact of a botanical agent that safely reduces appetite as a way to treat obesity and metabolic disease is very high.

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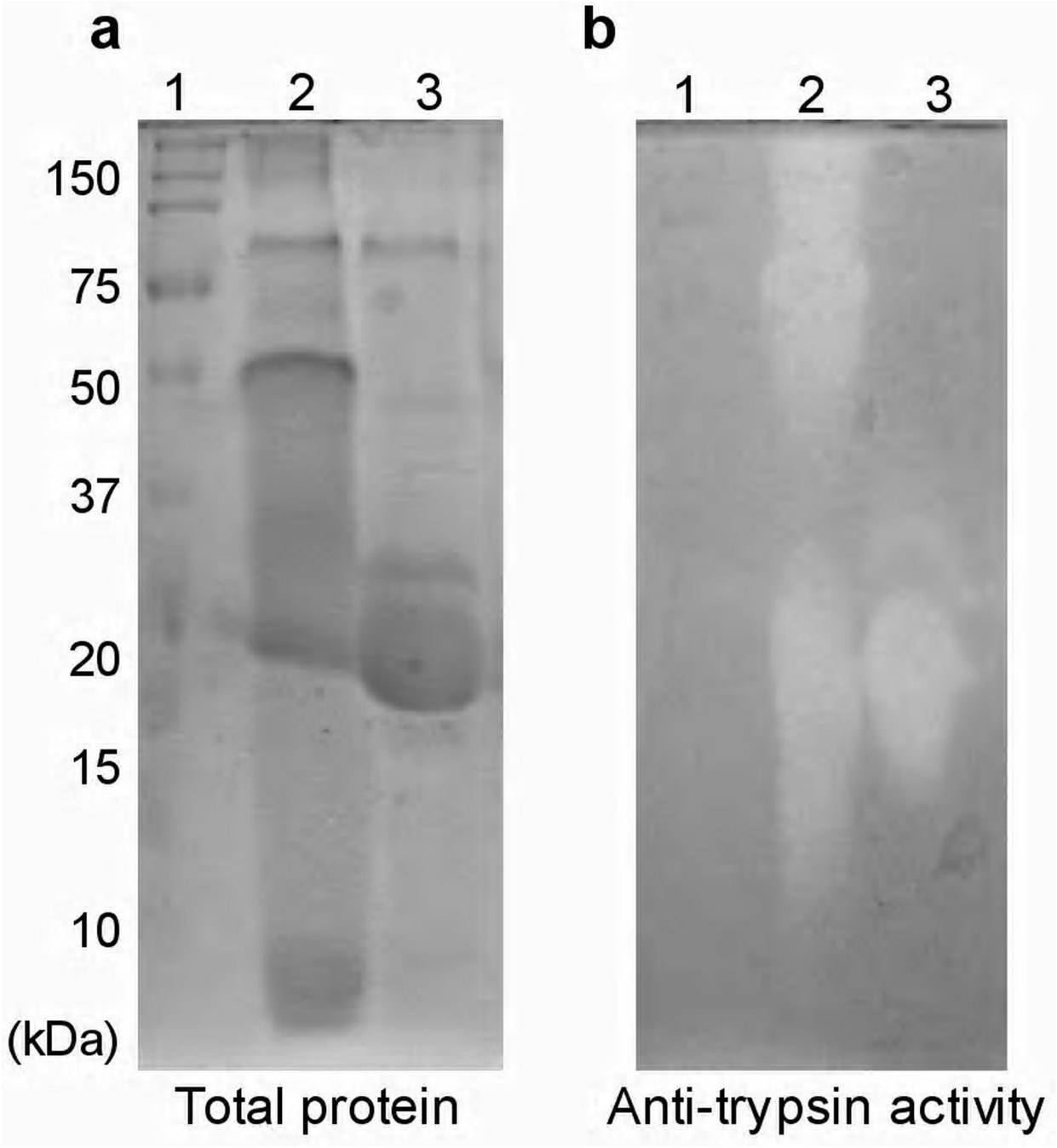

Figure 1.

Total protein content (a) and trypsin inhibitory activity (b) of PPIC (lane 2) and purified potato protease inhibitor II (lane 3). Twenty μg of total protein was loaded per well. Samples were separated on 10% PAGE and stained with Coomassie Blue for total protein; or treated with trypsin and developed in the presence of *N*-Acetyl-phenylalanine-naphthylester and o-dianisidine tetrazotized to visualize trypsin inhibitor activity (light bands). Lane 1, broad range protein molecular weight marker (kDa).

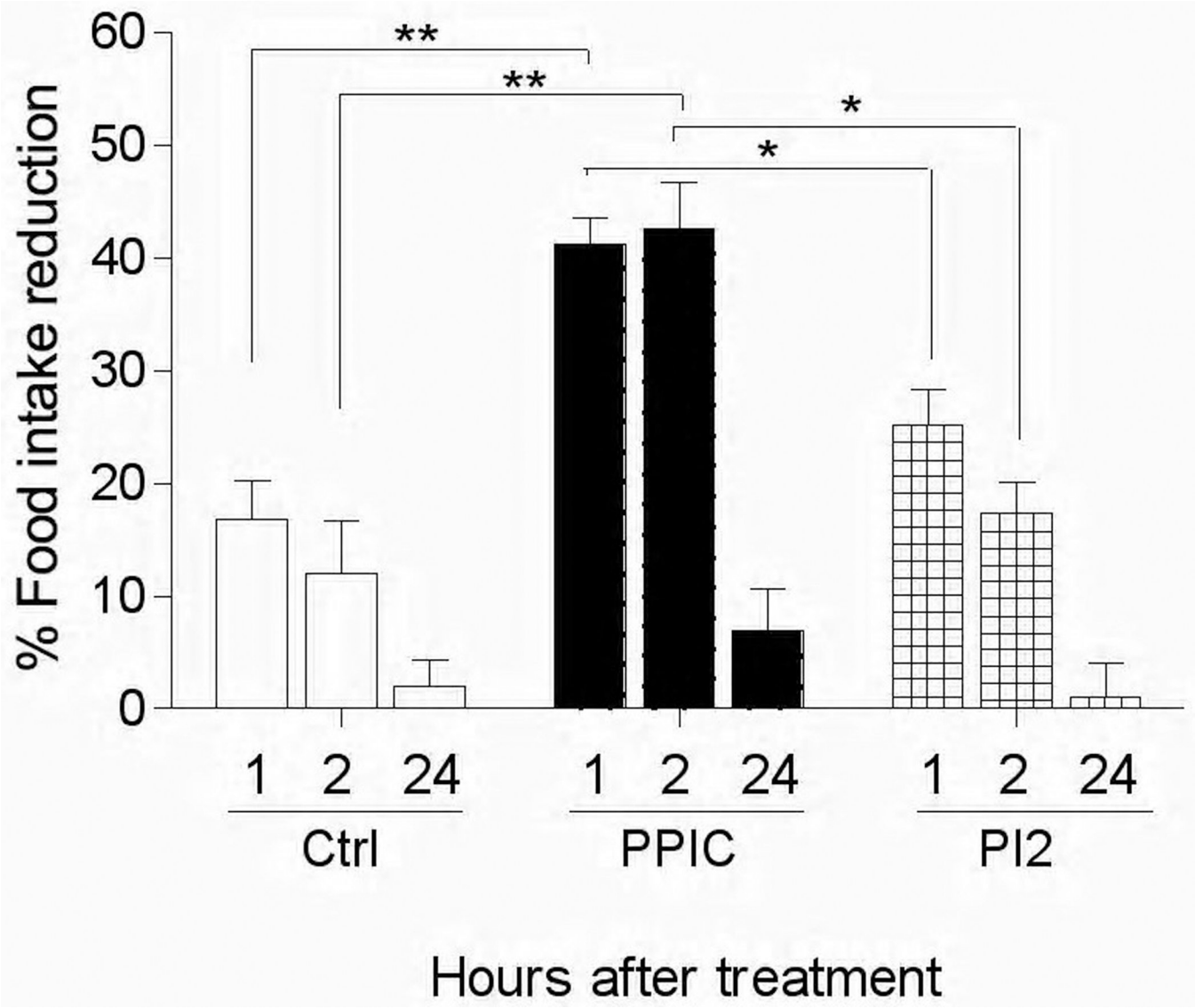

Figure 2.

Changes in food intake 1, 2, and 24 h after oral administration of a single dose of 100 mg/kg PPIC or purified potato protease inhibitor II to healthy rats (n=6–8). Results are expressed as % of food intake reduction relative to baseline food intake in the absence of treatment (means ± SEM). Asterisks indicate a significant difference (*p<0.05, **p<0.01) from control animals receiving 100 mg/kg casein.

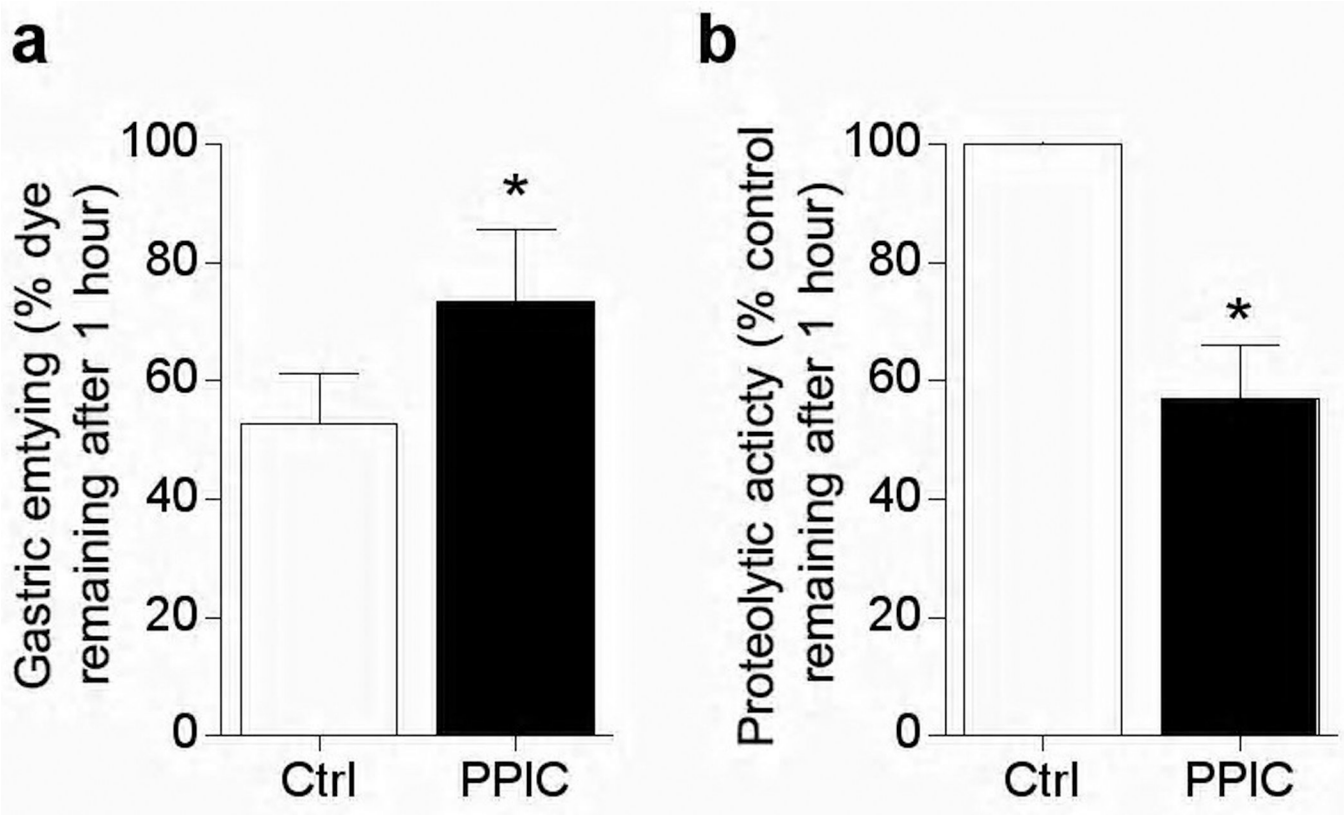

Figure 3.

Effect of a single dose of PPIC (100 mg/kg) on gastric emptying (a) and trypsin proteolytic activity in the duodenum (b) of healthy rats (n=6–8). All values are means ± SEM. Asterisks indicate a significant difference (*$p<0.05$) from control animals receiving 100 mg/kg casein.

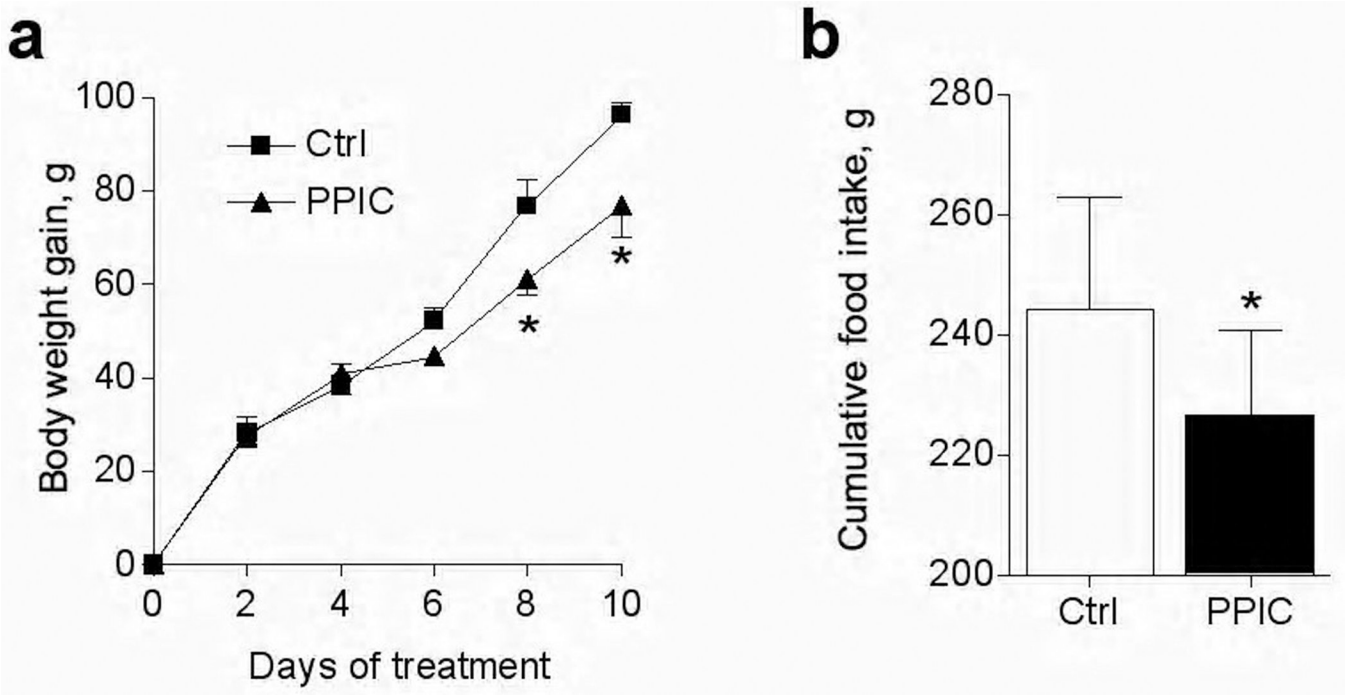

Figure 4.

Oral administration of 100 mg/kg PPIC for 10 days decreases body weight gain (a) and cumulative food intake (b) in healthy rats. All values are means ± SEM (n=6–8). Asterisks indicate a significant difference (*p<0.05) from control animals receiving 100 mg/kg casein.

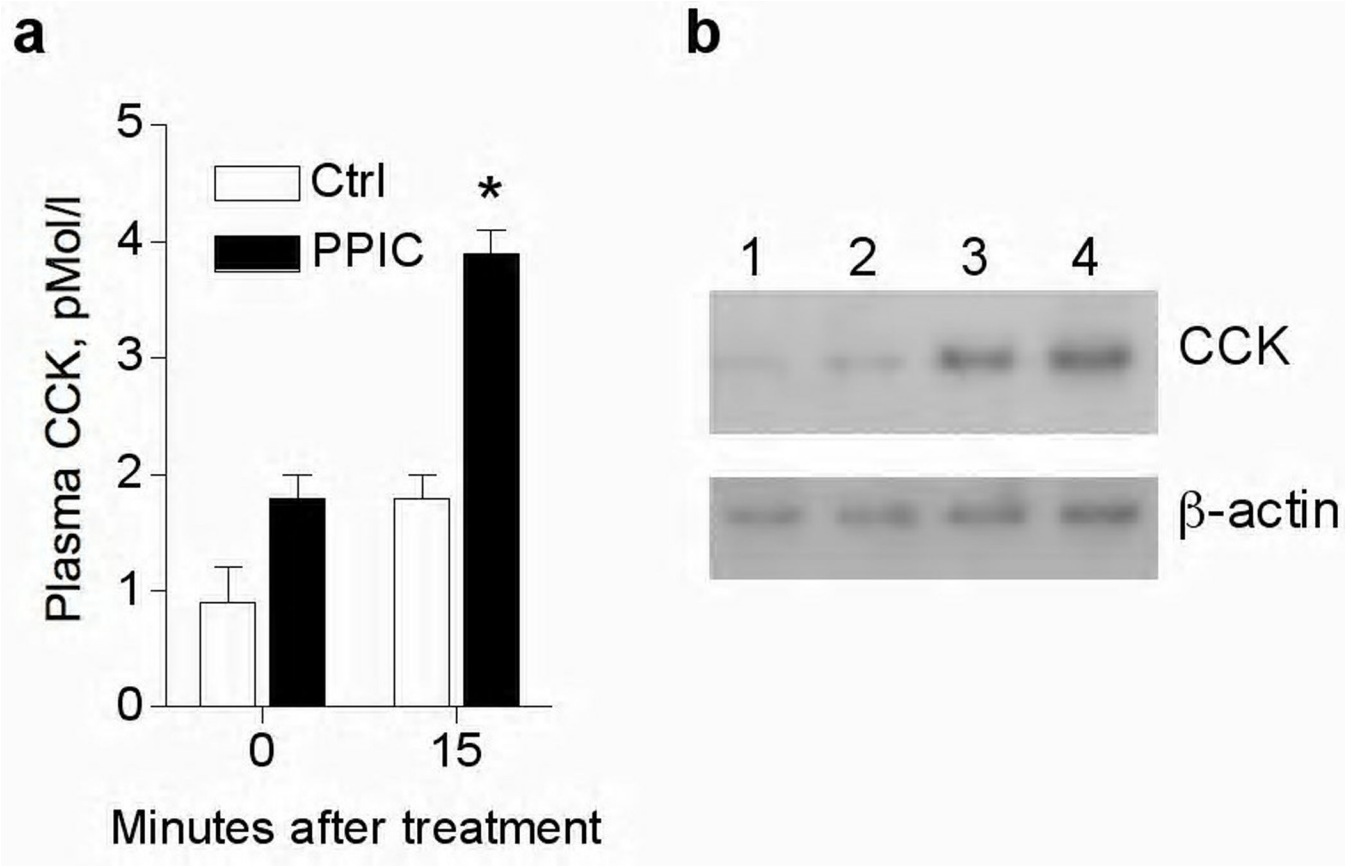

Figure 5.

Increased plasma CCK (a) and CCK mRNA levels (b) in the duodenal mucosa associated with PPIC administration. On the last day of the 10-day experiment, plasma CCK levels were measured in submundibular vein blood samples immediately before and 15 min after the animals were dosed with 100 mg/kg PPIC. Two control animals (lanes 1–2) and two PPIC-treated animals (lanes 3–4) were randomly selected to generate representative Northern blot results for CCK and β-actin expression levels. All values are means ± SEM (n=6–8). Asterisks indicate a significant difference (*$p<0.05$) from control animals receiving 100 mg/kg casein.

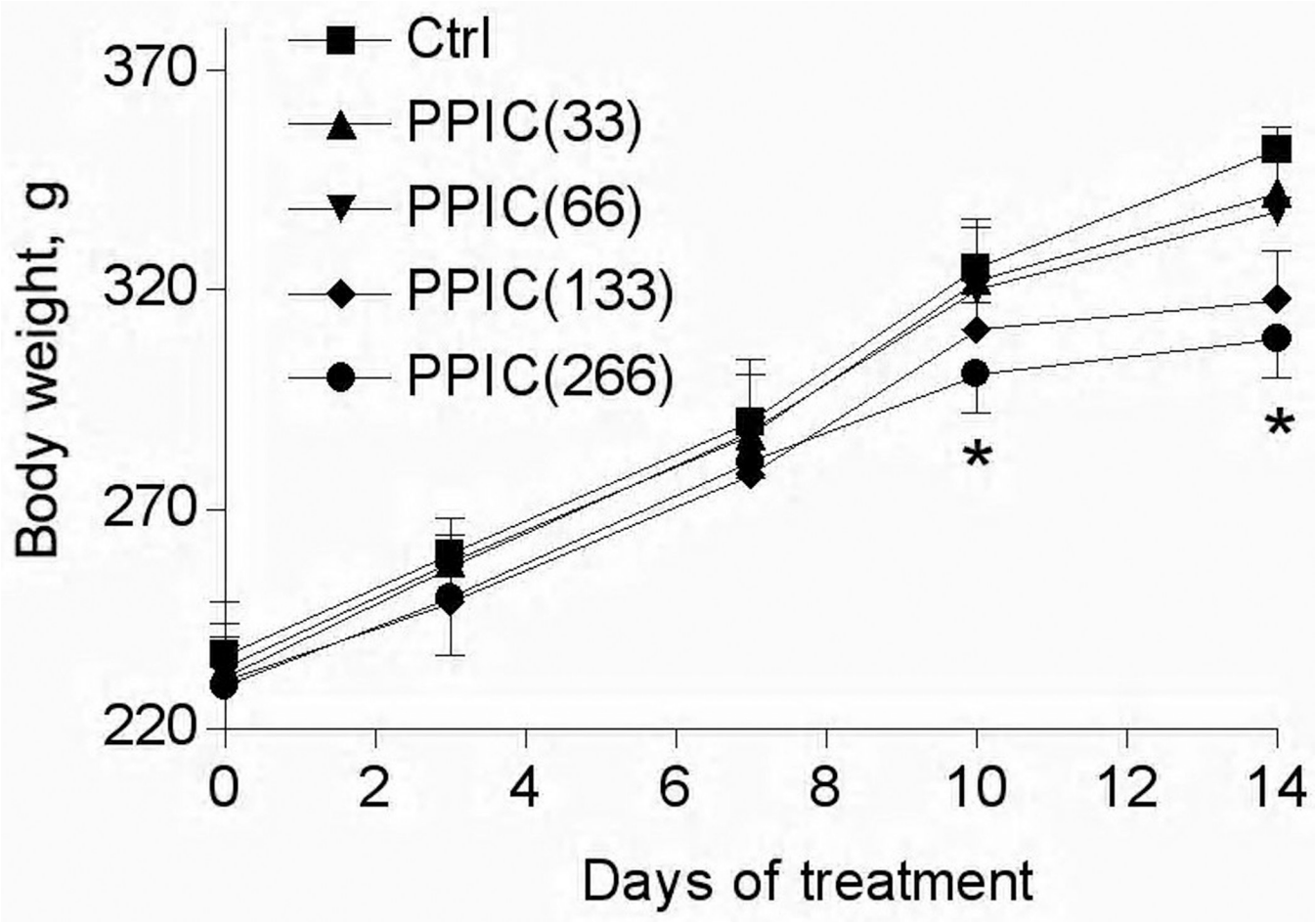

Figure 6.

Dose-response curve of body weight changes to PPIC administration in male rats during the 14-day ascending dose range study aimed at evaluation of safety and toxicity of oral PPIC (33–266 mg/kg per day). All values are means ± SEM (n=5). Asterisks indicate a significant difference (*$p<0.05$) from control animals receiving 100 mg/kg casein.