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Calcium-enriched casein phosphopeptide stimulates release of IL-6 cytokine in human epithelial intestinal cell line

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Phosphopeptides derived from digests of milk casein possess bioactive properties with gastrointestinal, immunological, vasoregulatory and nutritional activities (Clare & Swaisgood, 2000; Kitts & Weiler, 2003). Products of tryptic digestion of casein, yielding caseinphosphopeptides (CPP), bind to divalent minerals such as iron and calcium by ionic interactions that involve phosphoseryl residues (Kitts & Yuan, 1992; Aït-Oukhartar, 2000). Distribution of phosphoserine moieties varies with the individual native caseinates, and the extent of phosphorylation directly influences CPP mineral binding affinity (e.g. α_{s2}>, α_{s1}>β-caseins). The anionic pentapeptide (SerP-SerP-SerP-Glu-Glu) is the distinctive feature for the major fractions of casein phosphopeptides (CPP) characterized both *in vitro* and *in vivo*. Common CPP derived from tryptic digests of whole bovine casein *in vitro* include, β-casein-4P (1–25), α_{s1}-casein-5P (59–79), α_{s2}-casein-4P (1–21) and α_{s2}-casein-4P (46–70) (Kitts & Kwong, 2004).

Several CPP derived from β-casein have been recovered from the stomach and duodenum of adult humans after milk digestion (Chabance et al. 1998), and in the intestinal digests of miniature pigs (Meisel & Frister, 1988; Hirayama et al. 1992), and rats (Naito et al. 1972; Nagasawa et al. 1991) following ingestion of casein. Much attention has been given to the ability of CPP to prevent precipitation of calcium ions as insoluble complexes, thereby enhancing the amount of soluble calcium ion in the intestine for absorption (Kitts et al. 1992). There is also evidence that CPP may act as calcium ionophores and carry calcium across the intestinal cell membrane without interfering with membrane-bound receptors or ion channels (Ferraretto et al. 2001).

CPP may also induce immunostimulatory activity in a variety of cells including the intestine. Intestinal epithelial cells have a role in mucosal immune responses by stimulating cytokine release (Ng et al. 2003). Tryptic digests of α_{s1}-casein peptides rich in phosphoseryl residues have been shown to have humoural immunostimulatory activity by significantly inhibiting mitogen-induced proliferation of mouse spleen (Hata et al. 1998). Similarly, a commercially available CPP-III also exhibited a dose response immunostimulatory activity in mouse spleen cells, which was not influenced by digestive enzyme treatment but was lost following treatment with acid phosphatase (Hata et al. 1999). There is more recent evidence that the commercial source of CPP-III modulates the intestinal immune system by triggering cytokine secretion (Otani et al. 2003). For example, the CPP-III preparation obtained from Meiji Seika (Tokyo, Japan), stimulated IgA production in cultured mouse spleen cells (Otani et al. 2003), the proposed feature for this activity being the SerP–X–SerP sequence derived from β-casein (1–28). The aim of the present study was to determine the relative potency of different CPP preparations that varied in composition, particularly calcium content, in eliciting IL-6 cytokine release from cultured intestinal epithelial cells. The Intestinal 407 (Int-407) cell line was used. Albeit not having differentiated functions that involve intestinal absorption or transport mechanisms, this cell line is useful for exhibiting typical epithelial morphology and growth (Henle & Deinhardt, 1957) as well as participating in mucosal immune responses (Ng et al. 2003).

Materials and Methods

CPP-I, CPP-II and CPP-III were prepared by dissolving bovine casein in water (e.g. 10% w/v; pH adjusted to 8·5). Crystalline trypsin from porcine pancreas was added to a 0·01% (w/v) final concentration against the substrate and the mixture was incubated at 50 °C for 6 h. CPP-I was obtained by spray-drying the whole hydrolysate. The hydrolysate was further purified through ion-exchange chromatography using a Dowex 50WX8 cation exchange column (Dow Chemical Co., Midland MI, USA), followed by an activated charcoal column (American Norit Co., Jacksonville FL, USA), and was labelled CPP-II. CPP-III

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enriched with calcium was obtained by filtrating the insoluble hydrolysate mass at pH 4·5 before precipitating the phosphopeptides by adding calcium chloride (1·1% w/v) and ethanol (50% v/v) to the filtrate.

Casein, CPP-I, CPP-II and CPP-III were analysed by electrophoresis using 16·5% acrylamide Tris–Tricine (N-tris [hydroxymethyl] methyl glycine) SDS ready gel (10 well), according to the method of Laemmli (1970) and a Mini-Protean I Mini-Cell slab gel electrophoresis unit (Bio-Rad Laboratories, Richmond CA, USA). A 4% acrylamide stacking gel, pH 6·8, was used. Protein samples (40 μg) and polypeptide molecular standards underwent electrophoresis at a constant voltage of 100 volts for 100 min using a Tris–Tricine running buffer. Gels were fixed in 40% methanol and 10% acetic acid for 30 min, stained in 0·025% Coomassie Blue G-250 solution (10% acetic acid) for 1 h and destained in 10% acetic acid. Mineral characterization of CPP was performed on 10 mg CPP-I, CPP-II and CPP-III by ICP-MS analysis for calcium, iron, magnesium, zinc and copper at Elemental Research Inc. Phosphopeptide content was measured according to the method of Nagasawa et al. (1991).

Int-407 cell culture

Int-407 cells (CCL-6; American Type Culture Collection (Rockville MD, USA) derived from a human embryonic intestinal epithelial cell line, were used to study the protective effect of CPP on IL-6 secretion. Although this particular cell line originates from normal human embryonic intestinal tissue, it is contaminated with HeLa cells. Cells were grown as monolayers at 37 °C with 5% CO_2 in Eagle's minimum essential medium (EMEM), supplemented with 2 mM-L-glutamine, penicillin at 100 U/ml, streptomycin at 100 μg/ml, 1·5 g sodium bicarbonate/l, 1% (v/v) essential amino acids and 10% (v/v) fetal calf serum (FCS). Culture media and supplements were purchased from Invitrogen Canada Inc. (Burlington ON). Cells were spilt at a ratio of 1 : 3 when confluency was reached and viability was assayed at a density of $5{\cdot}0 \times 10^4$ cells/100 μl in 96-well plates until a tight monolayer was formed (1–2 d following seeding). Cells were washed twice with phosphate-buffered saline solution (PBS) and re-incubated with FCS-reduced EMEM (Invitrogen Canada Inc.). Viability of Int-407 cells supplemented with 5–1000 μg CPP per ml of FCS-reduced EMEM was determined, to rule out any possible cytotoxic effects of the peptide alone. Viability was assayed by monitoring the inhibition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma Chemical Co., St. Louis MO, USA) reduction in the colourimetric Mitochondrial Tetrazollium (MTT) assay (Mosmann, 1983). Cells were first rinsed twice with PBS before the addition of 75 μl MTT stock solution at a concentration of 5 mg/ml PBS. Following a 4-h incubation at 37 °C, 100 μl of acid isopropanol (0·04 M-HCl in 2-propanol; Fisher Scientific, Fair Lawn NJ, USA) was added to all wells and vortexed thoroughly to dissolve the dark blue crystals. Viable cell count correlated directly with the concentration of formazan determined at absorbance 570 nm and was compared against a control to indicate the effect of various treatments on cell survival over a range of concentrations for 48 h.

Measurement of IL-6 levels

Immunoreactive IL-6 was measured in the culture supernatant by ELISA and confirmed by immunohistochemical staining. Int-407 cells were incubated with CPP-I, CPP-II and CPP-III over a range of concentrations (e.g. 10–1000 μg/ml) for 48 h. Quantification of immunoreactive IL-6 concentrations in culture supernatant was performed using commercially available antibodies (ChemiKine™ human interleukin-6 sandwich ELISA from Chemicon International Inc., Temecula CA, USA). The lower limit of detection of IL-6 in the ELISA method was 3·4 pg/ml.

Immunohistochemical staining

Human Int-407 cells were incubated with 500 μg/ml CPP-III for 48 h. Cells were fixed in 10% formalin at 4 °C overnight and then slides containing the cells were rinsed for 2 min in 100% alcohols (18 : 1 : 1 100% ethanol: 100% methanol: 100% isopropanol). Slides were placed in an 80% solution of 100% alcohol for 2 min, followed by a washing step with deionized water for 5 min. Slides were placed face-up in an incubation tray and treated with 1% SDS in TBS buffer (100 mM-Tris, pH 7·4, 138 mM-NaCl, 27 mM-KCl). Slides were then immersed with blocking buffer (goat serum diluted in 1 : 10 in TBS), prior to incubation at 37 °C for 3 h. Cells on slides were treated with rabbit-anti-human IL-6 polyclonal antibody (10 μg/ml) for 2 h, and then rinsed three times in TBS. A Goat anti-Rabbit conjugated-peroxidase was added to the sample for another 1 h and cells were rinsed in TBS again. Cells were exposed to a DAB substrate solution and colour changes were monitored. Cells were finally washed in deionized water and mounted for microscopic examination. The morphology of the cells was examined using a Zeiss confocal microscope at 200× power.

Results and Discussion

Molecular weight and mineral content of CPP

Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed the presence of peptides with apparent molecular weights of 2·8–3·5 kDa in CPP-I, CPP-II and CPP-III (Table 1). Our qualitative assessment of peptides is supported with findings from others that show tryptic digests of casein containing peptides corresponding to 3·125 kDa for β-casein (1–25)-4P (Ferraretto et al. 2001), 2·6 kDa α_{s2}-casein (2–21)-4P (Reynolds et al.

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Table 1. Composition of casein phosphopeptides (CPP)†

Parameter	CPPI	CPPII	CPPIII
Calcium	400	500	58600
Iron	300	200	400
Magnesium	37	59	163
Copper	<1	<1	1
Phosphopeptide	12	12	84
Peptides: kDa			
2·5	+	+	++
3·1	+	+	++

† Minerals (ppm); Phosphopeptide, %; +, ++, relative presence of peptides with apparent molecular weight of 2·5 and 3·1 kDa

Table 2. Effect of casein phophopeptide (CPP) on the viability of Int-407 cells following a 24-h incubation with 4 mM-$FeSO_4$ at 37 °C†

CPP concentrations, μg/ml	Viability‡, %
0	100
5	103·9 ± 5·3
10	105·5 ± 5·3
25	100·2 ± 4·5
50	100·4 ± 5·2
100	103·3 ± 4·6
250	98·5 ± 2·9
500	101·7 ± 3·6
1000	106·2 ± 4·0

† Results are expressed as mean ± SD, $n = 12$ (average values for CPP-I, CPP-II and CPP-III)
‡ Viability (% control) = $\frac{A570\,nm_{sample}}{A570\,nm_{control}} \times 100$

1994), 2·7 kDa α_{s1}-casein (59–79)-5P (Reynolds et al. 1994; Ono et al. 1998).

Each gram of CPP-I and CPP-II contained 400–500 μg of calcium, whereas CPP-III had a very much greater calcium content of 58·6 mg/g (Table 1). All CPP preparations had a similar iron and magnesium content per gram peptide. In contrast, CPP-III was saturated with calcium ion with the addition of calcium chloride in the final preparation step prior to precipitation with alcohol. Iron and magnesium were also in higher concentrations. CPP phosphopeptide content was 12, 12 and 86%, for CPP-I, CPP-II and CPP-III, respectively. HPLC measurement of phosphoserine concentration in CPP-I and CPP-III has given values of 0·16 and 1·22 mmol/g, respectively (Kasai et al. 1995). Moreover, a CPP-III has been reported to contain 83–93% (w/w) peptide content, mainly consisting of bovine α_{s2}-casein (1–32) and β-casein (1–28) residues (Hirayama et al. 1992b). In the present study, all three preparations of CPP had no significant cytotoxic effect on cultured Int-407 cells, maintaining 98·5–106·2% viability over a wide concentration of CPP sample (Table 2). The IL-6 cytokine response of cultured Int-407 cells to exposure to different CPP preparations is presented in Fig. 1. Low calcium-low phosphospeptide CPP-I and CPP-II preparations did not stimulate IL-6 release from cultured cells above basal

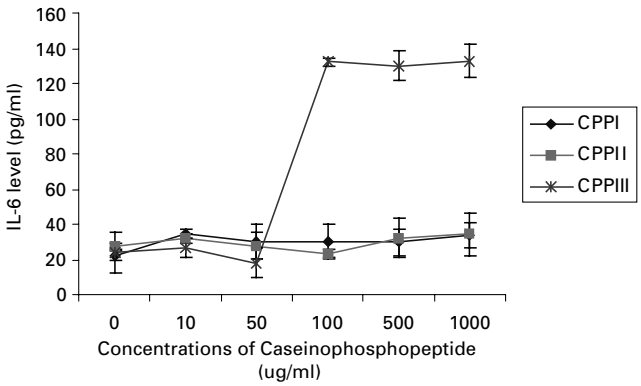

Fig. 1. Relative affinity of casein phosphopeptide (CPP-I, CPP-II and CPP-III)-induced Int-407 IL-6 secretion.

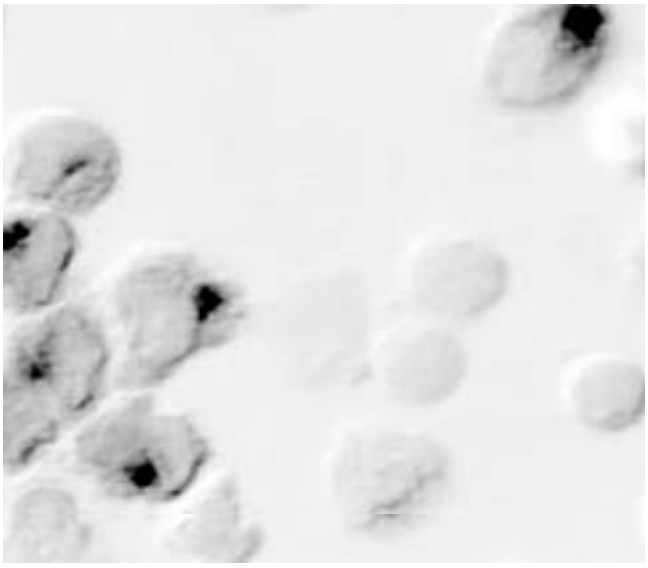

Fig. 2. Positively stained Int-407 cells for IL-6 on exposure to casein phosphopeptide (CPP-III). Magnification (×200).

levels. In contrast, CPP-III exposure to Int-407 cells produced a positive ($P<0·01$) response at inducing IL-6 secretion at a minimum concentration of 100 μg/ml culture media. The output of IL-6 induced by the presence of CPP-III in cultured intestinal cells plateaued at concentrations greater than 100 μg/ml. Cells positively stained for IL-6 are shown in Fig. 2 and confirm the ELISA results.

Mucosal immunity is an important part of the immune system with secretory IgA being a major component. Most of the IgA-producing B-cells are found in close proximity to the intestinal epithelial cells, which line the surface of the intestine. The epithelial cells are involved in transport of IgA from the intestinal lamina propria across to the intestinal lumen, and also have the capacity to produce several important immunoregulatory cytokines that can influence local B-cell Ig secretion (Ng et al. 2003). IL-6, a pleiotropic cytokine, can modulate several different biological events such as cellular differentiation, proliferation

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and apoptosis and is produced in both Int-407 and Caco-2 cells. Int-407 cells produce IL-6 cytokine on exposure to intestinal bacteria (Weinstein et al. 1997; Hosori et al. 2003) and viral infection, demonstrating a positive role in regulation of intestinal immune system.

Earlier studies show that the highly phosphorylated polar domain SerP–x–SerP of CPP can stimulate IgA production in mouse spleen cells (Otani et al. 2001) following enhancement of IL-6. CPP-III can also enhance intestinal mucosal IgA levels of mice when primed with LPS from *Salmonella typhimurium* (Otani et al. 2003). CPP-III weakly induces mRNA expression of IL-6 in Caco-2 cells after prolonged cell (e.g. 8-d) culture; however, the response is further enhanced when Caco-2 cells are exposed to heat-killed bacterial materials (Kawahara & Otani, 2004). In the present study, only the CPP-III preparation was effective in stimulating Int-407 secretion, whereas the other CPP-I and CPP-II preparations were not effective. In all cases cells were exposed to only CPP preparations without exposure to bacterial lipopolysaccharide. The primary difference between the three CPP preparations was the relative phosphopeptide content and amount of constituent calcium present with the peptide, which were both markedly higher in CPP-III. In human tumour HT-29 cells, CPP promotes extracellular calcium influx resulting in a transient rise of Ca^{2+} without altering intracellular calcium stores (Ferraretto et al. 2001), an effect attributed to the SerP–SerP–SerP–Glu–Glu motif, a common feature of two differently prepared CPP used in the study. On the other hand, it is also possible that in our study, the greater calcium content of CPP-III was the factor for triggering release of IL-6. This theory is strengthened by observations with human epithelial cells, which also show increases in stimulated IL-6 and TNFα cytokine transcripts with elevated soluble calcium (Veronesi et al. 1999). Other evidence for this conclusion exists with resveratrol, a phytochemical naturally present in grapes which acts to inhibit IL-6 biosynthesis in mouse peritoneal macrophage at 10^{-8}–10^{-5} mM concentrations by blocking calcium influx (Zhong et al. 1999). Finally, hydrogen peroxide treatment of cardiac fibroblasts producing transient changes in soluble Ca^{2+} can also induce IL-6 secretion (Colston et al. 2002).

Direct interaction of CPP with membrane receptor or endogenous ion channels has been ruled out in explaining the transient effect of CPP on transmembrane Ca^{2+} flux in cultured intestinal cells (Ferraretto et al. 2001). The present results suggest that the combined properties of enhanced calcium content and presence of phosphopeptides, two unique aspects of the CPP-III preparation, provided the necessary conditions for IL-6-induced immunostimulation of intestinal epithelial cells. This may be a reflection of the increased calcium-solubilizing capacity of CPP-III required for intestinal epithelial IL-6 secretion.

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