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IN VITRO AND ANIMAL STUDIES

Dietary A1 β-casein affects gastrointestinal transit time, dipeptidyl peptidase-4 activity, and inflammatory status relative to A2 β-casein in Wistar rats

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Abstract

We compared the gastrointestinal effects of milk-based diets in which the β-casein component was either the A1 or A2 type in male Wistar rats fed the experimental diets for 36 or 84 h. Gastrointestinal transit time was significantly greater in the A1 group, as measured by titanium dioxide recovery in the last 24 h of feeding. Co-administration of naloxone decreased gastrointestinal transit time in the A1 diet group but not in the A2 diet group. Colonic myeloperoxidase and jejunal dipeptidyl peptidase (DPP)-4 activities were greater in the A1 group than in the A2 group. Naloxone attenuated the increase in myeloperoxidase activity but not that in DPP-4 activity in the A1 group. Naloxone did not affect myeloperoxidase activity or DPP-4 activity in the A2 group. These results confirm that A1 β-casein consumption has direct effects on gastrointestinal function via opioid-dependent (gastrointestinal transit and myeloperoxidase activity) and opioid-independent (DPP-4 activity) pathways.

Keywords

β-casein, μ-opioid receptor, cow's milk, dipeptidyl peptidase 4, gastrointestinal transit, myeloperoxidase

History

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Introduction

Cows' milk contains a number of bioactive peptides derived from the enzymatic digestion of β-casein (Meisel, 2004). Studies of various breeds of cattle from different geographical regions have identified at least 11 variants of β-casein based on gene polymorphisms and protein sequences (Caroli et al., 2009). The two major variants, termed A1 and A2, have been the focus of research in recent years. These variants differ at a single amino acid at position 67, being a histidine in A1 β-casein and a proline in A2 β-casein. Because of this substitution, enzymatic digestion of A1 β-casein, but not of A2 β-casein, yields the peptide β-casomorphin-7 (BCM-7) (De Noni & Cattaneo, 2010). It is on this basis that the sub-variants may be termed A1- or A2-like. A1 β-casein and BCM-7 have been implicated in a wide range of clinical disorders, including abnormal gastrointestinal function (Zoghbi et al., 2006), atherosclerosis/ischemic heart disease (Steinerova et al., 2009), type 1 diabetes (Elliott et al., 1999; Pozzilli, 1999), abnormal respiratory function (Hedner & Hedner, 1987), and augmentation of the behavioural symptoms of schizophrenia and autism (Cade et al., 2000; Niebuhr et al., 2011). However, existing knowledge of the mechanisms by which A1 β-casein (and BCM-7) may cause these disorders is still limited. Therefore, this study focused on the gastrointestinal effects of A1 β-casein relative to A2 β-casein.

In vivo, casomorphins decrease gastrointestinal motility, at least in part by reducing the frequency and amplitude of intestinal contractions, thereby slowing gastrointestinal transit and increasing total gastrointestinal transit time (GITT) (Daniel et al., 1990; Schulte-Frohlinde et al., 1994). These effects are inhibited by naloxone, an opioid receptor antagonist with particular affinity for μ-opioid receptors, consistent with the evidence that BCMs are μ-opioid receptor agonists (Koch et al., 1985). It is also interesting to note that dietary hydrolysed casein may reduce GITT by reducing the release of opioid antagonists during digestion (Mihatsch et al., 2005). More recently, Ul Haq et al. (2013) reported inflammatory effects of A1 relative to A2 in rodent intestines following A1 consumption, as evidenced by increased MPO activity and proliferation of Th-2 cells.

It has been demonstrated that intestinal inflammation is associated with increased sensitivity to opioids via up-regulation of epithelial μ-opioid receptor expression (Pol et al., 2005; Puig & Pol, 1998), and that the effects of opioids on GITT are magnified in models of intestinal inflammation (Puig & Pol, 1998). It has also been demonstrated that BCM-7 up-regulates mucin production in gastrointestinal cells, an effect that was prevented by pre-treatment with a μ-opioid receptor antagonist (Zoghbi et al., 2006). Because intestinal mucin helps to protect the intestinal wall, it is possible that the mucin response to BCM-7 constitutes a protective mechanism against inflammatory effects of BCM-7. In turn, intestinal inflammation may lead to the suppression of brush border enzymes, and may affect nutrient absorption (Khanna et al., 1988).

Although several digestive enzymes are expressed in the brush border membrane, only dipeptidyl peptidase-4 (DPP-4) is currently known to be capable of catabolising BCM-7 (Tiruppathi et al., 1993). Other uncharacterised members of the DPP family may contribute to the hydrolysis of this exorphin (Pitman et al., 2009; Qi et al., 2003). Consequently, suppression of this enzyme

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because of low-grade inflammation may increase the half-life of BCM-7 in the intestinal tract, thereby increasing the potential for localised effects in the intestinal tract and/or systemic effects following its absorption and distribution. An alternative hypothesis is that DPP-4 expression may be up-regulated in the presence of BCM-7 as part of a homeostatic protective mechanism. This hypothesis is supported by the results of a study by Wasilewska et al. (2011), who reported that blood BCM-7 levels are positively correlated ($p<0.01$) with blood DPP-4 levels in healthy babies. By contrast, in a small subset of babies with respiratory-related acute life threatening events, BCM-7 levels were elevated and DPP-4 levels were abnormally low (Wasilewska et al., 2011).

Based on these prior findings, our first hypothesis was that the ingestion of A1 β-casein would increase GITT relative to A2 β-casein, and that this effect would be reversed by the opioid antagonist naloxone. Our second hypothesis was that the ingestion of A1 β-casein would increase gastrointestinal inflammation, as indicated by a range of markers. Finally, we assessed DPP-4 levels based on hypotheses that the net effect could either involve up-regulation through a positive feedback mechanism in response to increased substrate levels, or down-regulation because of localised inflammation attributed to A1-derived BCM-7.

Methods

Study design

This study involved direct comparisons of several aspects of gastrointestinal function in male Wistar rats ($n=48$, 4 weeks old; Animal Resources Centre, Canning Vale, WA, Australia) that were fed milk-based diets in which the β-casein component was of either the A1 or A2 type. The total β-casein content relative to other milk proteins was the same as the natural levels in bovine-sourced milk. The rats were obtained from the Animal Resources Centre (Canning Vale, WA, Australia) and were allowed to acclimatise for 7 d on a control diet (AIN-76A; Research Diets Inc., New Brunswick, NJ). They were then fed skim milk-based diets containing β-casein of either the A1 or A2 type for 36 or 84 h, together with water. Food and water were provided *ad libitum*. The nutritionally balanced diets were prepared by Research Diets Inc. (Table 1). All rats were orally gavaged 24 h before the end of the feeding periods with an inert tracer (titanium dioxide [TiO_2]). Half of the rats in each group were also injected with naloxone at this time. All rats were euthanised by CO_2 asphyxiation and cervical dislocation at the end of the feeding period (24 h after application of the TiO_2 tracer) for the tissue analyses described below. Food intake was not recorded during the study.

Table 1. Composition of the experimental diets.

	A1 diet		A2 diet	
	G	kcal	G	kcal
A1 skim milk powder	475	1691	0	0
A2 skim milk powder	0	0	468	1687
DL-methionine	3	12	3	12
Corn starch	150	600	150	600
Sucrose	288	1152	294	1176
Cellulose, BW200	50	0	50	0
Corn oil	45.2	406.8	43.0	387.0
Mineral mix S10001	35	0	35	0
Biotin, 1%	0	0	0	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Total	1058.2	3902.0	1055.0	3902.0

Ethics statement

This study was carried out in strict accordance with the recommendations of the New Zealand Animal Welfare Act 1999. The protocol was approved by the AgResearch Limited (Ruakura, New Zealand) Animal Ethics Committee (Ethics Approval No: 11692). All efforts were made to minimise suffering. The rats were observed daily to identify possible adverse effects of the treatment on their health.

Preparation of the experimental diets

Milk was collected from Friesian cows housed at the AgResearch Ruakura No. 1 Dairy Farm (Hamilton, New Zealand) that were either homozygous for A1 β-casein (A1/A1) or homozygous for A2 β-casein (A2/A2) based on genotyping of tail hair follicle material, which was performed at Genomnz™ (AgResearch Invermay Agricultural Centre, Mosgiel, New Zealand). The assay involves a manual amplification created restriction site procedure that introduces a restriction site in amplicons derived from A2 but not A1 β-casein template DNA. Genotypes were then identified by treating the samples with restriction enzymes followed by agarose gel electrophoresis. The milk from A1/A1 and A2/A2 cows (prepared separately) was then chilled and transported to the Institute of Food Nutrition and Human Health (Massey University, Palmerston North, New Zealand), where it was weighed, skimmed, re-weighed, and stored at −30 °C. Reversed-phase liquid chromatography coupled with mass spectrometry was performed to confirm that the β-casein content of the samples was either A1 or A2. Once the β-casein content had been confirmed, batches of milk were spray-dried and sent to Research Diets, Inc. to prepare each diet based on AIN-76A. The composition of each diet is summarised in Table 1. The three diets were balanced in terms of energy and macronutrient content.

Assessment of GITT

Rats were fed the allocated diet for 12 or 60 h. Then, half of the rats in each group were intraperitoneally injected with naloxone (Sigma, St. Louis, MO). For injection, 6 mg of naloxone was dissolved in 20 mL of normal saline and administered at a dose of 333 μL per 100 g of body weight, giving a final dose of 1 mg/kg body weight. The equivalent volume of normal saline was administered to control rats. The rats were then orally gavaged with 150 mg of TiO_2 (99% purity; AppliChem GmbH, Darmstadt, Germany) suspended in 700 μL of phosphate-buffered saline as a non-digestible tracer. Rats were then returned to their individual cages and continued feeding on their allocated diet for 24 h. Faecal and urine samples were collected using metabolic cages with a wire floor at 0, 4, 6, 8, 11, 14, 21, and 24 h after TiO_2 administration and were stored at −20 °C (faecal) or −80 °C (urine) until used for analysis. TiO_2 was chosen for this study because it has previously been successfully used as an indigestible marker in various animals (Bell et al., 2011; Boguhn & Rodehutscord, 2010; Woyengo et al., 2010).

To measure GITT, colorimetric analysis of TiO_2 content was carried out at the Nutrition Laboratory (Institute of Food, Nutrition and Human Health, Massey University). Briefly, faecal samples were dried, ground, digested in sulphuric acid and diluted to 10 mL with H_2O. Then, 50 μL of this solution was diluted in 1 mL of 30% H_2O_2 and 3.5 mL of H_2O, and centrifuged at 2500 rpm for 7 min. The absorbance was measured at 410 nm and TiO_2 content was determined using a standard curve. The cumulative recovery of TiO_2 was then calculated based on the faecal TiO_2 content at each time-point.

Blood and tissue samples were collected at euthanisation for the procedures described below. Intestinal tissue samples for

histological analyses were placed in 4% phosphate-buffered formalin, while other samples were snap-frozen in liquid nitrogen and stored at −80 °C. Measurements of total intestinal area, intestinal morphology, serum amyloid A (SAA), colonic myeloperoxidase (MPO), and DPP-4 enzyme activities were determined using samples obtained after the GITT studies, corresponding to 36 or 84 h of feeding the experimental diets.

Measurement of the total intestinal area

In interleukin-10 gene-deficient mice, a model of inflammatory bowel disease, the total area of the gastrointestinal tract is correlated with the degree of inflammation (Russ et al., 2013). Therefore, as a measure of overall intestinal inflammation, photographs of the entire length of the intestinal tract were taken, and these photographs were used to measure the area of the gastrointestinal tract. Images were prepared and converted into black and white images using PaintShop Pro (Corel, Ottawa, ON, Canada), and the gastrointestinal area was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Intestinal morphology

Intestinal morphology was assessed using 5-μm-thick formalin-fixed colon tissue sections prepared from rats fed the A1 or A2 β-casein diets. The prepared sections were stained with haematoxylin and eosin (H&E) and a histological injury score (HIS) was determined as previously described (Barnett et al., 2010; Dommels et al., 2007; Knoch et al., 2009). In brief, tissue sections were scored based on the presence of inflammatory lesions (mononuclear cell infiltration, neutrophil infiltration, lymphocyte/plasma cell infiltration), morphologic features associated with damage to the intestinal crypts (crypt hyperplasia, aberrant crypts/villi, crypt injury, crypt loss, goblet cell loss, crypt abscesses), and features associated with tissue architecture (lymphoid aggregates, aberrant submucosa, surface loss). Each of these factors was scored on a scale ranging from 0 to 10, and the sum score (inflammatory lesion score × 2 + crypt damage score + tissue architecture score) was calculated. The inflammatory lesion score was multiplied by 2 to give more weight to this value and to emphasise the major feature of inflammation in the intestine. Representative images of formalin-fixed H&E-stained colon tissue sections are shown in Figure 1.

Colonic MPO activity

Colonic MPO activity was assessed as a marker of neutrophil activity (Krawisz et al., 1984) because it was previously shown to be increased in mice with colonic inflammation (Dommels et al., 2007). In the present study, MPO activity was measured using an established method (Grisham et al., 1990). Briefly, 50 mg of colonic tissue was homogenised, centrifuged, ultrasonicated, and subjected to a freeze–thaw cycle. Endogenous MPO catalyses H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine, which can be monitored enzymatically at 562 nm. MPO activity was normalised to the total protein content measured by the bicinchoninic acid method.

SAA assay

SAA is primarily secreted during the acute phase of inflammation (Lu et al., 1994) and its plasma concentrations have been assessed as a marker of inflammation in a mouse model of colonic inflammation (Barnett et al., 2010). Therefore, in this study, we determined plasma SAA concentrations as a marker of gastrointestinal inflammation, using a commercially available enzyme-linked immunosorbent assay (Tridelta Development Ltd., Maynooth, County Kildare, Ireland).

DPP-4 assay

Because low-grade inflammation can cause flattening of the mucosal brush border, which transiently suppresses the production of digestive enzymes in the brush border (Khanna et al., 1988), we measured DPP-4 activity in the jejunum and colon using a commercially available kit (BML-AK498; Enzo Life Sciences, Plymouth Meeting, PA). This kit uses H-Gly-Pro-*p*-nitroaniline as a substrate, which is hydrolysed by DPP-4 to yield *p*-nitroaniline that can be measured colorimetrically at 405 nm.

Statistical analyses

All data were first tested for normality using the Kolmogorov–Smirnov and Shapiro–Wilk procedures. As the TiO_2 recovery data were highly skewed, data were statistically analysed using the Mann–Whitney *U* test. All other data were analysed using parametric tests. In some situations, the presence of outliers, although insufficient to invalidate parametric analyses, led to stronger results when the non-parametric Mann–Whitney *U* test

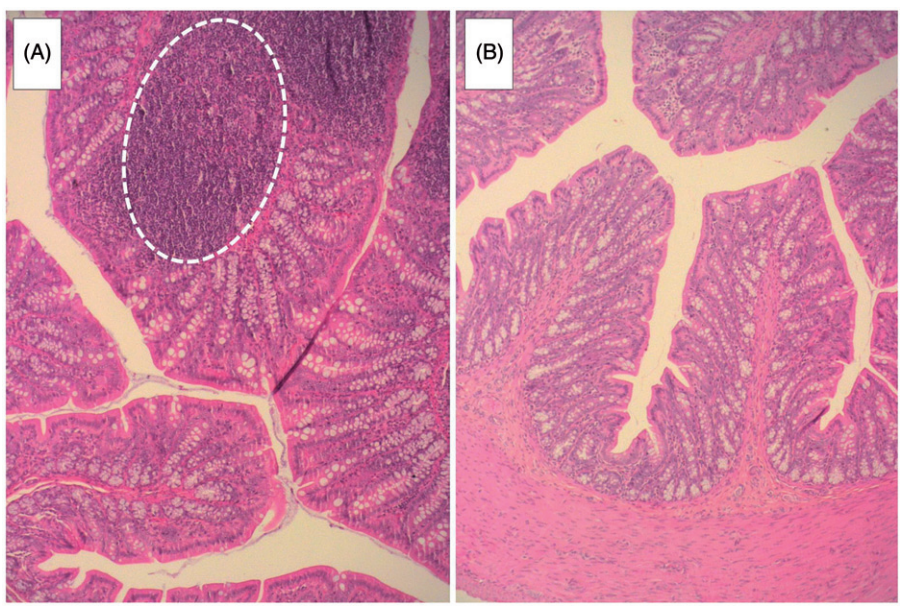

Figure 1. Representative images of formalin-fixed H&E-stained colon tissue sections (×100 magnification). As described in detail in the Methods section, intestinal morphology was assessed using 5-μm-thick formalin-fixed colon tissue sections prepared from rats fed the A1 (panel A) or A2 (panel B) β-casein diets. The sections were stained with H&E and a histological injury score (HIS) was determined based on the presence of an inflammatory infiltrate, and alterations to typical crypt morphology and expected tissue architecture, to yield a total HIS value for each section. While there was some evidence of increased HIS (particularly because of inflammatory cell infiltration, highlighted in panel A) in animals fed the A1 β-casein diet compared with those fed the A2 β-casein diet, this effect was not significant ($p = 0.36$).

Table 2. Cumulative recovery of TiO_2 at 8, 11, and 14 h relative to cumulative recovery at 24 h in rats fed diets containing A1 or A2 β-casein.

					Mann–Whitney *U* test relative to A1S at the same time period for A1N and A2S (one tailed), and relative to A2S for A2N (two-tailed)	
	N	Median	Interquartile range	Range	Mean rank	*p*
A1S (8 h)	11	0.02	0.00–0.03	0.00–0.13	—	—
A1N (8 h)	10	0.11	0.03–2.0	0.00–23.7	14.2:8.1	0.01
A2S (8 h)	12	0.11	0.02–0.18	0.00–7.94	14.8:9.0	0.02
A2N (8 h)	12	0.12	0.02–0.30	0.00–5.91	13.3:11.6	0.55
A1S (11 h)	11	0.07	0.02–0.11	0.0–32.7	—	—
A1N (11 h)	10	0.23	0.08–8.6	0.0–69.3	13.4:8.9	0.049
A2S (11 h)	12	0.22	0.22–8.7	0.0–27.5	15.6:8.1	0.003
A2N (11 h)	12	0.29	0.05–11.7	0.0–48.5	12.2:12.8	0.84
A1S (14 h)	11	25.7	0.0–44.8	0.0–55.1	—	—
A1N (14 h)	10	39.1	3.7–50.5	0.0–84.9	12.4:9.8	0.17
A2S (14 h)	12	45.2	13.9–64.7	0.1–69.4	14.4:9.5	0.046
A2N (14 h)	12	21.7	0.14–54.5	0.0–100	11.2:13.8	0.38

All data are percentages.

was applied. In these situations, both parametric and non-parametric levels of significance are reported. Analyses were performed using IBM SPSS Statistics version 20 (IBM, Armonk, NY).

For all datasets, we first tested for differences between the 36 and 84 h feeding durations using generalised linear model (GLM) analysis of variance (ANOVA). For GITT, DPP-4, and MPO, there were no significant differences (all, $p > 0.20$) between the two feeding durations, nor was there a significant interaction between feeding duration and naloxone/saline. These findings allowed us to combine the data for both feeding durations. Accordingly, the subsequent analyses focused on the direct effects of A1 versus A2 β-casein, and whether the effects were altered by naloxone, giving four experimental groups (A1 plus saline [A1S], A1 plus naloxone [A1N], A2 plus saline [A2S], and A2 plus naloxone [A2N]). For intestinal area and histological morphology, there were no significant differences between the naloxone and saline groups, but there were differences between diets and between feeding durations. Therefore, in analyses of intestinal area and histological morphology, the rats were grouped by diet (A1 versus A2 β-casein) and feeding duration (36 versus 84 h).

In all analyses, $p < 0.05$ was considered statistically significant and all tests were two-tailed except where specifically reported otherwise.

Results

Two rats died during the course of the trial for reasons that were not determined nor investigated. Another rat was excluded because insufficient TiO_2 was introduced by oral gavage. Depending on the analyses, data were available for 41–45 rats, with 4–6 rats analysed in each of the initial eight groups.

GITT

It has previously been demonstrated that 24 h is sufficient time to recover 100% of the administered tracer dose (Daniel et al., 1990). Consistent with this, 67% of rats (A1S = 7/11; A1N = 5/10; A2S = 10/12; A2N = 8/12) defecated no TiO_2 in the final collection period (21–24 h), and the mean hourly recovery in this period was only 10.7% of the hourly recovery rate in the preceding period (14–21 h), suggesting that recovery was almost complete by 24 h. However, the mean total recovery of TiO_2 in all four experimental groups was only 61% of the nominally applied volume (A1S = 60%, A1N = 65%, A2S = 57%, A2N = 63%), with no significant differences in TiO_2 recovery among the four groups. Whether the under-recovery was due to application spillage or retention of tracer within the gastrointestinal tract is unclear. Because of this uncertainty, we analysed the cumulative recoveries at various times relative to the cumulative recovery at 24 h and in absolute terms.

In saline-treated rats, TiO_2 recovery relative to cumulative recovery over 24 h was significantly lower in the A1 group than in the A2 group at 8 h (mean rank 9:15, $p = 0.02$), 11 h (mean rank 9:15, $p = 0.003$), and 14 h (mean rank 10:14, $p = 0.046$) (all Mann–Whitney *U* test, one-tailed) (Table 2). In rats fed the A1 β-casein diet, TiO_2 recovery was also significantly lower in the saline-treated group than in the naloxone-treated group at 8 h ($p = 0.01$) and 11 h ($p = 0.049$), but not at 14 h ($p = 0.17$) (Mann–Whitney *U* test, one-tailed). By contrast, in rats fed the A2 β-casein diet, the cumulative recovery of TiO_2 was not significantly different between the naloxone-treated and saline-treated groups at 8, 11, or 14 h ($p = 0.55$, $p = 0.84$, and $p = 0.38$, respectively) (Mann–Whitney *U* test, two-tailed).

The above analyses were repeated using absolute recovery values for TiO_2 and yielded very similar results, indicating that the delayed transit to 11 h, at least, is independent of any assumption of whether clearance of TiO_2 was complete at 24 h. In terms of absolute excretion, TiO_2 recovery was significantly lower in the A1 group than in the A2 group at 8 h (mean rank 9:15, $p = 0.03$) and 11 h (mean rank 9:15, $p = 0.01$), but not at 14 h (mean rank 10:14, $p = 0.06$) (all Mann–Whitney *U* test, one-tailed) (detailed data not shown). In rats fed the A1 β-casein diet, TiO_2 recovery was also significantly lower in the saline-treated group than in the naloxone-treated group at 8 h ($p = 0.01$) and 11 h ($p = 0.049$), but not at 14 h ($p = 0.23$) (Mann–Whitney *U* test, one-tailed). Consistent with the earlier results using relative recovery, the absolute cumulative recovery of TiO_2 in rats fed the A2 β-casein diet was not significantly different between the naloxone-treated and saline-treated groups at 8, 11, or 14 h ($p = 0.48$, $p = 0.80$; and $p = 0.55$, respectively) (Mann–Whitney *U* test, two-tailed).

The delayed recovery of TiO_2 in rats fed the A1 β-casein diet can also be shown in terms of the proportion of rats that started to excrete TiO_2 in different time periods. By comparing the initiation curves for the A1S group relative to the A1N, A2S, and A2N groups, it is apparent that TiO_2 excretion was delayed by approximately 3 h in the A1S group (Figure 2A). Similarly, the time to recovery of 10% of the TiO_2 was delayed by 2–3 h in the A1S group compared with the other three groups (Figure 2B). Comparisons at higher levels of recovery, such as 50%, were not possible because of the 7 h collection period between 14 and 21 h.

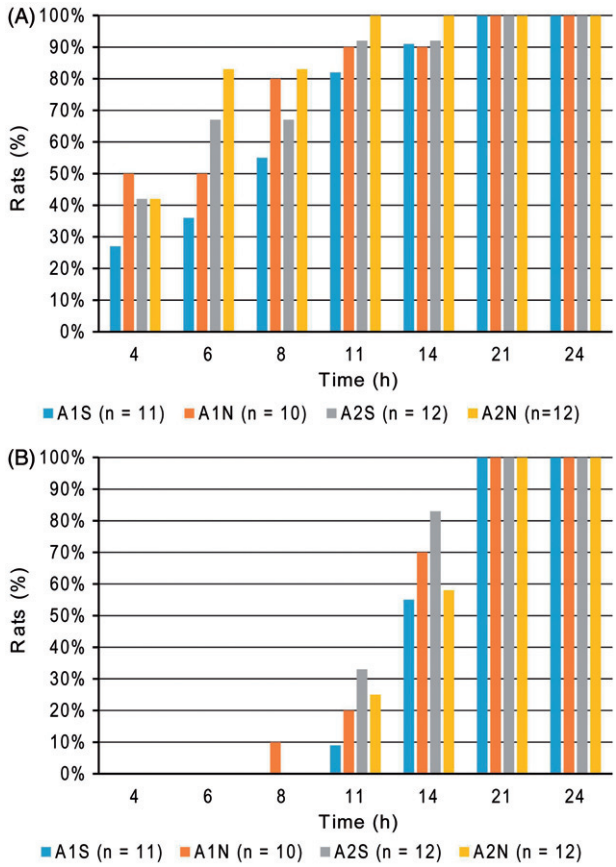

Figure 2. Proportion of rats that excreted some TiO_2 (A) or > 10% (B) of the total recovered TiO_2 at various times after oral gavage. A1S: A1 β casein plus saline; A1N: A1 β-casein plus naloxone; A2S: A2 β-casein plus saline; A2N: A2 β-casein plus naloxone.

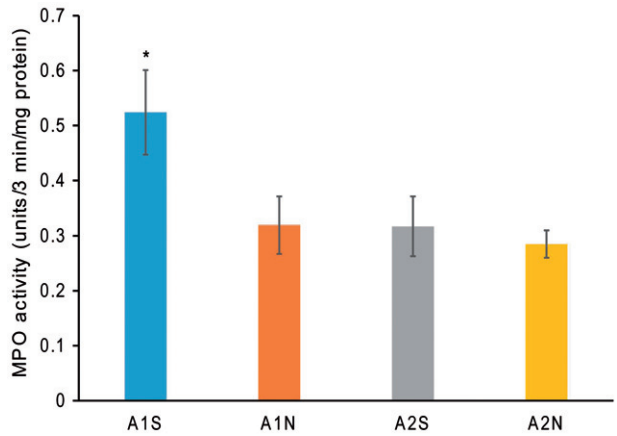

Figure 3. Myeloperoxidase activity in the jejunum. Data are means ± standard error of the mean. MPO: myeloperoxidase; A1S: A1 β casein plus saline; A1N: A1 β-casein plus naloxone; A2S: A2 β-casein plus saline; A2N: A2 β-casein plus naloxone. *$p < 0.05$ versus all other groups.

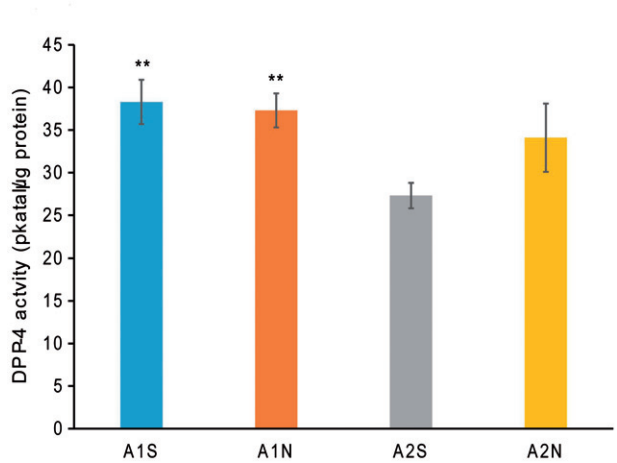

Figure 4. DPP-4 activity in the jejunum. Data are means ± standard error of the mean. DPP-4: dipeptidyl peptidase 4; A1S: A1 β casein plus saline; A1N: A1 β-casein plus naloxone; A2S: A2 β-casein plus saline; A2N: A2 β-casein plus naloxone. **$p < 0.01$ versus A2S.

Markers of intestinal inflammation

MPO activity was 65% higher in the A1S group than in the A2S group (0.52 versus 0.32 units/3min/mg protein, $p = 0.04$, independent samples *t*-test) (Figure 3). MPO activity was also 64% higher in the A1S group than in the A1N group (0.52 versus 0.32 units/3 min/mg protein, $p = 0.04$). The statistical significance of differences in MPO activity between the A1S and A2S groups increased further when we used non-parametric methods (i.e. distribution-free analyses) (median 0.49 versus 0.31 units/3 min/mg protein; mean rank 14:7; $p = 0.02$; Mann–Whitney *U* test).

The HIS inflammation scores were 55% higher in the A1S group than in the A2S group; however, this difference was not statistically significant (17.45 versus 11.33; $p = 0.36$, independent samples *t* test). Similarly, although the HIS inflammation score in the A1S group was more than double that in the A1N group, the difference was not statistically significant (17.45 versus 8.20; $p = 0.17$). Considering the possible influence of feeding duration within the A1S group, the value at 84 h was 2.37 times greater than that at 36 h (26.00 versus 10.33), but the numbers of rats were too low ($n = 11$) to achieve statistical significance ($p = 0.23$).

Intestinal area was evaluated using a GLM univariate model in which feeding duration (36 and 84 h) and treatment (saline versus naloxone) were initially considered as covariates for diet type. However, treatment (saline versus naloxone) was eliminated from the model at $p = 0.71$. In the amended model, which included feeding duration (36 versus 84 h) as a covariate, diet was statistically significant ($F = 3.6$, $P = 0.04$) and intestinal area was 8.2% greater in the A1 diet group than in the A2 diet group (3971 versus 3686; $p = 0.03$, one-tailed; both feeding durations combined). Feeding duration (36 versus 84 h) was not correlated with diet ($r = -0.03$), but intestinal area was 8.2% higher in the 84 h group than in the 36 h group (3977 versus 3693; $p = 0.03$, one-tailed; both diets combined).

SAA levels were close to the lower limit of detection in all samples, with values ranging from 0 to 0.049 µg/mL. Therefore, the SAA levels could not be reliably analysed (data not shown).

DPP-4 activity

Jejunum

DPP-4 activity was 40% higher in the A1S group than in the A2S group (38.3 versus 27.3 pkatal/µg protein; $p = 0.002$; independent samples *t*-test) (Figure 4). DPP-4 activity was 37% higher in the A1N group than in the A2S group ($p = 0.001$). Consistent with this, jejunal DPP-4 activity was not significantly different between the A1N and A1S groups (38.3 versus 37.3 pkatal/µg protein, $p = 0.75$), indicating that the effects of A1 β-casein on DPP-4 activity do not directly involve µ-opioid signalling pathways.

Although DPP-4 activity was apparently higher in the A2N group than in the A2S group (Figure 4), this was primarily driven

by an outlier that was approximately three standard deviations higher than the mean of all other data values. These differences were analysed non-parametrically and were not statistically significant ($p = 0.28$, Mann–Whitney U test).

Given the overall lack of effect of naloxone, it was also possible to combine the saline and naloxone groups with feeding duration to compare the overall effects of both diets. These data were analysed non-parametrically using the Mann–Whitney U test because of the presence of outliers. The median DPP-4 activities in the A1 and A2 groups were 37.9 ($n = 21$) and 28.8 ($n = 20$) pkatal/μg protein, respectively, and the distributions were significantly different (mean rank: 26.6:15.2; $p = 0.002$).

Colon

DPP-4 activity was much lower in the colon than in the jejunum. The individual values were tightly grouped within a range of 5.0–8.7 pkatal/μg protein, with an overall mean of 6.8 pkatal/μg protein. There were no between-group differences in colonic DPP-4 activity.

Discussion

The slower recovery of TiO_2 in the A1S group relative to the other groups at 8, 11, and 14 h, as a proportion of the cumulative 24 h recovery, provides clear evidence that A1 β-casein increases GITT (i.e. delays transit), and that this is at least partly controlled by opioid receptors. The results obtained using absolute recovery rather than relative recovery show that the statistical validity of the results to 11 h, at least, is independent of the assumption of whether or not excretion is complete at 24 h. Although delayed transit was hypothesised in prior reports from theoretical and empirical perspectives (Defilippi et al., 1995; Schulte-Frohlinde et al., 1994), our study is the first to provide experimental evidence that A1 β-casein directly increases GITT relative to A2 β-casein *in vivo*. This effect of A1 β-casein may also have consequences for other aspects of gastrointestinal function, including increased fermentation of other dietary components, especially in people with enzyme deficiencies.

The effects of A1 β-casein on intestinal inflammation are multifaceted. MPO activity was increased by 65% in the A1S group relative to the A2S group. This increase in MPO activity, as a marker of neutrophil activation, is indicative of up-regulation of inflammatory responses. This finding is consistent with those of a recent study showing a significant increase in intestinal MPO activity in mice fed an A1 β-casein-containing diet compared with mice fed an A2 β-casein-containing diet (Ul Haq et al., 2013). The significantly greater (64%) MPO activity in the A1S group than in the A1N group provides clear evidence that opioid receptor activation plays a role in this up-regulation of MPO activity (Saccani et al., 2012). The trends towards higher HIS inflammation scores (by 55%) and greater intestinal area (by 8.2%) in the A1 group than in the A2 group also support the notion that the A1 β-casein diet had pro-inflammatory effects. Furthermore, these effects seemed to be mediated by μ-opioid receptors, because naloxone greatly reduced HIS inflammation scores in the A1 group. However, these differences in HIS were not statistically significant, and longer feeding durations may be needed to fully examine the pro-inflammatory effects of A1 β-casein.

Jejunal DPP-4 activity was approximately 40% greater in the A1S group than in the A2S group ($p = 0.002$). Given that the difference was not attenuated by naloxone, it is evident that the impact on DPP-4 activity is largely, if not completely, independent of opioid signalling pathways. The specific design of this trial as a direct comparison of A1 versus A2 β-casein means that, although DPP-4 levels are clearly higher in the A1 group, we were are unable to conclude whether the DPP-4 levels were up-regulated in response to A1 β-casein or down-regulated in response to A2 β-casein. Although we found no reports describing normal DPP-4 levels in the jejunum of rats, we suggest that they are being up-regulated in response to BCM-7 released by A1 β-casein. Either way, it is well understood that DPP-4 also acts on a range of other substrates, including incretins such as glucagon-like peptide-1 (GLP-1), which regulate insulin secretion and modify glucose metabolism (Holst & Gromada, 2004; Lambeir et al., 2003; Schirra et al., 2006). Accordingly, DPP-4 inhibition is a key pharmacological target for modifying glucose metabolism in the treatment of type 2 diabetes (Chahal & Chowdhury, 2007; Gallwitz, 2011). Therefore, further studies are warranted to compare the effects of A1 and A2 β-casein on glucose metabolism *in vivo*, and the involvement of DPP-4 in these effects. It is notable that elevated DPP-4 levels have also been linked to other conditions, including cardiomyopathy (Chaykovska et al., 2011) and atherosclerosis (Matsubara et al., 2012).

In this study, we used feeding durations of 36 and 84 h to test the hypothesis that the effects of A1 β-casein may differ between acute and chronic feeding states. However, there were no differences between these two feeding durations in terms of the current experiments, allowing the data from these two feeding durations to be pooled for statistical analyses. The only exception to this was intestinal area, which was slightly greater after 84 h of feeding. However, this apparent difference may be due to the rapid growth of rats at this age (4 weeks old), resulting in a growth-related increase in intestinal size between 36 and 84 h of feeding. It is possible that a longer duration of feeding may be necessary to examine the chronic effects, if any, of the diets used in this study.

The results reported here were derived from two-way comparisons between A1 and A2 β-casein contained within predominantly milk-based diets fed to recently weaned Wistar rats. As an animal model, the trial has analogies both to recently weaned children fed milk formula and to pre-weaned children fed infant formula. It is of particular relevance that the levels of β-casein were not augmented relative to its natural levels in bovine milk. It is also relevant that the diets were equivalent in terms of whey and other casein protein components, and that the concentrations of β-casein in the diets were in the same proportions to whey and other casein proteins found naturally in both milk sources.

A number of follow-on studies are proposed. These include development of a set of standards in Wistar rats for the various parameters reported in this trial, based on a further control group fed a standard rat chow. Although the percentage differences in MPO activity were large and statistically significant, the circulating levels of SAA were very low, suggesting that the changes observed in this study represent a pre-inflammatory state or sub-clinical phase. Accordingly, a longer feeding duration is required to provide additional insights into the pro-inflammatory effects of A1 β-casein and to determine the possible molecular mechanism involved. We are also examining the effects of A1 and A2 β-casein on the activities of brush border enzymes, including lactase and sucrase. Finally, it is possible that other opioid receptors may play some role in the effects observed in this study. Therefore, studies using δ- and κ-opioid receptor antagonists are necessary to confirm the involvement of opioid receptors, and help assess the contributions of non-opioid receptor signalling pathways, especially in the context of DPP-4 activity, which was apparently unaffected by naloxone.

It has been noted that A2 β-casein contains a range of exorphins encoded in its primary sequence (Shah, 2000) which, if released during digestion, could theoretically impart a physiological effect on tissue within the gut lumen. Our experimental data indicate that, at least in the rodent model, there is insufficient

production of these exorphins from the digestion of A2 β-casein to elicit GITT, DPP-4 or MPO responses that are reversible by naloxone. This is in contrast to the digestion of A1 β-casein, which resulted in marked responses in the measured aspects of digestive function. From this, it can be hypothesised (and subsequently tested in humans) that the digestion of A1 β-casein, but not of A2 β-casein, leads to the release of exorphins at levels that are sufficient to impart a physiological effect on gut function, possibly as a result of differences in digestion rate. This difference may also influence the homeostasis of the intestinal microbiota, which may respond directly to milk-derived bioactive peptides (Selhub et al., 2014), or indirectly owing to changes in the environment of the gut lumen.

Conclusions

In conclusion, we have demonstrated that consumption of A1 β-casein in rats relative to the consumption of A2 β-casein causes a delay in gastrointestinal transit (i.e. an increase in GITT), together with increases in MPO activity in the colon and DPP-4 activity in the jejunum. The effects of A1 β-casein involve both opioid and non-opioid signalling pathways, with the effects on GITT and MPO being opioid receptor-mediated, whereas the effect on DPP-4 activity was apparently independent of opioid receptors. Additional studies are now warranted in both animal and human subjects to fully understand the underlying mechanisms, particularly the mechanisms involved in the effects of A1 β-casein relative to A2 β-casein on jejunal DPP-4 activity, together with the pro-inflammatory effects of A1 β-casein in the colon. It will also be essential to examine the possible involvement of other opioid receptors and non-opioid signalling pathways in these effects of A1 β-casein, as well as the possible long-term implications of these effects. Exclusion of A1 β-casein from the diet may prevent a range of potentially adverse interactions with aspects of digestive function.

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