Whey protein and sphingomyelin but not casein contribute to α-tocopherol bioaccessibility in skim milk

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Abstract

Bioaccessibility, or the extent to which nutrients can be taken up by enterocytes, is an important predictor of nutrient bioavailability. Despite being fat-soluble, the relatively high bioaccessibility of α-tocopherol (α-T) is unaffected by the fat content of dairy milk. This suggests that physiochemical properties of dairy milk independent of fat are functionally responsible for promoting α-T bioaccessibility. We therefore hypothesized that the emulsifying properties of whey protein (WP) and micellarized casein (CAS) and an amphiphilic phospholipid, sphingomyelin (SM), are responsible for α-T bioaccessibility. To test this, simulated digestions in vitro were performed to define the independent and additive contributions of WP, CAS, and SM relative to non-fat milk on α-T bioaccessibility. Digestions containing 15 mg α-T were performed in non-fat milk (245 mL) or water (245 mL) containing milk-matched levels of WP (1.6 g), SM (16.1 mg), and CAS (6.6 g), alone or in combination (WP+SM+CAS). α-T recovery was evaluated by HPLC-ECD following the gastric through intestinal phases of digestion. α-T bioaccessibility was expressed as the ratio of α-T recovered in the aqueous fraction relative to that in chyme. α-T bioaccessibility differed in response to treatments as follows (means ± SEM; $P<0.05$): WP (82.0 ± 1.4%) = SM (81.3 ± 3.9%) > skim milk (57.4 ± 1.8%) > CAS (35.9 ± 2.3%) = WP+SM+CAS (33.6 ± 1.1%). Lower bioaccessibility in WP+SM+CAS treatment compared to skim milk suggests that other components of milk may also contribute to α-T bioaccessibility. Relative to skim milk, isolated SM and WP potentiate α-T bioaccessibility while CAS is inhibitory. These findings suggest that WP and SM partially contribute to α-T bioaccessibility while other factors may also have a potentiating role.

Introduction

Dairy milk is nutrient-dense, rich in bioactive components, and an economical food that is commonly consumed in the Western diet. In the US, Americans consume ~95 kg/y/person of milk (1). Consistent with its high consumption, specifically its saturated fat component, reducing dairy product intakes are often recommended to lower the risk of obesity-related disorders despite evidence from prospective studies supporting that higher intakes actually *lower* the risk of cardiometabolic disorders (e.g. diabetes, metabolic syndrome) (2). Controlled studies in humans provide clear evidence that dietary saturated fat intakes are not correlated to circulating levels, but rather increasing dietary carbohydrate incrementally increases circulating saturated fatty acids (3). Therefore, additional work is needed to more fully define the functional benefits of dairy products in order to address long-standing controversy for their health benefits in humans

Vitamin E plays an essential role in human health and metabolism, acting as an antioxidant to remove free radicals and other oxidative agents from the human body (4). Although there are several forms of vitamin E, only one of them, α-tocopherol (α-T), can be utilized to reverse vitamin E deficiency in humans, an issue that affects more than 92% of Americans (5). This creates a challenge of delivering vitamin E and other fat-soluble nutrients without significantly increasing dietary fat nor calories consumed.

Previous work has shown that dietary fat dose-dependently increases the absorption of vitamin E (6). In contrast, recent studies have shown that when consumed with dairy milk, dairy-fat has no impact on α-tocopherol *bioavailability*, or the quantity of α-tocopherol that reaches systemic circulation. This suggests that a component of milk bother than fat is capable of potentiating micellarization thus increasing *bioaccessibility*. Due to their physiochemical properties, the

proteins casein (CAS) and whey (WP), and the lipid, sphingomyelin (SM), were selected to be investigated.

Casein is an amphiphilic protein that composes ~80% of dairy protein (7). Casein is amphipathic and naturally forms micelles in when suspended in milk (8). Whey comprises the remaining ~20% of protein in milk and is a natural emulsifier (7). Alternatively, sphingomyelin is the main constituent of the milk fat globule membrane (MFGM) and is an amphiphilic sphingolipid (9). Although sphingomyelin is a lipid, it is left behind after the skimming process making it a possible candidate for potentiating α-T bioaccessibility (9).

Materials and Methods

Materials

All HPLC-grade solvents and most chemicals were purchased from Fisher Scientific. Whey protein was purchased from Glanbia, casein from Erie Foods International, and sphingomyelin from Avanti Polar Lipids. Bile, pancreatin, pepsin, and lipase were purchased from Sigma.

Study Design

1.5 mg α-T were added to non-fat milk (50 mL) or water (50 mL) containing milk-matched levels of WP (1.6 g), SM (16.1 mg), and CAS (6.6 g), alone or in combination. The solutions underwent *in vitro* digestion and were analyzed for vitamin E content.

Micellarization of Casein

Casein micelles were formed according to previous studies (10) with minor modification (11). In brief, a 5% (w/v) dispersion of sodium caseinate was made, prior to the addition of tri-potassium

citrate, potassium biphosphate, and calcium chloride. Samples were then placed in a water bath at 37ºC and eight consecutive additions of potassium biphosphate and calcium chloride were added at 15 minute intervals followed by an hour of incubation.

In Vitro Digestion

In vitro digestion was performed through the small intestinal phase as described by Garrett et. al (12), with minor modification. In short, each sample was spiked with 1.5mg of α-T before being acidified to pH 2.5 with hydrochloric acid and pepsin being added. Following incubation (37ºC, 85rpm, 1hr), sodium bicarbonate was used to adjust the pH to 6 and lipase, pancreatin, and bile were added and the pH was raised to 6.5 using sodium hydroxide before incubation (37ºC, 85rpm, 2hr). After incubation, an aliquot of chyme was taken and centrifuged (40,000 x g, 4ºC, 45 min) to yield the mixed micelle aqueous phase which was collected and filtered.

Vitamin E Extraction and Analysis

Vitamin E was extracted in hexane following saponification by saturated potassium hydroxide. Extracted samples were injected into an ultra-high performance liquid chromatography system and separation was performed using 10mM lithium perchlorate in 95% methanol on a Kinetex column (75 x 3 mm, 2.6 µm; Phenomenex). Vitamin E was quantified using electrochemical detection and was integrated on channels three and four (275, 400 mV). Bioaccessibility was expressed as the proportion of α-T recovered in the aqueous phase to that in the chyme.

Statistical Analysis

Each treatment group was run in quintuplet and statistical analysis was completed on Graphpad using a 1-way ANOVA with Tukey's post test.

Results

α-T bioaccessibility differed in response to the treatments as follows (means ± SEM; $P<0.05$): whey protein (82.0 ± 1.4%) = sphingomyelin (81.3 ± 3.9%) > skim milk (57.4 ± 1.8%) > casein (35.9 ± 2.3%) = whey + sphingomyelin + casein (33.6 ± 1.1%). These results indicate that relative to skim milk, whey protein and sphingomyelin potentiate α-tocopherol bioaccessibility while casein inhibits micellarization

Conclusion

Micellarization is an early but critical step in the absorption and utilization of fat soluble nutrients such as vitamin E, however, dietary fat is necessary in order to promote the formation of these micelles. The findings of this study have shown that non-triglyceride components of dairy milk are capable of forming micelles thus potentiating α-T bioaccessibility. The data from this study suggest that both whey protein and sphingomyelin can be used as novel vehicles to deliver lipophilic nutrients and pharmaceuticals without significantly increasing caloric intake through dietary fat.

The potentiating effects of whey protein on α-T bioaccessibility can be attributed to the selective cleavage of whey protein by pepsin. In a previous study conducted by White et al, it was found that pepsin selectively cleaves the α-lactalbumin subunit of whey while leaving the β-lactoglobulin unit largely intact (13). This partial digestion, even under acidic conditions, allows

the whey protein to maintain its emulsifying properties and explains the potentiating role of whey on α-T bioaccessibility. This partial enzymatic breakdown could also be used to explain why the bioaccessibility of α-T in both whey protein and sphingomyelin is not statistically different, even though there is a significantly more whey protein than sphingomyelin present (1.6 g vs 16.1 mg).

Unlike WP and SM, CAS was found to inhibit α-T bioaccessibility. We hypothesize the inhibitory of effect of CAS on the bioaccessibility of α-T is due to the enzymatic breakdown of the casein micelle. Previous studies have shown that hydrophobic molecules, such as curcumin, are able to bind to casein micelles increasing delivery to caco-2 cells in *in vitro* cell culture models (7,14). These studies, however, did not examine the effects of enzymatic digestion on emulsifying properties of casein. We hypothesize that during enzymatic digestion, the micelle structure is disrupted decreasing α-T micellarization. However, we are unsure if the resulting peptide fragments form micelles which exclude α-T or if they are unable to form micelles at all. Further experimentation is needed to evaluate this question.

Our study also found that in combination WP+SM+CAS had a lower bioaccessibility than skim milk alone. This suggests that some other component of nonfat milk has a potentiating role in α-T bioaccessibility. We believe that another lipid component of the MFGM could be playing this role, however, further experiments must be conducted to elucidate the role of these compounds on α-T bioaccessibility. Lastly, these experiments were conducted *in vitro*, creating limitations to the applicability of the results. Further testing, including human clinical trials, are required to better understand the health benefits of dairy compounds and their derivatives.

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