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Hypaphorine Is Present in Human Milk in Association with Consumption of Legumes

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Supporting Information

ABSTRACT: In metabolomic analysis of human milk amines, we found a previously unidentified compound. This was tentatively identified as hypaphorine, an indole alkaloid composed of tryptophan and three methyls, and with neurological and glucose-lowering effects in rodents. Hypaphorine identity was confirmed by hypaphorine synthesis, and then a fluorometric method was developed to quantify hypaphorine in milk and foods. Using dietary records, we identified peanut products as probable sources of hypaphorine. Milk from 24 lactating women showed widely varying hypaphorine, with a mean ± SD 0.34 ± 0.33 μM, and the highest concentration of 1.24 μM. Peanuts showed high hypaphorine of 70 μg/g compared to 60 and 100 μg/g in dried chickpeas and lentils. Dietary challenge in lactating women with hypaphorine-rich foods demonstrated transfer of hypaphorine into milk with hypaphorine appearance peaking 5−18 h after consumption and prolonged disappearance indicative of slow excretion or metabolism. The potential functional roles of hypaphorine in human nutrition remain to be addressed.

KEYWORDS: *hypaphorine, peanuts, legumes, human milk, methyl, tryptophan, electrospray ionization mass spectrometry*

INTRODUCTION

Human milk is a complex biological fluid that provides all of the nutrients needed for healthy growth and development of breast-fed infants, with additional crucial roles in facilitating colonization and metabolism of the large intestinal microflora.[1] Advances in the understanding of human milk have made it increasingly apparent that the essential and beneficial roles of human milk go far beyond, and are not replicated by, mixtures of classical dietary essential nutrients.[2] Human milk is composed of components synthesized by the lactating mammary gland and derived by uptake from the maternal plasma, some of which reflect transfer from the maternal diet or other environmental exposures. To date, much of the focus on maternal diet in lactation has been on the essential nutrients, proteins and their components, vitamins, and essential fatty acids. More recently, there has been increased interest in the unusual milk fat globule membrane, including glycerophospholipids, sphingolipids, and complex glycolipids, and milk oligosaccharides and their immunomodulatory and protective roles for the infant.[3−7] Potential effects of transfer of environmental contaminants into human milk on the young infant are also areas of increasing interest.[8,9] However, little work has as yet been done on the transfer of naturally occurring components in foodstuffs that may confer physiological benefit or risk to breast-fed infants, including the intestinal microflora.

In studies applying targeted metabolomic analysis to further elucidate different amino acids, amines, and phosphorylated amines in human milk, we observed a previously unidentified component in milk from some, but not all, lactating women, which was present in concentrations approaching that of some free amino acids. This report describes this incidental finding, the identification of this compound as hypaphorine (also named lenticine, betaine of tryptophan, or *N,N,N*-trimethyltryptophan) and subsequent work to identify dietary sources, and the demonstration of its transfer into human milk.

Hypaphorine is an indole alkaloid composed of three methyl groups and a single tryptophan as shown in Figure 1. Hypaphorine was first identified in the seeds of the tropical pea plant *Erythrina hypaphorus* by Greshoff while working in the Botanic Gardens at Buitenzorg, Java, from 1889 to 1891.

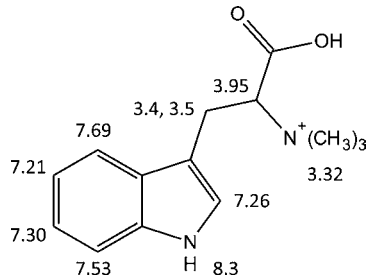

Figure 1. Structure of hypaphorine with assigned ^{1}H NMR signals obtained from analysis of the synthesized standard compound. The original and integrated NMR spectra with zoomed in aryl- and alkyl sections are provided as a Supporting Information.

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The first synthesis of hypaphorine was reported over 20 years later by van Romburgh and Barger, who suggested that hypaphorine could be used "to test the view expressed by Winterstein and Trier that betaines are merely waste products rendered innocuous by methylation".[10,11] Notably, it is now well-known that betaine (*N*,*N*,*N*-trimethylglycine) and related quaternary amines, such as choline, are important sources of methyl groups for the one carbon folate pool and remethylation of homocysteine, subsequently contributing methyl groups for DNA and RNA methylation, neurotransmitter and phosphatidylcholine metabolism, and synthesis of metabolites such as creatine.[12]

Although the hypaphorine structure with tryptophan and three methyl groups suggests physiological relevance, little is known regarding its metabolism or physiological effects. Early studies focused on possible toxic effects of hypaphorine-containing plants. Early concerns over toxic effects found in goats of feeding on *Astragalus lusitanicus*, a pea plant found in the Mediterranean area, initially ascribed to hypaphorine were subsequently refuted by studies showing lack of toxicity of high oral doses (up to 2 g/kg) of synthetic and purified hypaphorine.[13] Studies on the role of hypaphorine in plants have shown that it influences actin organization[14] and inhibits the effects of indole-3-acetic acid via competitive binding to horseradish peroxidase-C,[15] leading to suggestions of potential therapeutic implications in cancer.[16] Although tryptophan is a precursor to serotonin and melatonin,[17] only indirect evidence is available to link hypaphorine to physiological effects linked to this amino acid. In this regard, hypaphorine from *Erythrina velutina*, a Brazilian medicinal plant, has been reported to have sleep-inducing effects and to increase nonrapid eye movement (NREM) sleep-time in mice.[17] More recent studies have described significant reductions in plasma glucose in diabetic rats given hypaphorine from the seeds of *Impatiens niamniamensis*.[18] More relevant to human diets, hypaphorine has been identified in several legumes,[19] including lentils[20,21] and chickpeas,[22] although no information on dietary intakes in human populations has as yet been reported.

In the present report, we describe our discovery and confirmation of hypaphorine in human milk and work to elucidate potential dietary sources in our population, followed by a dietary challenge to show that hypaphorine is rapidly transferred into human milk when lactating mothers consume foods containing the compound. Analyses of infant formulas based on soy protein and cow milk protein were included because of the finding of hypaphorine in human milk.

MATERIALS AND METHODS

Chemicals and Reagents. Deionized water (HPLC-grade) was obtained from an EASYpure RoDi system (Barnstead International, Dubuque, IA, USA). HPLC-grade solvents and acids (methanol, acetonitrile, hydrochloric acid, and glacial acetic acid) were from Fisher Scientific (Mississauga, ON, Canada). DL-Tryptophan and iodomethane were purchased from Sigma-Aldrich (Oakville, ON, Canada). All chemicals were used without further purification.

Discovery of Hypaphorine in Human Milk. Human milk samples were collected at 30 days postpartum from mothers exclusively breast-feeding one full-term infant with no known health problems. The milk samples were collected during an infant feeding by manual expression by interrupting the infant's feeding. The samples were stored frozen at −75 °C. For analysis, the samples were thawed on ice, and then 400 μL of milk was placed in an Amicon Ultra 3 kDa molecular weight cutoff microcentrifugal filter device (Millipore, USA) and centrifuged for 30 min at 2500*g* at room temperature. Sample filtrates (milk plasma) from 24 women were used without further treatment for targeted metabolomic analysis focused to the separation and identification of amino acids, amines, and phosphorylated amines. The separation of hydrophobic milk plasma components was achieved by HPLC with a C_{18} reversed-phase Agilent Zorbax Eclipse AAA column, 2.1 × 150 mm, with 3.5 μm particles with a column flow rate of 0.45 mL/min. A gradient program using two buffers A (10 mM ammonium acetate at pH 7.5) and B (50% 20 mM ammonium acetate, 25% methanol, 25% acetonitrile) was employed to elute the different components (see details in the Supporting Information). The eluent was directly fed into the electrospray ionization (ESI) source of a Waters QTOF micro mass spectrometer (MS), with a regular electrospray source, and operated in positive mode. A sample of milk plasma was sent for accurate mass measurement and determination of the atomic composition of the unknown peak.

Dietary information had been collected for each of the women using a food frequency questionnaire that included information on the intakes of all foods and beverages, with the frequency of intake and portion sizes, over the previous 30 days. Due to the method of dietary data collection, intake data specific for the days or day immediately preceding milk collection was not available.

Identification of Hypaphorine. Hypaphorine hydroiodide was synthesized using a method adapted from van Romburgh and Barger[10] and Jones and Tiekink.[23] Briefly, L-tryptophan (1.0076 g, 0.0098 mol) was dissolved in ∼32 mL of methanolic sodium hydroxide (0.015 g/mL). Methyl iodide (17.67 g, 0.1245 mol) was added slowly in 0.6−1.0 mL aliquots to the refluxing mixture over 1 h with further additions of methanolic sodium hydroxide as required to maintain an alkaline reaction mixture (pH ∼9−10), and then the mixture was refluxed for additional 7 h, during which a pH of ∼9−10 was maintained through addition of methanolic sodium hydroxide. Methyl iodide is toxic and was handled in a certified fume hood. At the end of the reflux, the condenser was removed and the reaction mixture heated with stirring for 25 min to evaporate excess methyl iodide. This was followed by cooling and acidification with 10% HCl to pH 2. This acidic reaction mixture was rotary evaporated to yield off-white to beige colored crystals. Recrystallization, first in hot aqueous solution, then in 10% ammonia in methanol overnight, yielded white crystals of the pure L-hypaphorine hydroiodide (1.945 g, 0.0052 mol, 53% yield). This compound was used to confirm the identity of hypaphorine in milk and food samples by comparison of retention time and fragmentation spectra in the described LC−MS analyses. ^{1}H NMR spectra for the synthesized product were recorded on a Bruker AV-400 (400 MHz) NMR spectrometer (Billerica, MA, USA) with δ referenced to the deuterated solvent (D_2O) at room temperature.

Dietary Sources of Hypaphorine. Foods analyzed for the presence of hypaphorine (Table 1) were purchased from local stores. Solid foods were ground, and 1 g was extracted with 10 mL of 30:70 v/v acetonitrile:deionized water mixture, under ultrasonication for 30 min, and then left undisturbed for 5 min, while solid particles settled.

Table 1. Tested Foods and Their Hypaphorine Content[a]

food item	hypaphorine content in [μg/100g]
lentils (dried, freshly ground)	10000
peanuts (freshly ground)	7000
peanut butter	4500
chickpeas (dried, freshly ground)	6000

[a]Foods analyzed, but hypaphorine not detected: ancient grains (containing red and white quinoa, oats, millet, buckwheat, amaranth, kaniwa), carrots (freshly pressed juice), green beans, black beans, kidney beans, lima beans, green peas, yellow peas, walnuts, cashews, almonds, coconut water, cumin seeds, cardamom, caraway seeds, sesame seeds, nutmeg, pine nuts, red beets (stems, leaves, and juice from body), fennel (stem, body, fresh, juice), pistachio nuts, cow's milk (Lucerne Foods, Canada), Enfamil A+ (cow milk based infant formula, Mead Johnson, USA), Isomil (soy based infant formula, Abbott, USA).

Subsequently, 400 μL of supernatant was filtered with an Amicon Ultra 3 kDa molecular weight cutoff microcentrifugal filter device (Millipore, USA) and centrifuged for 30 min at 2500*g*. Extract filtrates were diluted as necessary with deionized water before HPLC fluorometry and LC−MS analyses.

Transfer of Hypaphorine from Diet in Lactating Women into Human Milk. Studies to demonstrate the transfer of hypaphorine from the diet into human milk were undertaken with three women who were breast-feeding full-term gestation infants. These women all had well-established lactation; neither the mothers nor their infants had any known risks of food allergies. Each mother consumed peanut butter products and/or lentils once. When possible, samples of milk were collected each time the infant was fed from one day before the dietary challenge, then for up to 3 days after the hypaphorine-containing foods were consumed. Milk samples were frozen upon collection in sample vials provided and labeled with the date and time of collection.

HPLC Conditions and Fluorescence Detection for Quantitative Studies of Hypaphorine in Milk and Foods. Hypaphorine was separated using gradient chromatography with a Zorbax Eclipse AAA HPLC column (, 3.0 × 150 mm, 3.5 μm particle size) in an Alliance 2695 HPLC system (Waters, Milford, MA, USA) with as solvent A (0.1% acetic acid in deionized water) and solvent B (100% acetonitrile). After 2 min of 95% A, B was increased to 40% for the next 18 min, then returned to 5% prior to the next analyses. The injection volume was 4 μL, and the column flow rate was maintained at 0.6 mL/min. The effluent was analyzed with a Waters 474 fluorescence detector (Milford, MA, USA); the excitation wavelength was set to 286 nm and the measured emission wavelength was set to 366 nm with a bandwidth of 10 nm.

Mass Spectrometric Analysis of Pure Hypaphorine Standard and Hypaphorine in Milk and Foods. Liquid chromatography combined with electrospray ionization (ESI) (tandem) MS (LC−(MS/) MS) in positive mode was employed to confirm the identity of hypaphorine in food and milk samples, and our synthesized hypaphorine standard compound. Both fragmentation analysis (MS/MS) and accurate mass measurements for elemental composition determination were used for confirmation of identity as further described. Briefly, due to the potential presence of coeluting and similar fluorescent components, we also used LC−ESI-MS to confirm the identity and obtain quantitative results using HPLC−fluorometry for analyses of hypaphorine in milk and food samples. Accurate mass measurement for the synthesized hypaphorine was done on a Waters/Micromass LCT time-of-flight mass spectrometer (Milford, MA, USA) located in the Department of Chemistry at the University of British Columbia. Accurate mass measurements for human milk samples and fragment ions were done on an Agilent Technologies 6220 oaTOF instrument (Santa Clara, CA, USA), located in the Department of Chemistry at the University of Alberta. Low-resolution tandem mass analyses were performed on either a Waters/Micromass Quattro Micro triple-quadrupole MS or a QTOF Micro (both Milford, MA, USA), in our laboratories at the Child & Family Research Institute, University of British Columbia. Raw data were further processed with IGOR Pro software (Version 6, Wavemetrics, Lake Oswego, OR, USA). Chemical structures were drawn with ChemSketch (Version 10.0, ACD Laboratories, Toronto, ON, Canada).

RESULTS AND DISCUSSION

Our analysis of amine and phosphorylated amines by HPLC revealed a peak in the total ion chromatogram that could be not be readily identified (Figure 2). This peak was present in most, but not all, samples, and in varying concentrations, some of which approached that of free amino acids. The component was tentatively identified as hypaphorine, although the reason for its occurrence was not known. There were no other components in the total ion chromatogram that could not be identified, and we did identify, for example, caffeine, acetylsalicylic acid, acetaminophen, and polyethylene glycol

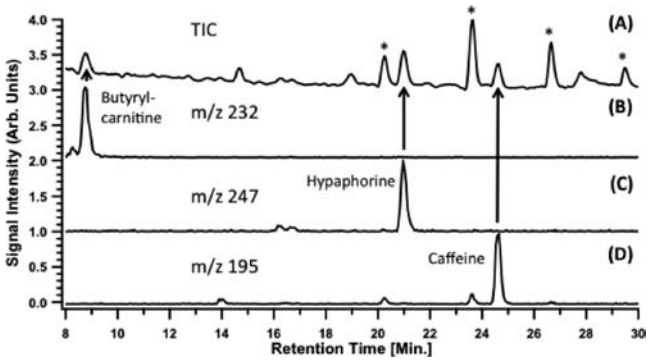

Figure 2. Selected HPLC−MS chromatograms of milk plasma. All chromatograms are normalized to the highest peak. (A) Total ion chromatogram (TIC) of milk plasma. The "*" labeled peaks belong to mass-to-charge-ratios 239, 283, 327, and 371 respectively (according to retention time), which indicates a polyethylene glycol-type interference (repeating unit [C_2H_4O], 44 Da).[24] This contamination is also found in system blanks. (B) Single ion chromatogram for *m*/*z* 232, with the main signal from butyrylcarnitine at retention time 8.75 min. (C) Single ion chromatogram for *m*/*z* 247 with the main signal from hypaphorine at retention time 20.95 min. (D) Single ion chromatogram for *m*/*z* 195 with the main signal from caffeine at retention time 24.6 min. Experimental details are provided in the Supporting Information.

related contaminants[24] (see Figure 2) in addition to several methylated and phosphorylated amines. Since hypaphorine had been described in legumes, we reviewed the diet records for the most logical source(s) of hypaphorine in the diets for the women in our study; from this we speculated that peanuts and peanut-containing products were likely candidates. We thus proceeded with studies to confirm the identity of hypaphorine in human milk, which required synthesis of the pure compound, and to measure hypaphorine in foods, including peanut products (*Arachis hypoaea*), which is a species in the legume family. With the synthesis of the pure compound and comparison of the retention time and fragmentation spectra of the previously unidentified hypaphorine in human milk with the pure compound, we confirmed its identity. We next developed a simple HPLC−fluorometry method to quantitatively measure hypaphorine in foods that could potentially be sources of hypaphorine in our population, provided the data to show hypaphorine is present in peanuts, and quantified the hypaphorine content in human milk from 24 lactating women. Finally, we conducted a dietary challenge study to demonstrate that hypaphorine consumed in peanut-containing foods and lentils is transferred from the diet of lactating women.

Hypaphorine Hydroiodide Standard. The melting point (mp) of the synthesized hypaphorine was 219−223 °C, consistent with the previously reported mp range of 220−221 °C.[25] NMR and MS assignments: ^{1}H NMR (400 MHz, D_2O-d_2) δ 3.32 [9H, s, $N(CH_3)_3$], 3.4, 3.5 [2H, dd, CH_2], 3.95 [1H, dd, $R_2N(CH)$], 7.21−7.69 [4H, m, aromatic CH], 7.26 [1H, s, aromatic N−CH], 8.3 [1H, s, aromatic NH]; MS (+HRESIMS) obsd *m*/*z* 247.1443 (calcd for $C_{14}H_{19}N_2O_2$, *m*/*z* 247.1447; Δ ppm, −1.6). ^{1}H NMR assignments are included in the structure shown in Figure 1, and the original integrated NMR scans are in the Supporting Information.

Mass Spectrometric Characterization of Hypaphorine. Figure 3 shows the tandem mass spectra of hypaphorine found in human milk and a proposed fragmentation pathway with accurate mass measurements of the observed fragment ions.

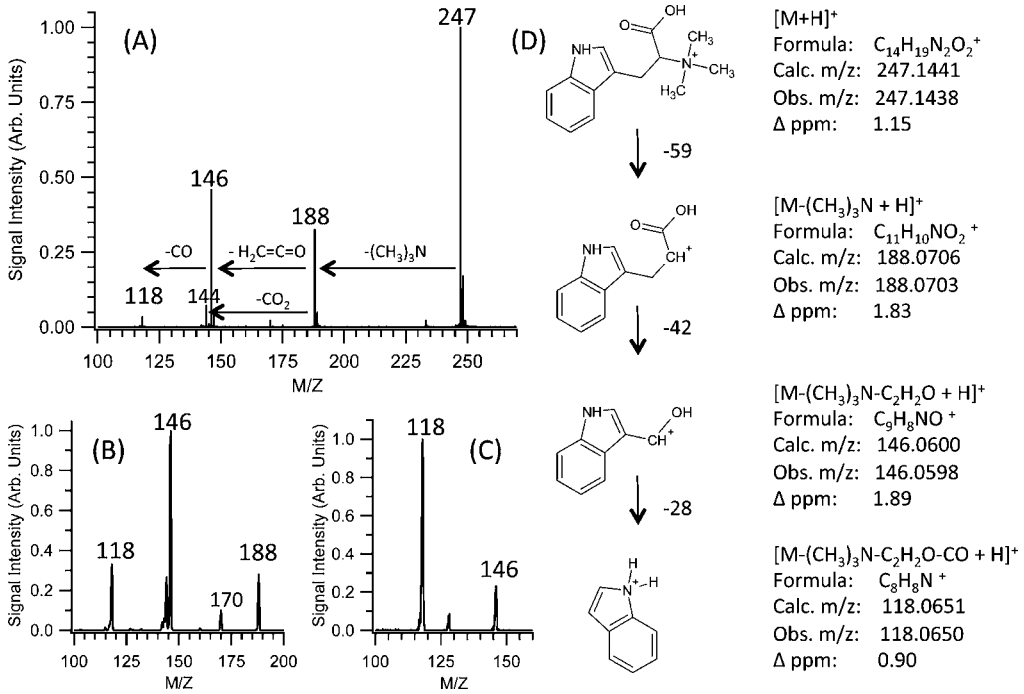

Figure 3. Tandem MS analysis of hypaphorine standard compound. (A) MS/MS spectrum of hypaphorine with main fragments assigned. (B) Pseudo-MS^3 spectrum of the fragment with m/z 188. (C) Pseudo-MS^3 spectrum of the fragment with m/z 146. (D) Proposed fragmentation pathway with calculated and observed accurate masses and mass errors for fragment and precursor ions. Detailed explanations are given in the text.

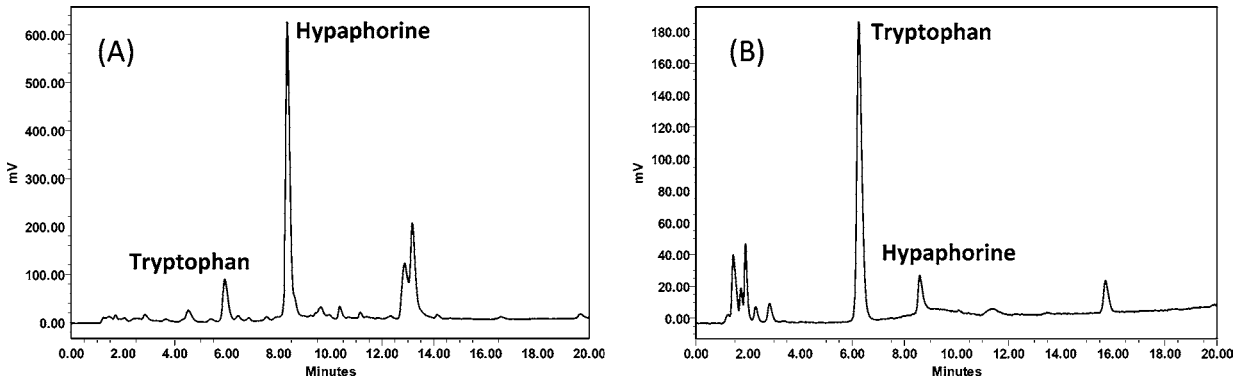

Figure 4. HPLC–fluorograms. (A) Peanut butter extract. One gram of peanut butter was mixed with 40 mL of a 30:70 acetonitrile:deionized water mixture, sonicated, and filtered through a 3 kDa MW cutoff centrifugal device. (B) Human milk plasma. Results are explained in the text.

The first intense fragment ion at m/z 188 is the product of a neutral loss of trimethylamine (59 Da), which is typical for some quaternary amine ions, such as acylcarnitines.[26] The next intense fragment at m/z 146 can be explained by a neutral loss of $H_2C{=}C{=}O$ (42 Da) and rearrangement of the ion structure. The final major signal at m/z 118 is proposed to be an ion formed through a further loss of CO (28 Da) and proton transfer to nitrogen. The pseudo-MS^3 spectra (Figure 3B/C), achieved through elevating the nozzle/skimmer voltage in the ESI source,[27] support the suggested fragmentation pathway, as they show consecutive losses from the ions with m/z 188 and 146, now selected as respective precursor ions, and are similar to tryptophan fragmentation (see www.massbank.jp, MassBank Record: KO004072). For the ion with m/z 188, there are two side reactions observed: (1) neutral loss of water yielding an ion with m/z 170 (18 Da), (2) neutral loss of CO_2 (44 Da), yielding an ion with m/z 144 (Figure 3A/B). Pseudo-MS^3 analysis of the ion with m/z 144 does not yield any further fragment ions in the observed range under the employed conditions. The proposed fragmentation pathway is further supported by the accurate mass measurements performed for the individual fragment ions that show less than 2 ppm mass errors and thus confirm the indicated ion elemental compositions (Figure 3D). A similar fragmentation spectrum was observed with the synthesized hypaphorine standard (see Supporting Information). During the synthesis of hypaphorine we analyzed the reaction mixture by HPLC–MS before the final purification steps and observed leftover tryptophan, *N*-monomethyltryptophan, *N*,*N*-dimethyltryptophan, and *N*,*N*,*N*-trimethyltryptophan methyl ester in the reaction mixture, besides the main product hypaphorine. We compared this data with all milk and food extract samples and confirmed that *N*-monomethyl tryptophan, *N*,*N*-dimethyltryptophan, and *N*,*N*,*N*-trimethyltryptophan methyl ester is not detectable in any of the analyzed samples.

HPLC–Fluorometry Analysis. Figure 4 shows fluorograms for an extract of peanuts and one of the human milk samples that contained high levels of hypaphorine. Fluorescence

chromatograms of standard solutions of tryptophan and synthesized hypaphorine iodide were used for calibration (see calibration curves and fluorogram example in Supporting Information). When compared to tryptophan, hypaphorine showed almost twice the fluorescence response under the employed conditions. Both compounds showed a linear calibration curve (r^2 = 0.9999) over a 2-order of magnitude concentration range (0.1−10 μM or 0.025−2.47 μg/mL). The LOQ for hypaphorine was calculated to be 0.02 μM (0.005 μg/mL). For the shown example in Figure 4, in peanut butter tryptophan levels were calculated to be 20 μg/g (detected concentration was 0.50 μg/mL in the 40 mL volume) and hypaphorine levels were calculated to be 45 μg/g (detected concentration was 1.12 μg/mL in the 40 mL volume). For human milk plasma, we calculated concentrations of 1.52 μM free tryptophan (~0.31 μg/mL) and 0.05 μM free hypaphorine (~0.01 μg/mL). To rule out potential coeluting and interfering components with similar fluorescence, the samples were also analyzed by LC−MS. Coeluting fluorescent components of unknown origin were observed for some foods analyzed that contained no hypaphorine (Table 1). The identity of these coeluting components is not known and needs to be elucidated; at this point we only know that our analysis by LC−MS did not identify any known tryptophan-related metabolites. There was no evidence for the presence of these coeluting and interfering components in any human milk sample or in the hypaphorine-containing foods (lentils, chickpeas, peanuts).

Hypaphorine Containing Foods. Table 1 shows the foods analyzed and the concentration of hypaphorine. We found that peanuts and peanut butter contain significant amounts of hypaphorine, and to our knowledge this is the first such report. Since peanuts and peanut products are widely consumed in many countries, and commonly eaten in snacks, confectionary, and peanut butter in North America, this finding is of special interest. Previous reports have documented hypaphorine in lentils[20,21] and chickpeas,[22] albeit with lower quantities than determined by us. Our quantitative analyses found 100 μg/g and 60 μg/g hypaphorine compared to previous reports of 50 μg/g[22] and 4 μg/g[19] in chickpeas and lentils, respectively. Reasons for the higher concentrations in our analyses could include differences in growing conditions or processing, or improved extraction efficiency of our method employing ultrasonication and a water/acetonitrile mixture. Our results also emphasize that breast-fed infants are exposed to hypaphorine through human, but neither cows' milk nor cow-milk-based or soy-based infant formulas showed detectable levels of this compound. However, it is possible that the absence of hypaphorine in cows' milk reflects feeding practices of dairy cattle, since it is possible that cattle grazing pasture with vetch or clover, which are members of the leguminous pea family, might have hypaphorine in their milk, if hypaphorine is not affected by metabolism in the rumen.

Hypaphorine Content of Human Milk from Lactating Women without Dietary Intervention. The mean ± SD hypaphorine concentration in the human samples was 0.34 ± 0.33 μM (Figure 5). The highest concentration of milk hypaphorine found was 1.24 μM and was in milk from a woman of East Indian background who reported eating 1 cup/day of chickpeas in addition to peanuts. For comparison, the concentration of free tryptophan in the milk samples was 2.23 ± 1.1 μM, and no correlation was observed between the milk hypaphorine and tryptophan concentrations (Figure 5). We grouped the women as high consumers or low/nonconsumers

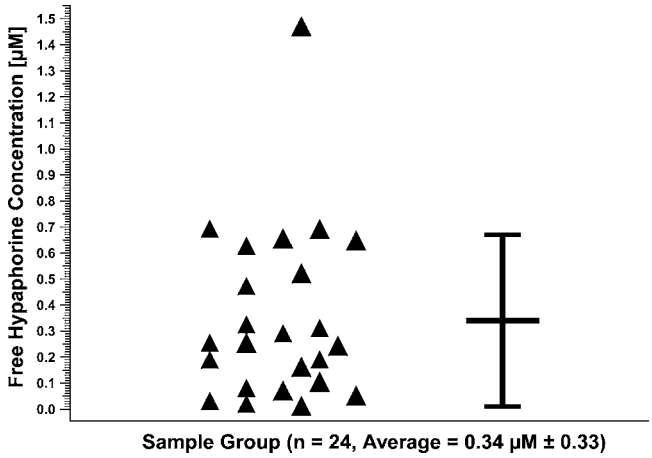

Figure 5. One-dimensional scatter plot of hypaphorine in human milk. Not all "high" levels could be explained by the reported intake of known hypaphorine-rich foods from the food questionnaires. The highest value was from a mother of East India background who ate 1 cup of chickpeas/day in addition to peanuts. The concentration of hypaphorine (1.46 μM) approaches the range of the milk free tryptophan, average for the group 2.23 ± 1.1 μM (n = 24). There is no observed correlation between hypaphorine and tryptophan concentration (correlation factor = 0.07).

of foods known to contain hypaphorine, based on their dietary intakes of lentils, chickpeas, and peanuts as reported in the dietary data. However, this yielded two groups with overlapping ranges of milk hypaphorine (Figure 5). Possible explanations were hypaphorine in other unidentified foods; the difficulty in identifying all exposures to peanuts, for example, in confectionary, cookies, and snack items in the food records; rapid changes in milk hypaphorine in response to very recent intake; and our dietary record, which was based on average intakes over the preceding month. Nevertheless, our results show that some lactating women secreted milk with high hypaphorine concentrations.

Dietary Challenge To Demonstrate Transfer of Hypaphorine from the Diet of Lactating Women into Human Milk. The purpose of these studies was to demonstrate transfer of hypaphorine from the maternal diet into human milk, and to offer insight into the time course of its appearance and disappearance from milk. We did not attempt analyses of quantitative transfer, which would require either use of a stable isotope tracer or quantitative collection of milk, thus interfering with infant feeding. No foods known to contain hypaphorine were consumed for 3 days before and after the dietary challenge. Figure 6 shows the rapid appearance of free hypaphorine in milk for one volunteer who consumed peanut containing foods and lentil soup in one meal. Free hypaphorine increased in the milk within 4 h of consumption and showed a peak at about 5-fold above the baseline concentration within 18 h, followed by a decline over 2−3 days, again with no correlation to the changes in milk free tryptophan (Figures 6A, 6B). The relatively constant low baseline milk hypaphorine before the dietary challenge and slow decline in milk hypaphorine following consumption suggest slow metabolism and/or excretion of hypaphorine in humans. However, given the limited knowledge on the hypaphorine content of foods, absorption, tissue distribution, and metabolism in humans, any conclusion remains tenuous. We have added hypaphorine time courses for 2 more volunteers after a similar dietary challenge in

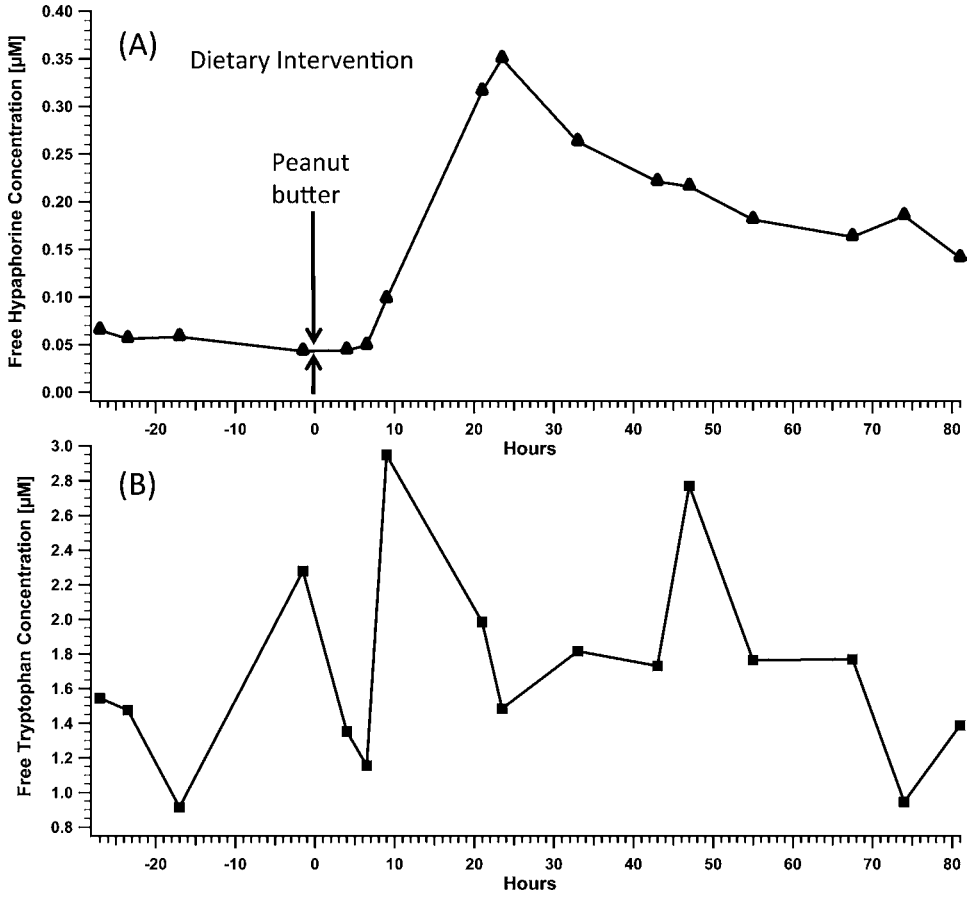

Figure 6. Illustrative time course for one volunteer of change in concentration of hypaphorine in human milk before and after a dietary challenge with hypaphorine-rich foods (peanut butter and a small cup of lentil soup). The time 0 h was set from the first diet challenge with peanut butter. Two other volunteers with similar diet challenges showed also increased free hypaphorine (see Supporting Information). (A) Free hypaphorine concentrations of 16 time course milk samples collected over a period of 110 h. (B) Free tryptophan concentrations for comparison. The correlation factor between the sets equals 0.16.

the Supporting Information. Approximate exposure of the breast-fed infant to hypaphorine from mother's milk can be estimated assuming an average daily intake of 780 mL, or about 100 mL of human milk per feeding. Previous studies of the transfer of stable isotope labeled fatty acids from the maternal diet into human milk have shown appearance by 6 h after ingestion, with a peak at 12−18 h, followed by a decline in milk levels over the following 24 h.[28] Such a pattern is similar to the appearance and decline of hypaphorine in milk following ingestion by lactating women in the present study. Our study does not address quantitative transfer of hypaphorine for the mother via breast milk to the breast fed infant; nevertheless our findings make it clear that hypaphorine is present in human milk, and increases on ingestion of hypaphorine containing foods by the lactating mother. Notably, hypaphorine is a quaternary amine and as such positively charged and very water-soluble.

In summary, the plant metabolite hypaphorine is present in human milk as a result of ingestion of hypaphorine-containing foods by the lactating mother. The rate of transfer of dietary hypaphorine to milk following ingestion indicates a relatively rapid absorption and transfer profile, peaking about 6−18 h after maternal intake. Chickpeas and lentils have been previously identified as hypaphorine-containing foods. The present report has identified substantial amounts of hypaphorine are also present in peanuts. Whether hypaphorine is present in other foods is not known, and current food databases do not include this nitrogen-containing amine. The hypaphorine content of human milk varies widely, with high levels approaching those of free tryptophan in the milk of some women in our population in western Canada. This is the first report of hypaphorine in human milk, and the potential concentration in milk of lactating women following traditional diets high in plant proteins such as lentils, chickpeas, and peanuts (groundnuts) is not known. The potential physiological effects of hypaphorine in either breast-feeding women or their infants are also unknown, as are the potential functional roles of hypaphorine which remain to be further investigated.

■ ASSOCIATED CONTENT

Supporting Information

Copies of original NMR scans for hypaphorine, calibration curves for fluorometric tryptophan and hypaphorine determination, fluorogram and mass spectra for synthesized hypaphorine, time courses of free hypaphorine concentration in human milk from 2 more volunteers after a dietary intervention, and details of the employed HPLC−MS gradient program. This material is available free of charge via the Internet at http://pubs.acs.org.

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