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Ruminant meat and milk contain δ-valerobetaine, another precursor of trimethylamine N-oxide (TMAO) like γ-butyrobetaine

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ABSTRACT

Quaternary ammonium compounds containing *N*-trimethylamino moiety, such as choline derivatives and carnitine, abundant in meat and dairy products, are metabolic precursors of trimethylamine (TMA). A similar fate is reported for N^{ε}-trimethyllysine and γ-butyrobetaine. With the aim at investigating the metabolic profile of such metabolites in most employed animal dietary sources, HPLC-ESI-MS/MS analyses on ruminant and non-ruminant milk and meat were performed. Results demonstrate, for the first time, the presence of δ-valerobetaine, occurring at levels higher than γ-butyrobetaine in all ruminant samples compared to non-ruminants. Demonstration of δ-valerobetaine metabolic origin, surprisingly, showed that it originates from rumen through the transformation of dietary N^{ε}-trimethyllysine. These results highlight our previous findings showing the ubiquity of free N^{ε}-trimethyllysine in vegetable kingdom. Furthermore, δ-valerobetaine, similarly to γ-butyrobetaine, can be degraded by host gut microbiota producing TMA, precursor of the proatherogenic trimethylamine N-oxide (TMAO), unveiling its possible role in the biosynthetic route of TMAO.

1. Introduction

Ruminant meat and milk, particularly those from cattle, represent worldwide fundamental aliments of paramount importance to humans for the remarkable content of substances with high nutritional value. However, it is known that excessive consumption of meat and dairy products, owing to the high content of cholesterol and saturated fatty acids, is serious risk factor of cardiovascular diseases (CVD) (Micha, Wallace and Mozaffarian, 2010; Guo et al., 2017; Drouin-Chartier et al.,2016). Moreover, in the last years studies have highlighted further risk factors of CVD, represented by the presence of quaternary ammonium compounds containing the *N*-trimethylamino moiety, such as choline derivatives and carnitine, present in a wide range of foods, such as red meat, eggs, and cheese. As matter of fact, these compounds are transformed by the host's intestinal microbiota, producing trimethylamine (TMA), which is easily absorbed and converted in the host's liver by flavin monooxygenases into trimethylamine N-oxide (TMAO) (Koeth et al., 2013), a substance believed to be both proatherogenic and associated with cardiovascular risks (Wang et al., 2011; Subramaniam & Fletcher, 2018; Zeisel & Warrier, 2017).

More recently, γ-butyrobetaine, the immediate biosynthetic precursor of carnitine, has been shown as the major gut microbial metabolite formed from dietary carnitine in mice, and is converted into TMA and TMAO in a gut microbiota-dependent manner (like dietary carnitine), thus accelerating atherosclerosis (Koeth et al., 2014; Skagen et al., 2016). Furthermore, it was also shown that chronic dietary exposure to carnitine or γ-butyrobetaine promotes the development of functionally distinct gut microbial communities optimized for the metabolism of carnitine or γ-butyrobetaine, respectively (Koeth et al., 2014).

Carnitine is a metabolite essential for the fatty acid transport and utilization in mitochondria. In mammals, it is biosynthesized through a sequence of four enzyme catalyzed reactions, starting from N^{ε}-trimethyllysine (TML), a particular alpha-amino acid bearing also a quaternary ammonium group. In the first reaction, TML is converted into 3-

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hydroxy-TML (HTML) by N^{ε}-trimethyllysine hydroxylase. Then, HTML is cleaved by a specific aldolase into glycine and 4-*N*-trimethylaminobutyraldehyde, which is successively oxidized to 4-*N*-trimethylaminobutyrate (γ-butyrobetaine). The pathway ends with γ-butyrobetaine hydroxylation which produces 3-hydroxy-4-*N*-trimethylaminobutyrate, also called carnitine (Vaz & Wanders, 2002). In mammals, especially carnivores, carnitine is largely introduced with the diet. However, animals can synthesize carnitine endogenously and a biosynthetic route alternative to that reported above is not known. The source of TML necessary for carnitine biosynthesis has been a puzzling problem for long time. As attempts aimed to demonstrate in higher organisms the TML formation from direct methylation of free lysine were unsuccessful, it is believed that it originates from the hydrolysis of proteins containing TML as a post-translational modification of lysine residues, such as, for example, histones, calmodulin, myosin and cytochrome *c* (Huszar, 1975; Morse, Vergnes, Malloy & McManus, 1975). On the other hand, as TML was believed to be rare in its free form, it was difficult to hypothesize a different origin for it. However, that view was strongly questioned by our recent findings that the main provenience of free TML could be from vegetables, which represent an important source of daily alimentation for most mammals, and the only source of food for herbivorous animals (Servillo, Giovane, Cautela, Castaldo & Balestrieri, 2014). In fact, we found that TML, believed to be rare in plants as free amino acid, is, on the contrary, ubiquitous in them and at not negligible levels. Therefore, it can be introduced in conspicuous amounts as free amino acid in animals, especially herbivorous, through vegetable food (Servillo et al., 2014). Under this respect, our finding raises the question of whether a tortuous and energy expensive route, as the one of TML formation from the breakdown of endogenous proteins, is really preferred when the substance is so easily available in vegetable foods. Consequently, we proposed that in mammals, especially herbivorous, TML introduced through diet might be further utilized for carnitine biosynthesis (Servillo et al., 2014). In light of this, here, we sought to investigate the occurrence of other TML metabolites, bearing the alleged “dangerous” trimethylammonium moiety, focusing our attention on meat and milk from various herbivorous mammals.

2. Materials and methods

2.1. Reagents

N^{ε}-trimethyllysine, valine, 5-aminovaleric acid, γ-butyrobetaine hydrochloride, Nα-(*tert*-butoxycarbonyl)-l-lysine, trifluoroacetic acid, and iodomethane-d3 were from Sigma-Aldrich (Milan, Italy). Milli-Q water was used for all the preparations of solutions and standards. The solution of formic acid 0.1% in water used for the HPLC-ESI-MS/MS analyses was from Sigma-Aldrich (Milan, Italy).

2.2. Synthesis and purification of valine betaine, δ-valerobetaine, N^{δ}-trimethyl-d9-valerobetaine and N^{ε}-trimethyl-d9-lysine

The preparation of valine betaine, δ-valerobetaine, N^{δ}-trimethyl-d9-valerobetaine (δ-valerobetaine-d9), and N^{ε}-trimethyl-d9-lysine (trimethyllysine-d9) was performed following the procedure of Chen & Benoiton (1976), based on a heterogeneous phase reaction in the presence of $KHCO_3$ using iodomethane (or iodomethane-d3) as a methylating agent. Briefly, about 100 mg of valine or 5-aminovaleric acid, or Nα-(*tert*-butoxycarbonyl)-l-lysine were dissolved in 20 mL of methanol, added with 1 g of $KHCO_3$, 10 mL of iodomethane (or iodomethane-d3), and stirred 12 h at room temperature. The addition of $KHCO_3$ (1 g) and iodomethane (or iodomethane-d3) (10 mL) was repeated twice more. Finally, the mixture was centrifuged and the supernatant was evaporated to dryness at 40 °C in a rotavapor. The residue, containing the product, was dissolved in 10 mL of Milli Q grade water and applied on 10 cm column filled with a mixed-bed resin of Dowex-1-OH^- and Biorex-70-H^+ (1:1 v/v), able to retain amino acids but not betaines (Wood, et al., 2002). The aqueous wash from this column was then applied to a 10 × 2 cm column with AG50WX8-H^+ resin, and washed with 20 mL of water. Finally, the product was eluted with 30 mL of 6 M NH_4OH and evaporated to dryness under a stream of air. In the case of N^{α}-(*tert*-butoxycarbonyl)-l-lysine labelling with iodomethane-d3, the dried product was treated for 2 h at room temperature with neat trifluoroacetc acid (1:10 w/w) in order to remove the protecting group and then evaporated again to dryness.

2.3. Preparation of meat extracts

Fresh meat samples (n = 6 for each species in duplicate), purchased in local markets, were from animals raised in Campania (Italy). Meat specimens were cut in small pieces and homogenized on ice chamber with three parts of 0.1% (w/w) formic acid precooled at 4 °C. The homogenate was centrifuged at 10,000 × *g* at 4 °C for 10 min and the clarified supernatant filtered sequentially through a 5 μm and 0.45 μm Millipore filters. All extracts were prepared in triplicate for each type of meat and then stored frozen in aliquots until used. Before mass spectrometric analysis, aliquots were melted and filtered through Amicon Ultra 0.5 mL centrifugal filters with 3 kDa molecular weight cutoff.

2.4. Preparation of milk samples

Milk from all animal species (n = 6 for each species in duplicate) were provided by Department of Veterinary Medicine and Animal Production, Federico II University, Naples, Italy. Three samples of human milk were provided by voluntary donors and analyzed in duplicate.

Milk specimens were first skimmed by centrifugation at 3000 × *g* for 15 min a 4 °C to remove the fat globules. The skimmed milk was filtered through a 5 μm Millipore filters and then stored frozen in aliquots until used. Before mass spectrometric analysis, aliquots were melted and filtered through Amicon Ultra 0.5 mL centrifugal filters with 3 kDa molecular weight cutoff.

2.5. Analysis by HPLC-ESI-MS/MS

The analyses were performed by HPLC-ESI MS/MS according to Servillo et al. (2016, 2017) with an Agilent 1100 series liquid chromatograph using a Supelco Discovery C8 column, 250 × 3.0 mm, particle size 5 μm. The chromatography was conducted isocratically with 0.1% formic acid in water at flow rate of 100 μL/min. Volumes of 10 μL of standard solution or sample were injected. Compounds were identified on the basis of their retention times and MS^2 fragmentation patters. Quantification of each substance was generally obtained by comparison of the peak area of its most intense MS^2 fragment with the respective calibration curve built with standard solutions. HPLC-ESI-MS/MS analyses were performed with an Agilent LC-MSD SL quadrupole ion trap, in positive multiple reaction monitoring (MRM) using for each analyte the most intense MS^2 transitions. Matrix effect in quantitative determinations was assessed by the standard addition method. The mass spectrometer was operated utilizing nitrogen as the nebulizing and drying gas. The instrumental conditions were as follows: nebulizer pressure, 30 psi; drying temperature, 350 °C; drying gas 7 L/min. The ion charge control (ICC) was applied with target set at 30,000 and maximum accumulation time at 20 ms. The concentrations of each compound were determined by comparison with the relative calibration curve. Standard stock solutions of each analyte were prepared at 2 mg/L. Additional calibration levels (0.2, 0.1, 0.05, 0.02, 0.002 and 0.001 mg/L) were prepared by serial dilution with water containing 0.1% formic acid. The calibration curves were build using these standard solutions. The linear regression analysis was carried out by plotting the peak areas of the monitored fragment ions versus the concentrations of the analyte standard solutions. The linearity of the instrumental response was assessed by correlation coefficients