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In vivo investigation of homocysteine metabolism to polyamines by high-resolution accurate mass spectrometry and stable isotope labeling

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ABSTRACT

Polyamines are essential polycations, playing important roles in mammalian physiology. Theoretically, the involvement of homocysteine in polyamine synthesis via *S*-adenosylmethionine is possible; however, to our knowledge, it has not been established experimentally. Here, we propose an original approach for investigation of homocysteine metabolites in an animal model. The method is based on the combination of isotope-labeled homocysteine supplementation and high-resolution accurate mass spectrometry analysis. Structural identity of the isotope-labeled metabolites was confirmed by accurate mass measurements of molecular and fragment ions and comparison of the retention times and tandem mass spectrometry fragmentation patterns. Isotope-labeled methionine, spermidine, and spermine were detected in all investigated plasma and tissue samples. The induction of moderate hyperhomocysteinemia leads to an alteration in polyamine levels in a different manner. The involvement of homocysteine in polyamine synthesis and modulation of polyamine levels could contribute to a better understanding of the mechanisms connected with homocysteine toxicity.

The deleterious effects observed in hyperhomocysteinemia (HHcy)[1] have attracted a great deal of interest during recent decades, and a number of studies were undertaken to understand homocysteine (Hcy) metabolism and the mechanism of its toxicity [1–3]. However, the pathogenic culprit in HHcy is still obscure. The controversial results about the deleterious effect of elevated plasma Hcy again return the interest to the metabolism and metabolites of Hcy in pathogenesis of HHcy [4–6].

Hcy is a sulfur-containing, non-proteinogenic amino acid, the levels of which are regulated mainly by two metabolic pathways: remethylation and transsulfuration (Fig. 1). The transsulfuration of Hcy to cysteine via cystathionine is mediated by the consecutive actions of the enzymes cystathionine-β-synthase and cystathionine-γ-lyase. Both enzymes require vitamin B_6 as cofactor. Through the remethylation pathway, Hcy is converted to methionine (Met) by means of either methionine synthase or betaine-homocysteine *S*-methyltransferase. The first enzyme requires vitamin B_{12} as cofactor and acts outside the mitochondria, whereas the second enzyme requires betaine as co-substrate and acts inside the mitochondria. In the next step, Met is activated by adenosine triphosphate (ATP) and the enzyme methionine adenosyltransferase, leading to the formation of *S*-adenosylmethionine (SAM) [7,8]. The latter compound is a key substrate in two essential metabolic reactions. It can donate methyl group to a variety of transmethylation reactions or undergo decarboxylation by the enzyme *S*-adenosylmethionine-decarboxylase and then be included in polyamine synthesis [7,9]. A product of the methylation reactions is *S*-adenosylhomocysteine (SAH), which is further metabolized to Hcy by the enzyme SAH-hydrolase [7,8]. In the polyamine synthesis, the propylamine moiety delivered from SAM is transferred to putrescine (or spermidine) and producing spermidine or spermine, respectively. The enzymes involved in these conversions are spermidine synthase and spermine synthase [7,9].

In contrast to the large number of reports concerning the influence of Hcy on the cellular methylation via alteration of SAM levels [10,11], there is still a lack of information about its impact on polyamine synthesis, although theoretically Hcy could affect both pathways.

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[1] *Abbreviations used:* HHcy, hyperhomocysteinemia; Hcy, homocysteine; Met, methionine; ATP, adenosine triphosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; d_8-Hcys, isotope-labeled homocystine; HRAM, high-resolution accurate mass; MS/MS, tandem mass spectrometry; FAESC, 2-[*N*-(2-fluoroacridonyl)]ethyl-*N*-succinimidyl carbonate; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; FAESC, 1-({[2-(2-fluoro-9-oxoacridin-10(9H)-yl)ethoxy]carbonyl}oxy) pyrrolidine-2,5-dione; HCD, higher energy collision dissociation; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

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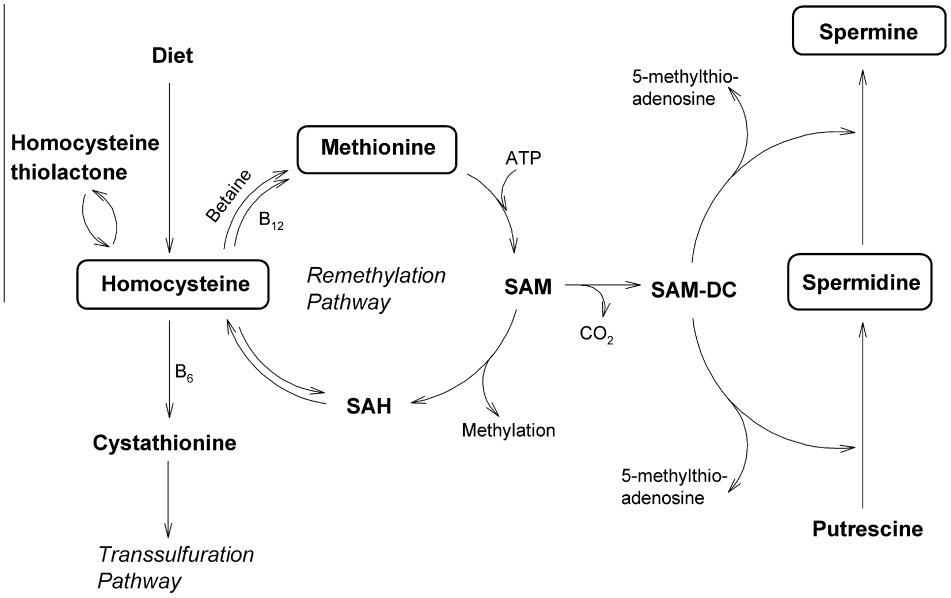

Fig.1. Metabolism of homocysteine (Hcy). ATP, adenosine triphosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

The polyamines are ubiquitous compounds in all living cells. They are essential mediators in various physiological processes, such as cellular proliferation, progression, and differentiation, and also are involved in intracellular signaling and apoptosis [9,12]. Furthermore, they have a critical role in cardiac pathophysiology [12] and central nervous system function [13], which are also associated with HHcy [14–16].

The aim of this study was to develop an original approach for investigation of Hcy involvement in spermidine and spermine synthesis. For this purpose, we made two innovations: design of a short-term in vivo experiment with stable isotope-labeled homocystine (d_8-Hcys) supplementation and design and synthesis of a new amino-specific derivatization reagent. The verification of the labeled aminopropyl moiety incorporation in the metabolite molecules was made using high-resolution accurate mass (HRAM) mass spectrometry analysis by accurate mass measurements of molecular and fragment ions and comparison of the chromatographic retention times and the tandem mass spectrometry (MS/MS) fragmentation pattern of all isotope-labeled metabolites with their unlabeled analogues.

Materials and methods

Animal study

Experiments were carried out on healthy adult albino rats of Wistar strain (250–300 g body weight). The animals were adapted under laboratory conditions for 2 weeks before starting the experiment. They were kept in animal houses by maintaining standard conditions of temperature (22–25 °C) and humidity (50%) with an alternating 12 h light/dark cycle. The study was performed in accordance with the institutional care and use of laboratory animal guidelines of the Medical University of Sofia and is in conformity with the European convention of animal protection (directive 2010/63/EU of the European Parliament and European Council).

The experiments were performed on 12 albino rats of Wistar strain kept in metabolic cages. They were divided into two different groups of equal average body weight as follows:

Group I: Control group animals received drinking water for 5 days.

Group II: Animals received 0.05 mg of d_8-Hcys per milliliter drinking water for 5 days.

The average volume of drinking water per day was in the range of 30 to 35 ml.

Isotope-labeled homocystine was chosen because it is the most stable homodimer derivative of Hcy. Except for the added d_8-Hcys, all diets consisted of an identical standard composition. Body weight, chow consumption, and fluid intake were monitored daily.

Materials

DL-Homocystine-3,3,3′,3′,4,4,4′,4′-d_8 was purchased from C/D/N Isotopes (Canada). Perchloric acid and acetonitrile (MS grade) were obtained from Merck (Germany). High-purity water was generated using a Purelab UHQ II system from ELGA (The Netherlands). Ethyl bromoacetate, $NaBH_4$, and disuccinimidyl carbonate were obtained from Sigma–Aldrich (Germany). 2-Fluoroacridone was synthesized in the laboratory as described previously [17]. All other reagents were of the highest purity available in the laboratory.

Synthesis of 2-[N-(2-fluoroacridonyl)]ethyl-N-succinimidyl carbonate

Synthesis of ethyl [N-(2-fluoroacridonyl)] acetate

To a solution of 2-fluoroacridone (545 mg, 2.5 mmol) in 20 ml of dimethylformamide (DMF), anhydrous potassium carbonate (3.46 g, 25 mM) and ethyl bromoacetate (1.5 ml, 13 mM) were added. The reaction mixture was microwave radiated for 60 s and then cooled to room temperature. The microwave radiation was repeated until the 2-fluoroacridone was completely reacted (5 × 60 s). Then, 70 ml of water was added to the reaction and the mixture was acidified to approximately pH 3.0 with 6 M hydrochloric acid. The formed precipitate was filtered off, washed with water, and dried under vacuum. Yield = 83%. ESI–MS, $[M+H]^+$ *m*/*z* 300.1025, calc. $[M+H]^+$ 300.1030, −1.66 ppm.

Synthesis of 2-[N-(2-fluoroacridonyl)] ethanol

The ethyl [N-(2-fluoroacridonyl)] acetate (2.07 mmol, 620 mg) was dissolved in 15 ml of tetrahydrofuran, and $NaBH_4$ (4.15 mM, 157 mg) and methanol (27 mM, 1.2 ml) were added. The time course of the reaction was followed by means of high-performance