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Myocardium proteome remodelling after nutritional deprivation of methyl donors☆,☆☆

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Abstract

Methyl donor (MD: folate, vitamin B12 and choline) deficiency causes hyperhomocysteinemia, a risk factor for cardiovascular diseases. However, the mechanisms of the association between MD deficiency, hyperhomocysteinemia, and cardiomyopathy remain unclear. Therefore, we performed a proteomic analysis of myocardium of pups from rat dams fed a MD-depleted diet to understand the impact of MD deficiency on heart at the protein level. Two-dimension gel electrophoresis and mass spectrometry-based analyses allowed us to identify 39 proteins with significantly altered abundance in MD-deficient myocardium. Ingenuity Pathway Analysis showed that 87% of them fitted to a single protein network associated with developmental disorder, cellular compromise and lipid metabolism. Concurrently increased protein carbonylation, the major oxidative post-translational protein modification, could contribute to the decreased abundance of many myocardial proteins after MD deficiency. To decipher the effect of MD deficiency on the abundance of specific proteins identified in vivo, we developed an in vitro model using the cardiomyoblast cell line H9c2. After a 4-day exposure to a MD-deprived (*vs.* complete) medium, cells were deficient of folate and vitamin B12, and released abnormal amounts of homocysteine. Western blot analyses of pup myocardium and H9c2 cells yielded similar findings for several proteins. Of specific interest is the result showing increased and decreased abundances of prohibitin and α-crystallin B, respectively, which underlines mitochondrial injury and endoplasmic reticulum stress within MD deficiency. The in vitro findings validate the MD-deficient H9c2 cells as a relevant model for studying mechanisms of the early metabolic changes occurring in cardiac cells after MD deprivation.

Keywords: Methyl donor deficiency; Hyperhomocysteinemia; Myocardium; H9c2 cells; Proteomics

1. Introduction

Hyperhomocysteinemia (HHcy) has been invoked in addition to other major recognized risk factors (i.e., age, sex, diabetes…) for cardiovascular diseases (CVD), the chief cause of death in the world [1,2]. HHcy is clinically defined by plasma homocysteine (Hcy) levels higher than 15 μM. Hcy is a sulfur amino acid produced through the one-carbon cycle from transmethylation of different compounds like hormones, neurotransmitters, nucleic acids, and phospholipids. S-adenosylmethionine is a critical substrate for these reactions, especially those related to epigenetic regulation [3]. Hcy requires methyl donors (MDs) such as folate (vitamin B9), vitamin B12 (cobalamin) or choline to be intracellularly remethylated into methionine [4]. One major consequence of MD deficiency is a mild or moderate HHcy characterized by circulating total Hcy concentrations in the 15–30 or 31–100 μM range, respectively. This condition has long been associated with an elevated risk of chronic pathologies like atherosclerosis, ischemic heart disease and stroke in humans [5]. MD deficiency and subsequent HHcy can occur in individuals with increased requirement and/or inadequate intake of either MD, e.g. during pregnancy or aging [6]. Supplementation with folic acid and/or other vitamins B reduces the concentration of circulating total Hcy in patients prior CVD, but has no beneficial effects on the development or onset of

Abbreviations: CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CVD, Cardiovascular diseases; C, Control; 2-D, two-dimension; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; ERR-α, estrogen-related receptor-α; FBS, fetal bovine serum; FC, Fold change; HHcy, hyperhomocysteinemia; Hcy, homocysteine; IPA, Ingenuity Pathway Analysis; p*I*, isoelectric point; MD, methyl donor; MDD, MD-deficient; MS, mass spectrometry; MW, molecular weight; PGC-1α, PPAR-γ co-activator-1 α; PPAR, peroxisome proliferator-activated receptor; PC, protein carbonyl; PTM, post-translational modification; sHSP, small heat shock protein.

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cardiovascular events [7]. The mechanisms of the association between cardiomyopathy and MD deficiency are not yet fully understood. Atherogenic effects of HHcy include a variety of cellular changes including endothelial dysfunction, inflammation, oxidative stress and endoplasmic reticulum (ER) stress [8]. MD deficiency has been shown to affect tissue structure, function or metabolism in rodents especially when occurring during pre- or perinatal periods [9–11]. However, data are currently limited on the impact of MD deficiency on the heart. Garcia et al. reported recently myocardial hypertrophy with cardiomyocyte enlargement, mitochondrial disorganization and dysfunction, and altered fatty acid oxidation and energy metabolism in weaning rats from dams fed a diet deprived of vitamin B12, folate and choline during gestation and lactation [12]. This was concomitant with abnormal myocardial accumulation of Hcy and lipids, and impaired expression of the peroxisome proliferator-activated receptors (PPARs) α and γ, PPAR-γ coactivator-1α (PGC-1α) and estrogen-related receptor-α (ERRα). Altered expression of these important functional proteins prompted us to investigate further the impact of maternal MD deprivation on the myocardial proteome of rat pups. Deficiency of one or several MDs, as well as intracellular Hcy accumulation, has been shown to cause imbalanced redox state in rat tissues, a condition favoring the development of CVD [13,14]. Thus, we first evaluated changes in protein carbonylation, a major oxidative post-translational modification (PTM) of proteins, in myocardium of rat pups after maternal MD-deprivation. Then, we used a large-scale proteomic approach to bring new information on the effect of this deficient condition on the protein pattern of the myocardium of the offspring. Finally, aiming to develop a cell study model of MD deficiency, we used the H9c2 cardiomyoblast cell line to evaluate the impact of this condition on the abundance of specific proteins identified in vivo by the proteomic analysis. This cell line deriving from embryonic rat heart, has been shown to retain many characteristics of rat cardiomyocytes and is used as a model of cardiac hypertrophy [15,16], one major feature of MD deficiency in rat pups [12].

2. Materials and methods

2.1. Rat dietary model

Dietary experiments on rats were performed at INSERM U954 (Nancy, France) according to local internal guidelines for animal care and housing and the National Institute of Health Guide for the care and use of laboratory animals. As previously described [12], adult female Wistar rats (Charles River, Les Oncins, France) were constantly maintained under standard laboratory conditions, with food and water available ad libitum. One month before mating, they were fed either standard food containing vitamins B12 (0.04 mg/kg), folate (0.9 mg/kg), and choline (2.1 g/kg) (control diet: C diet) according to the supplier (Maintenance diet M20, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) or a MD-deficient (MDD) diet deprived of vitamins B12, folate, and choline (Special Diet Service, Saint-Gratien, France) [12]. The content of all other ingredients as well as the protein sources were identical in both diets. MDD and C diets were respectively maintained in both groups of dams until weaning of their offspring, i.e., at 21 days after birth, as described previously [12]. The 21 day-old pups were killed by decapitation after exposure to halothane and their heart was rapidly collected, washed in Ringer's buffer solution, freeze-clamped in liquid nitrogen and stored at −80°C until analysis.

2.2. Myocardium protein carbonylation

Protein carbonyls (PC) levels were determined using the 2,4-dinitrophenylhydrazine (DNPH) assay as described in [17]. A standard curve of carbonylation was performed with protein samples from adult rat myocardium using a spectrophotometry method. The PC content of these samples was calculated using the molar extinction coefficient of 22,000 $M^{-1}.cm^{-1}$ and expressed as nmol/mg protein. A dot-blot immunoassay was performed on PVDF membrane to quantify the total carbonylation of myocardial proteins in MDD *vs* C rats. Adducts of 2,4-dinitrophenyl hydrazone with PCs were probed with a specific primary rabbit antibody and a secondary goat HRP-conjugated anti-rabbit IgG antibody provided with the OxyBlot oxidized protein detection kit (Chemicon International, Temecula, CA). Densitometry analyses of the dots were done using the ImageJ®1.37v software (Wayne Rasband, NIH, USA).

2.3. Myocardium proteomics analysis

Proteomic analysis was carried out as previously described, with a few changes [18]. Freeze-clamped whole myocardium was homogenized (using a mini-potter) in an extraction buffer made of 5 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonic acid (CHAPS), 40 mM Tris, 0.2% (v/v) biolytes pH 3–10 and 50 mM dithiothreitol (DTT). After homogenate centrifugation (18,000×*g* for 15 min) and measurement of the supernatant protein concentration (RC DC™ protein assay kit, Bio-Rad Laboratories, Marnes-La-Coquette, France), supernatant proteins were separated by two-dimension (2-D), 12% polyacrylamide gel electrophoresis. For the first dimension of analytical and preparative gels, 500 μg of proteins were loaded onto 17 cm, pH 3 — 10 non-linear, Bio-Rad ReadyStrips by inclusion of an adequate volume of extract in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 0.2 % biolytes pH 3–10, 0.0002% (w/v) Bromophenol blue) (BioRad, Sigma). Following the second dimension, separate protein spots were visualised on gels by 0.02% (w/v) colloidal Coomassie Blue staining and images were captured on an image scanner (Amersham ImageScanner, GE Healthcare, Orsay, France). Quantification of protein changes across triplicates of each protein sample from MDD or C pups (n=6 per condition) was captured via image alignment and analysis, and integrated analysis of expression profiles using the Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne UK). Results were expressed as means±S.D. Tailored multivariate statistical analysis facilities incorporated into the Progenesis SameSpots software were used for image analysis with P<.05 considered significant. Spots showing a significant change in abundance between the two conditions were subsequently analysed by mass spectrometry (MS) (Matrix-Assisted Laser Desorption/Ionisation–Time-of-Flight, MALDI-ToF) and when necessary for validation of the identification, LC-MS/MS) at the Metabolism Exploration Platform (http://www5.clermont.inra.fr/plateforme_exploration_metabolisme/).

2.4. Generation of functional networks

MS-identified proteins were evaluated by Ingenuity Pathway Analysis, IPA (Ingenuity Systems, Mountain View, CA, USA), a software application that notably enables to recognize in silico networks of hypothetically interacting proteins based on a regularly updated "Ingenuity Pathways Knowledge Base". Highly connected networks generated by IPA contain protein relationships with evidenced direct physical contact and/or indirect interactions explicitly described in scientific sources. IPA networks are ranked by scores representing the log probability that they were found by random chance alone. Scores ≥2 have at least a 99% confidence of not being generated from the Global Molecular Network by random chance alone and are therefore usually attributed to a valid network. Furthermore, we manually inspected all protein interactions generated by IPA to eliminate any possible spurious results.

2.5. Model of MD-deficient cardiac cells

H9c2 cells from the clonal myoblastic cell line derived from embryonic rat heart (ATCC, LGC Standards, Molsheim, France) were cultured between passages 13 and 23 at 37°C under a 5:95 CO_2:air atmosphere, in a DMEM medium containing 4.5 g/L glucose, 3.7 g/L $NaHCO_3$, stabilized glutamine and sodium pyruvate (Dutscher, Brumath, France), and supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA Laboratories, Les Mureaux, France), 1% nonessential amino acids (Invitrogen, Cergy-Pontoise, France) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). The culture medium was refreshed 24 h after seeding, then every 2 days. Cells were grown in this culture medium until 70% of confluence to maintain them as cardiomyoblasts.

For MD depletion experiments, a MCDB-131 medium lacking folic acid, vitamin B12 and choline was synthesized in the lab and designed as the MDD medium. Complete (C) MCDB-131 medium was synthesized concurrently by supplementing the MDD medium with amounts of the lacking MDs similar to those in commercial MCDB-131 [19]. Both C and MDD MCDB-131 media were supplemented with stabilized glutamine, nonessential amino acids, antibiotics and 10% (v/v) dialysed FBS. Subsequently, there was no folic acid, vitamin B12 and choline in the MDD culture medium vs. 1.2 μM, 10 nM and 100 μM in the C culture medium, respectively. After growth to about 50% confluence in DMEM, cells were adapted overnight in the C MCDB-131 medium and then exposed either to the latter or to the MDD MCDB-131 medium for 4 days. In order to maintain the culture below 70% confluence during the depletion time, cells were sub-cultured after two days in fresh C or MDD MCDB-131 medium, respectively. At each passage or medium renewal, the culture medium was sampled and kept at −80°C for further measurement of extracellular Hcy concentrations (see below).

2.6. Assays of extracellular Hcy and intracellular folate and vitamin B12

Measurement of extracellular Hcy concentration was performed by reverse phase-HPLC coupled with fluorescence detection, after Hcy reduction by Tris(2-carboxyethyl phosphine hydrochloride) and derivatization with 7-fluorobenzofurane-4-sulfonic acid [20]. Intracellular concentrations of both vitamins were determined by using a radio-dilution isotope assay (simulTRAC-SNB, ICN Pharmaceuticals).