Journal of Molecular and Cellular Cardiology 52 (2012) 753–760

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Homocysteine induces cardiomyocyte dysfunction and apoptosis through p38 MAPK-mediated increase in oxidant stress

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ARTICLE INFO

Article history:
Received 14 October 2011
Received in revised form 13 December 2011
Accepted 20 December 2011
Available online 29 December 2011

Keywords:
Homocysteine
Hyperhomocysteinemia
Cardiomyocytes
Contractility
Apoptosis
Antioxidant
Ischemia reperfusion injury
Oxidative stress

ABSTRACT

Elevated plasma homocysteine (Hcy) is a risk factor for cardiovascular disease. While Hcy has been shown to promote endothelial dysfunction by decreasing the bioavailability of nitric oxide and increasing oxidative stress in the vasculature, the effects of Hcy on cardiomyocytes remain less understood. In this study we explored the effects of hyperhomocysteinemia (HHcy) on myocardial function *ex vivo* and examined the direct effects of Hcy on cardiomyocyte function and survival *in vitro*. Studies with isolated hearts from wild type and HHcy mice (heterozygous cystathionine-beta synthase deficient mice) demonstrated that HHcy mouse hearts had more severely impaired cardiac relaxation and contractile function and increased cell death following ischemia reperfusion (I/R). In isolated cultured adult rat ventricular myocytes, exposure to Hcy for 24 h impaired cardiomyocyte contractility in a concentration-dependent manner, and promoted apoptosis as revealed by terminal dUTP nick-end labeling and cleaved caspase-3 immunoblotting. These effects were associated with activation of p38 MAPK, decreased expression of thioredoxin (TRX) protein, and increased production of reactive oxygen species (ROS). Inhibition of p38 MAPK by the selective inhibitor SB203580 (5 μM) prevented all of these Hcy-induced changes. Furthermore, adenovirus-mediated overexpression of TRX in cardiomyocytes significantly attenuated Hcy-induced ROS generation, apoptosis, and impairment of myocyte contractility. Thus, Hcy may increase the risk for CVD not only by causing endothelial dysfunction, but also by directly exerting detrimental effects on cardiomyocytes.

1. Introduction

Hyperhomocysteinemia (HHcy) is a pathological condition caused by multiple factors including dietary deficiencies of B vitamins and genetic defects, leading to reduced function of enzymes required for the metabolism of homocysteine (Hcy) such as cystathionine beta synthase (CBS) [1]. Since McCully proposed HHcy as a risk factor for cardiovascular disease (CVD) over 40 years ago [2], epidemiological studies have associated HHcy with an increased risk of coronary artery disease [3], peripheral vascular disease [4], venous thrombosis [5], myocardial infarction [6], and stroke [7]. However, Hcy-lowering therapies in subjects with established CVD were not shown to reduce the risk of recurrent CVD events despite substantial reduction in total Hcy levels with vitamin treatment [8–10]. Thus, further exploration is needed to elucidate the effects of HHcy on CVD.

Several mechanisms have been suggested for the link between HHcy and CVD, including HHcy-induced functional perturbation of endothelial cells, vascular smooth muscle cells, and platelets, as well as effects on components of the coagulation and/or fibrinolytic systems [11–13]. Although the precise mechanisms are not completely understood, HHcy has been shown to promote endothelial dysfunction by decreasing the bioavailability of nitric oxide (NO) and increasing oxidative stress in the vasculature [14]. In addition, emerging evidence suggests that HHcy may also have adverse effects on the myocardium. A positive correlation between left ventricular mass index and plasma total Hcy was observed in patients with end-stage renal disease [15]. Ten weeks of HHcy was shown to elicit adverse cardiac remodeling and diastolic dysfunction in normotensive rats [16], and to exacerbate remodeling and diastolic dysfunction in hypertensive hearts [17]. Recently, HHcy was shown to inhibit NO-dependent regulation of cardiac O_2 consumption *in vitro* through increased superoxide ($O_2^{\bullet -}$) production via activation of NADPH

Abbreviations: Hcy, homocysteine; HHcy, hyperhomocysteinemia; tHcy, total homocysteine; TRX, thioredoxin; CBS, cystathionine β-synthase; CVD, cardiovascular disease; NO, nitric oxide; ROS, reactive oxygen species; % CS, percent cell shortening; $[Ca^{2+}]_i$, intracellular calcium; WT, wild type; TTE, trans-thoracic echocardiography; EDP, end diastolic pressure; ESP, end systolic pressure; I/R, ischemia reperfusion; TUNEL, terminal dUTP nick-end labeling.

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doi:10.1016/j.yjmcc.2011.12.009

oxidase [18], which reduced the bioavailability of NO and altered cardiac substrate metabolism [19]. However, whether HHcy has direct effects on cardiomyocytes, the major cellular components of the heart, is not well elucidated.

To complement the previously described vascular effects of HHcy and provide additional mechanisms for the association of HHcy and CVD, in this study we assessed the direct effects of HHcy on cardiomyocyte function, survival, and redox status, and examined the underlying mechanisms involved. We found that the p38 MAPK signaling pathway plays a critical role in mediating Hcy's effects on cardiomyocytes via modulation of cellular thioredoxin (TRX) expression.

2. Materials and methods

This study was performed in accordance with the guidelines of the Animal Care and Use Committees of Boston University School of Medicine and Harvard Medical School, and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.1. Hyperhomocysteinemic mouse models

Mice heterozygous for targeted disruption of the CBS gene (CBS mice) [20] were obtained from the Jackson Laboratory and subsequently bred at Boston University and Harvard Medical School. The animals were fed standard chow. The genotype was identified by PCR for sequences in intron 3 and the Neo insert in CBS mice [20]. Plasma total Hcy (tHcy) was measured using a commercial kit from Bioanalytical Systems following the manufacturer's protocol. After addition of an internal standard followed by chemical reduction and protein precipitation, samples were separated on a BAS 200A HPLC using a reverse-phase C18 column (100×2 mm) with 3 μm beads. Thiols were measured using an electrochemical detector. Quantitation was achieved by comparison with various concentrations of homocysteine standards that were similarly processed and used to generate a standard curve. Columns and mobile phase (MP-4) were obtained from Bioanalytical Systems.

2.2. Mouse cardiomyocyte isolation

Mouse cardiomyocytes were isolated as previously described [21].

2.3. Isolated mouse heart ischemia/reperfusion studies

Hearts were isolated from mice and perfused in the Langendorff mode (retrograde) as previously described [22]. Ventricular tissue samples were obtained at the end of each experiment and cut into two parts. One part was snap-frozen in liquid nitrogen and then stored at −80 °C for Western blotting analysis. The other part was fixed with 4% paraformaldehyde and used for *in situ* TUNEL analysis.

2.4. Adult rat cardiomyocyte isolation, culture, and treatment

Ventricular cardiomyocytes were isolated from male Wistar rats (200 to 250 g) (Charles River Laboratories) using the collagenase perfusion method as previously described [23]. DL-Homocysteine was purchased from Sigma, stored at −20 °C and fresh stock solution prepared for every experiment and added directly to the culture medium to obtain the desired concentration. In the p38 MAPK inhibition experiments, cells were treated with 5 μM SB203580 (A.G. Scientific) or vehicle (dimethylsulfoxide, 0.05% final concentration in medium) for 30 min, followed by Hcy treatment for 24 h.

2.5. Measurements of mouse and rat cardiomyocyte cell shortening and $[Ca^{2+}]_i$ transients

Cardiomyocyte cell shortening (CS) and intracellular calcium ($[Ca^{2+}]_i$) transients were recorded as previously described [23].

2.6. Terminal dUTP nick-end labeling (TUNEL) assay

Cardiomyocyte apoptosis was quantified with an *in situ* cell death detection kit (Roche Applied Science). In brief, cardiomyocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature, washed, and then permeabilized with 100% methanol for 30 min at −20 °C. The fixed cells were then labeled by TUNEL (37 °C, 1 h), washed, and mounted using antifade mounting media containing 4′,6 diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). For *ex vivo* study, 4 μm paraffin-embedded sections of ventricular tissues were assayed for cell death by TUNEL staining. Fluorescent images were taken in 10 to 15 random high-powered (40×) fields using a fluorescence microscope (Nikon Instruments). All cells were counted on each slide, and TUNEL-positive cells were expressed per 100 nuclei.

2.7. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS were determined using the method described previously [23].

2.8. Measurement of mitochondrial membrane potential

Inner mitochondrial membrane potential was assessed using the JC-1 dye (Molecular Probes). Cardiomyocytes were plated onto 12-well plates upon isolation. After treatment, cells were incubated in the dark for 2 h at 37 °C in phenol red-free DMEM containing the dye (3.5 μM). Mitochondrial membrane potential was determined using epi-fluorescent microscopy (SPOT Advanced). The red fluorescence (excitation 550 nm; emission 600 nm) and green fluorescence (excitation 485 nm; emission 535 nm) were quantified by subtracting the field background and normalizing to the traced cell area (SigmaScan Pro 4). Inner mitochondrial membrane potential normalized to the number of cells was calculated by dividing mitochondrial (red) fluorescence by cytosolic (green) fluorescence.

2.9. Western blotting

Protein was obtained from cardiomyocytes or heart homogenates, protein concentration determined [24], and expression of specific proteins determined through Western blotting, as previously described [23]. The following primary antibodies were used: rabbit anti-cleaved caspase-3, anti-p38 MAPK, mouse anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling), and rabbit anti-TRX (Abcam). Anti-β-actin antibody (Abcam) was used to confirm equal loading.

2.10. Adenovirus-mediated overexpression of TRX protein

Cardiomyocytes were infected with recombinant adenovirus expressing human TRX or control adenovirus Ad5Bg1 II (Gene Transfer Vector Core, University of Iowa) at an MOI of 20 for 24 h, followed by 100 μM Hcy treatment for 24 h.

2.11. Statistical analysis

All data are expressed as mean ± SE. Student *t* test and 1-way ANOVA were performed for comparison between two groups and among multiple groups, respectively. A p value <0.05 was considered statistically significant.