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Homocysteine Induces Cardiomyocyte Dysfunction and Apoptosis through p38 MAPK-Mediated Increase in Oxidant Stress

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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 hours 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 μ M) 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.

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DISCLOSURES

None.

CONFLICTS OF INTEREST

None.

Keywords

homocysteine; hyperhomocysteinemia; cardiomyocytes; contractility; apoptosis; antioxidant; ischemia reperfusion injury; oxidative stress

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 O₂ consumption *in vitro* through increased superoxide (O₂^{•-}) production via activation of NADPH 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.

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.

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.

Mouse cardiomyocyte isolation

Mouse cardiomyocytes were isolated as previously described. [21]

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.

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 hr.

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]

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 hr 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 hr), 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.

Measurement of intracellular reactive oxygen species (ROS)

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

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 hr at 37°C in phenol red-free DMEM containing the dye (3.5 μ M). 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.

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.

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 hr, followed by 100 μ M HHcy treatment for 24 hr.

Statistical analysis

All data are expressed as mean $\pm$ 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.

Results

Hyperhomocysteinemia impairs cardiomyocyte function

Plasma tHcy concentration increased by more than twofold in CBS mice compared with WT mice (Figure 1A). No differences in body weight (BW), heart weight (HW), or the ratio of HW-to-BW were observed between WT and CBS mice (data not shown). Cardiomyocytes isolated from CBS mice demonstrated impaired contractile function compared with those from WT mice, as shown by decreased % CS (Figure 1B) without a significant effect on the $[Ca^{2+}]_i$ transients (data not shown), indicating a calcium-independent mechanism for the reduced contractility.

We further determined whether HHcy may play a role in the myocardial response to I/R. To examine ventricular function in the absence of endogenous neurohormonal factors, *ex vivo* studies were performed in isolated Langendorff-perfused isovolumically beating hearts from WT and CBS mice. Baseline EDP and DP were similar between the two groups. During global zero-flow ischemia, EDP rose in both WT and CBS mouse hearts, with comparable values at peak contracture at the end of ischemia (70.44 $\pm$ 3.79 mmHg and 68.21 $\pm$ 4.14 mmHg, respectively) (Figure 1C). During the reperfusion period, however, cardiac relaxation was significantly impaired in CBS mouse hearts, with increased EDP compared with WT hearts (Figure 1C). Contractile function was also impaired in CBS mouse hearts during reperfusion, as demonstrated by decreased recovery of developed pressure (Figure 1D). In addition, although coronary flow was similar at baseline between the two groups, it was greatly reduced in CBS mouse hearts during reperfusion (Figure 1E), suggesting that HHcy induces vasoconstriction or indirectly impairs coronary perfusion via an increase in diastolic wall tension. These data suggest that HHcy increases ventricular and coronary vascular dysfunction following I/R.

Hyperhomocysteinemia increases apoptosis in the heart following I/R

To determine the role of HHcy in cell death during I/R, hearts were fixed at the end of reperfusion and stained by TUNEL (Figure 2A). For sham-operated WT and CBS hearts, only minimal TUNEL-positive cells were observed ($0.57 \pm 0.49/100$ nuclei and $0.70 \pm 0.26/100$ nuclei, respectively). After 15 min global zero-flow ischemia and 30 min reperfusion, the number of TUNEL-positive cells was found to be significantly increased in CBS mouse hearts compared with WT mouse hearts (CBS, $54.61 \pm 3.00/100$ nuclei, $p < 0.01$, versus WT, $37.52 \pm 2.77/100$ nuclei). Cleaved caspase-3 protein was detected using Western blotting to confirm apoptosis. The hyperhomocysteinemic hearts showed more cleaved caspase-3 than those from WT hearts after I/R (Figure 2B).

Hcy directly induces cardiomyocyte contractile dysfunction, apoptosis and intracellular ROS generation

To determine whether Hcy may directly cause cardiomyocyte dysfunction, we treated isolated adult rat cardiomyocytes with Hcy. As shown in Figure 3A, Hcy treatment for 24 hr directly reduced cardiomyocyte %CS in a concentration-dependent manner and the effect reached a plateau at 100 μ M. Hcy treatment did not change the $[Ca^{2+}]_i$ transients (data not shown), indicating a calcium-independent mechanism for the impaired contractility. Acute treatment of the cells with 100 μ M Hcy for up to 1 hr did not affect %CS (data not shown). To investigate whether the effect was thiol group-specific, cells were treated with Hcy, cysteine (Cys), and the Hcy-disulfide form, homocystine, each at 100 μ M for 24 hr. In contrast to vehicle and cysteine, both Hcy and homocystine reduced % CS (Figure 3B), suggesting that the effect was not a generic thiol effect.

To examine whether Hcy affects cardiomyocyte survival, cells were incubated with 100 μ M Hcy for 24 hr, and apoptosis was assessed by TUNEL. As shown in Figure 3C, percentage of TUNEL positive cells was significantly increased in Hcy-treated cardiomyocytes compared with that in control cells. Consistent with this observation, cells exposed to 100 μ M Hcy for 24 hr manifested a concomitant increase in cleaved caspase-3 expression (Figure 3D) compared with control cells.

We subsequently studied whether Hcy alters redox status in cardiomyocytes. Exposure to 100 μ M Hcy for 24 hr significantly enhanced DCF fluorescence compared with the control cells (Figure 3E), indicating that Hcy induced ROS production in cardiomyocytes.

Hcy induces cardiomyocyte dysfunction via activation of p38 MAPK

Owing to the fact that p38 MAPK has been implicated in cardiomyocyte dysfunction[25] and apoptosis[26], we investigated whether p38 MAPK plays a role in the adverse effects of Hcy on cardiomyocytes. A time course study revealed that Hcy induced p38 MAPK activation after incubation for 5 min, with a peak at 10 min and decrease thereafter (Figure 4A). Pretreatment of the cells with a p38 MAPK inhibitor, SB203580 (5 μ M), added 30 min prior to exposure to 100 μ M Hcy for 24 hr significantly attenuated Hcy-induced ROS generation (Figure 4B, DCF fluorescence), contractile dysfunction (Figure 4C, % CS), and apoptosis (Figures 4D, cleaved caspase-3, and 4E, percentage of TUNEL-positive cells). These results indicate that activation of p38 MAPK plays a critical upstream role in mediating Hcy-induced cardiomyocyte dysfunction and apoptosis.

To determine whether inhibition of Hcy-induced cardiomyocyte apoptosis by the p38 MAPK inhibitor was mediated by a mitochondrial pathway, mitochondrial membrane potential was assessed using the JC-1 dye. Hcy treatment decreased the ratio of red-to-green fluorescence, whereas inhibition of p38 MAPK activity attenuated the effect of Hcy (Figure

4F). These results suggest that p38 MAPK inhibition attenuates the effects of Hcy on mitochondrial membrane permeability.

Hcy induces down-regulation of TRX expression in cardiomyocytes

To determine whether Hcy-induced ROS accumulation affects cellular antioxidant proteins such as TRX, which has important roles in regulating oxidative stress and cardiac function as well as cell survival,[27] we examined TRX protein expression levels by Western blotting. As shown in the supplemental figure, TRX expression was decreased in hearts from CBS mice compared to WT mice. Following 24 hr treatment with 100 μ M Hcy, TRX protein levels were significantly decreased (Figure 5A) compared with controls. This effect of Hcy was completely prevented by the p38 MAPK inhibitor, SB203580 (Figure 5A). To test whether reducing ROS levels will attenuate Hcy-induced detrimental effects on cardiomyocytes, we increased the intracellular level of TRX by adenovirus transfer in cardiomyocytes before Hcy treatment. Infection with control adenovirus, Ad-TRX(-), altered neither Hcy-induced ROS generation nor Hcy-induced impairment of % CS and apoptosis (Figure 5B to F) in cardiomyocytes. In contrast, adenoviral-mediated overexpression of recombinant human TRX increased total cellular TRX level (Figure 5B) and significantly protected cardiomyocytes from Hcy-induced ROS generation (Figure 5C). The effect of Hcy on cardiomyocyte contractility was also prevented by overexpression of TRX (Figure 5D). Both cleaved caspase-3 levels (Figure 5E) and TUNEL staining (Figure 5F) revealed that overexpression of TRX protected cardiomyocytes from Hcy-induced apoptosis. These results suggest that Hcy-induced adverse effects were secondary to reduced antioxidant protein expression and increased oxidative stress, and that p38 MAPK activation plays a major role in this process.

Discussion

Our study demonstrates a direct adverse effect of HHcy on the myocardial response to I/R. In vitro studies suggest a potential mechanism of this adverse effect, i.e., that Hcy impairs contractile function and induces apoptosis in cardiomyocytes by downregulating specific antioxidant proteins and increasing ROS generation via activation of p38 MAPK.

The absence of CBS, the rate-limiting enzyme of the transsulfuration pathway in liver and kidney[28–30], limits the ability of cardiovascular tissue to lower intracellular Hcy.[30] Prior work in our laboratory has shown that Hcy inhibits the expression of GPx1 in endothelial cells *in vitro* and *in vivo*, leading to an increase in ROS that inactivate NO and promote endothelial dysfunction.[14, 31, 32] Recent studies demonstrate that HHcy also affects cardiac metabolism.[18, 19] These investigators demonstrated that O_2^- generated by NOX in HHcy reduced the ability of NO to regulate mitochondrial function in the myocardium.[18]

Our results show that Hcy impaired cardiomyocyte contractility without altering $[Ca^{2+}]_i$ transients. One possible mechanism to explain the effect of Hcy in decreasing contractility is that the effect is mediated by the reduced responsiveness of myofilaments to Ca^{2+} . The interaction between myofilaments and Ca^{2+} is suggested to be regulated by the phosphorylation status of myofilament proteins.[25] Dephosphorylation of α -tropomyosin by p38 MAPK has recently been associated with depression of myocardial sarcomeric tension and ATPase activity.[33] Hence it is possible that p38 MAPK induced changes in sarcomeric protein function may underlie Hcy-induced cardiomyocyte contractile dysfunction.

Apoptosis plays an important role in CVD including myocardial infarction and I/R injury, [34] and may contribute to the development and progression of cardiac dysfunction and

heart failure.[35] Accordingly, we examined the effect of Hcy on cardiomyocyte survival. Our results show that exposure to 100 μ M Hcy treatment for 24 hr increases apoptosis in cardiomyocytes. Furthermore, Hcy induces the dissipation of mitochondrial membrane potential, suggesting the involvement of the intrinsic apoptotic pathway. A similar effect of Hcy on mitochondrial membrane potential was recently reported in H9c2 cells (rat cardiomyoblast cells).[36]

Several studies have shown that ROS reduce cardiomyocyte contractility and impair cardiac function.[37, 38] ROS are also reported to mediate apoptosis in cardiomyocytes.[39] The generation of peroxynitrite is one of the mechanisms recently shown for Hcy-induced cell death in H9c2 cells.[40] Our investigation on cellular redox status revealed that 100 μ M Hcy treatment for 24 hr increases ROS generation in cardiomyocytes and decreases expression of antioxidant proteins that are important in detoxifying free radicals and preserving cardiac function. Increasing the intracellular TRX by adenovirus transfer protects cardiomyocytes against Hcy-induced ROS generation, and protects cardiomyocytes from Hcy-induced contractile dysfunction and apoptosis, suggesting that the adverse effects of HHcy are redox-sensitive and secondary to downregulation of antioxidant proteins.

Of several possible mechanisms underlying the effects of Hcy on cardiomyocytes, our findings suggest a key role for p38 MAPK activation, which is consistent with its role as a stress-activated kinase associated with cardiomyocyte apoptosis.[41] Furthermore, contractile dysfunction was reported in transgenic mice with cardiac-specific overexpression of p38 MAPK.[42] An effect of Hcy has also been suggested on the activation of p38 MAPK in various cardiovascular cells, including endothelial cells,[43] platelets,[44] and vascular smooth muscle cells.[45] Our results demonstrate that Hcy activates p38MAPK after 5 minutes of incubation and reaches a peak at 10 minutes. Hcy-induced ROS generation is attenuated by the p38 MAPK inhibitor, SB203580, possibly by preventing the Hcy-induced downregulation of TRX. Furthermore, cardiomyocyte function and survival analyses reveal that the suppression of contractility and induction of apoptosis by Hcy are prevented by pretreating cells with SB203580. Our results are in concert with the report that the negative inotropic effect of p38 MAPK on cardiomyocytes is mediated by altering myofilament sensitivity to intracellular Ca^{2+} . [25] Taken together, we conclude that Hcy induces contractile dysfunction and apoptosis by activating p38 MAPK. The proposed mechanism of the adverse effects of Hcy on cardiomyocytes is illustrated in Figure 6. The exact mechanism of p38MAPK activation by homocysteine needs further investigation. Since the activation of p38MAPK was rapid and preceded the increase in ROS, the most likely mechanisms are either an alternative activation pathway such as Ras-mediated activation of p38 MAPK as demonstrated in the case of interleukin-1 [46], or activation by a transient burst of ROS production on auto-oxidation of homocysteine. Further investigations will address potential mechanisms of interactions between hyperhomocysteinemia and the p38MAPK system in cardiomyocytes.

Similar to our *in vitro* results, cardiomyocytes from hyperhomocysteinemic mouse hearts displayed impaired contractility compared with that of WT hearts without alteration in $[\text{Ca}^{2+}]_i$ transients, although the *in vivo* cardiac function analysis (in the absence of I/R injury) by transthoracic echocardiography did not reveal any difference between the two groups (data not shown). The discordance between the *in vivo* results and that obtained in cardiomyocytes isolated from the same animals suggests that during and after the cardiomyocyte isolation process, in addition to the insult of physical dissociation and chemical digestion of heart tissue, exposure of cardiomyocytes to ambient air which has higher oxygen content than what the cells are exposed to *in situ*, might provide an oxidative insult. The impaired antioxidant system in the hyperhomocysteinemic hearts was likely unable to counterbalance the excessive ROS generation, resulting in disturbance in

intracellular redox homeostasis, which may have led to reduced responsiveness of myofilaments to Ca^{2+} .

Excessive ROS is known to form during I/R.[47] Therefore, we applied I/R as an oxidative insult to study the effect of HHcy on myocardial response to oxidative stress. Following I/R, hearts from hyperhomocysteinemic mice showed both impaired diastolic and contractile function compared with that of WT mice. Previous studies have shown that only a minimal amount of ROS or reactive nitrogen species was generated during ischemia while a massive generation of ROS took place during reperfusion.[48] Our results show that cardiac dysfunction was similar between the WT and CBS mouse hearts during ischemia and was exacerbated only during reperfusion, suggesting an association of myocardial dysfunction with increased ROS generation during reperfusion in hyperhomocysteinemic hearts. Not surprisingly, our results demonstrate increased vascular resistance upon reperfusion, since endothelial dysfunction has been a typical phenotype[14] in HHcy. Therefore, HHcy may sensitize hearts to I/R injury by decreasing antioxidant protein expression and increasing vasoconstriction.

One limitation of this study is that we do not yet know the dynamic change of intracellular Hcy concentrations after incubation with exogenous Hcy. Such knowledge would provide an understanding of how quickly Hcy enters cardiomyocytes, and whether Hcy exerts its adverse effects through membrane-bound proteins or by interacting with intracellular signaling molecules which may activate p38 MAPK.

In conclusion, this study demonstrates the direct effects of Hcy on cardiomyocytes in the absence of endothelial cells, previously believed to be central to the pathogenesis of HHcy-induced CVD. Our *in vivo* results also suggest that HHcy may increase the risk for CVD by sensitizing myocardial response to I/R injury. The mechanism of action of homocysteine *in vitro* and *in vivo* does not likely involve a direct effect of homocysteine or its thiol group. The effects are more likely the result of an alteration in the methionine-homocysteine cycle within the cardiomyocyte regardless of whether such changes are induced via exposure to homocysteine or its oxidized form homocystine (*in vitro* results), or due to a decrease in expression of an enzyme in the transsulfuration pathway of homocysteine metabolism (*in vivo* results). Multiple and synergistic pathogenic mechanisms such as increased ROS, altered methylation, or homocysteinylolation of proteins, which cumulatively may affect signaling mechanisms and redox balance may be operative in HHcy and explain our results. Overall, these data suggest that Hcy may increase the risk for CVD not only by causing endothelial dysfunction and promoting atherothrombosis, but also by impairing cardiomyocyte function and survival.

Highlights

- Effects of hyperhomocysteinemia on myocardium are not well understood.
- Ischemia-reperfusion caused worse injury in hyperhomocysteinemic mice.
- Homocysteine impaired contractility and promoted apoptosis in cardiomyocytes.
- Mechanism is via activation of p38MAPK and increase in oxidant stress.
- Overexpression of thioredoxin decreased oxidant stress and rescued cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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
[Ca²⁺]_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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Figure 1.

Impaired cardiomyocyte contractile function in CBS mouse hearts. A, Plasma total Hcy levels were higher in CBS mice than in wild type (WT) mice. B, Cardiomyocytes isolated from CBS mouse hearts showed impaired contractile function in comparison to the cells from WT mouse hearts. % CS was measured by real-time edge detection. Cells were paced at 5 Hz. $n=5$ (heart number) for WT group, $n=3$ for CBS group. C to E, Increased ventricular and vascular dysfunction in the hyperhomocysteinemic hearts following I/R. End diastolic pressure (C), developed pressure (D), and coronary flow (E) in hearts isolated from WT mice ($n=7$) and CBS mice ($n=7$) during 15 min zero-flow global ischemia and 30 min

reperfusion. Hearts were perfused with a constant pressure of 80 mmHg and paced at 7 Hz.
* $p < 0.05$ vs. WT.

Figure 2.

Hyperhomocysteinemic hearts have more cell death than wild type hearts following I/R. Isolated wild type (WT) and CBS heterozygous deficient (CBS) mouse hearts were subjected to 15 min global zero flow ischemia, followed by 30 min reperfusion. A, Representative figure of TUNEL staining on heart sections from sham-operated hearts and from the hearts following I/R. Bar-graph summarizes the number of TUNEL-positive cells/100 nuclei in sham-operated and I/R heart sections. Results are the mean $\pm$ SEM of 10–15 high-power (40 $\times$) fields from four samples for each group. B, Western blot analysis of cleaved caspase-3 levels in the heart following I/R demonstrating an increase in the CBS hearts after I/R.

Figure 3.

Hcy induces contractile dysfunction, apoptosis, and ROS generation in cardiomyocytes. Isolated rat cardiomyocytes were treated with Hcy (10–500 μ M) (3A) or with Hcy, cysteine, or homocysteine (100 μ M) for 24 hours. Hcy treatment impaired cardiomyocyte contractility as shown by percent cell shortening in the cells paced at 5 Hz after Hcy treatment ($n=4$ experiments). Homocysteine produced a similar effect while cysteine had no effect. C, Hcy treatment increased cardiomyocyte apoptosis as shown by TUNEL staining ($n=5$ experiments). D, Hcy treatment enhanced caspase-3 activation as shown by Western blot analysis of the expression of cleaved caspase-3. β -actin is shown to confirm equal loading ($n=6$ for each group). E, Hcy treatment enhanced ROS generation as shown by

representative fluorescent microscopy images of control and Hcy-treated cells (left panel). Bar-graph summarizing the relative intensities of DCF fluorescence averaged from 7 experiments each from fifty cells analyzed under each condition (right panel).

Figure 4.

Activation of p38 MAPK contributes to Hcy-induced contractility impairment, ROS generation, and apoptosis in cardiomyocytes. A, Hcy activates p38 MAPK in a time-dependent manner. Cardiomyocytes were incubated with 100 μ M Hcy for 0 to 20 minutes. Bar-graph shows results from 3 experiments. B to E, Cardiomyocytes were treated with or without Hcy (100 μ M) for 24 hrs in the presence of p38 MAPK inhibitor SB203580 (5 μ M) or vehicle DMSO added 30 minutes prior to Hcy. B, SB203580 decreased Hcy-induced ROS generation in cardiomyocytes, as estimated with DCF fluorescence ($n=5$ experiments). C, SB203580 prevented Hcy-induced cell contractility impairment. Cardiomyocytes were paced at 5 Hz ($n=4$ experiments). D and E, SB203580 prevented Hcy-induced apoptosis, as revealed by Western blotting cleaved caspase-3 and TUNEL staining ($n=5$ experiments). F, Inhibition of p38 MAPK activity prevented Hcy-induced dissipation of mitochondrial

membrane potential, assessed by JC-1 dye. Data are expressed as the ratio of mitochondrial (red) to cytosolic (green) fluorescence ($n=3$ experiments).

Figure 5.

Overexpression of TRX protects cardiomyocytes against Hcy-induced contractile dysfunction and apoptosis by reducing intracellular ROS generation. A, Hcy treatment (100 μ M, 24 hr) reduced TRX expression in rat cardiomyocytes, which was prevented by inhibition of p38 MAPK activity. B to F, Cardiomyocytes were infected with adenovirus containing recombinant human TRX or empty vector at MOI=20 for 24 hr, followed by incubation with or without 100 μ M Hcy for 24 hr. B, Western blot analysis of TRX levels in cells with adenovirus infection, followed by Hcy treatment (100 μ M, 24 hr), $n=5$ experiments. C, Overexpression of TRX prevented Hcy-induced intracellular ROS generation, determined by DCF fluorescence, $n=6$ experiments. D, Overexpression of TRX

attenuated Hcy-induced reduction in cell contractility, determined by percent cell shortening in cardiomyocytes paced at 5 Hz, $n=3$ experiments. E, Overexpression of TRX inhibited Hcy-induced activation of caspase-3, determined by Western blot analysis of cleaved caspase-3. β -actin was used to confirm equal loading. F, Overexpression of TRX prevented Hcy-induced apoptosis, determined by TUNEL staining, $n=4$ experiments.

Figure 6.
Proposed mechanism of Hcy-induced cardiomyocyte contractile dysfunction and apoptosis.