Published in final edited form as:

J Cell Physiol. 2011 October ; 226(10): 2712–2720. doi:10.1002/jcp.22621.

Folic Acid Improves Acetylcholine-induced Vasoconstriction of Coronary Vessels Isolated from hyperhomocysteinemic mice: An Implication to Coronary Vasospasm*

Natia Qipshidze, Naira Metreveli, David Lominadze, and Suresh C. Tyagi

Department of Physiology and Biophysics University of Louisville School of Medicine Louisville, KY 40202

Abstract

Human atherosclerotic coronary vessels elicited vasoconstriction to acetylcholine (Ach) and revealed a phenomenon of vasospasm. Homocysteine (Hcy) levels are elevated in the atherosclerotic plaque tissue, suggesting its pathological role in endothelial damage in atherosclerotic diseases. Accordingly, we examined the role hyperhomocysteinemia (HHcy) in coronary endothelial dysfunction, vessel wall thickness, lumen narrowing, leading to acute/chronic coronary vasospasm. The therapeutic potential and mechanisms of folic acid using hyperhomocysteinemic cystathionine beta synthase heterozygote (CBS-/-) and wild type (CBS+/+) mice were addressed. The CBS-/- and CBS+/+ mice were treated with or without a Hcy lowering agent folic acid (FA) in drinking water (0.03 g/L) for 4 weeks. The isolated mouse septum coronary artery was cannulated and pressurized at 60 mmHg. The wall thickness and lumen diameters were measured by Ion-Optic. The vessels were treated with Ach (10^{-8} - 10^{-5} M) and, for comparison, with nonendothelial vasodilator sodium nitroprusside (10^{-5} M). The endothelium-impaired arteries from CBS-/- mice constricted in response to Ach and this vasoconstriction was mitigated with FA supplementation. The level of endothelial nitric oxide synthase (eNOS) was lower in coronary artery in CBS-/- than of CBS+/+ mice. Treatment with FA increased the levels of Ach-induced NO generation in the coronary artery of CBS-/- mice. The results suggest that Ach induced coronary vasoconstriction in CBS-/- mice and this vasoconstriction was ameliorated by folic acid treatment. The mechanisms for the impairment of vascular function and therapeutic effects of folic acid may be related to the regulation of eNOS expression, NO availability and tissue homocysteine.

Keywords

CBS; MTHFR; eNOS; Nitric oxide; endothelial cell; nitroprusside

Introduction

Despite many large prospective studies which have established that hyperhomocysteinemia (HHcy) predicts vascular risk (Den Heijer M et al., 2005; Wilson PW et al., 2002), paradoxically, several interventional trials have failed to demonstrate any clinical benefit of homocysteine (Hcy)-lowering therapy (Albert CM et al., 2008; Bona KH et al., 2006). There were several limitations of these trials, including limitations of Hcy measurements.

*This study was supported in part by NIH grants; HL-71010; HL-74185; HL-88012 and (HL-80394 to DL).

Please address correspondence to: Suresh C. Tyagi, Ph. D., University of Louisville Dept. of Physiology & Biophysics School of Medicine, Bldg. A, Room 1215 500 South Preston Street, Louisville, KY 40202 Phone: (502) 852-4902 Fax: (502) 852-6239 sotyag01@gwise.louisville.edu.

Although folic acid treatment significantly lowered plasma Hcy, tissue levels of Hcy were never measured in these trials. Folic acid may promote tissue uptake of Hcy, as has been seen with other hormone and growth factor such as insulin (Fukagawa NK et al., 1986), where insulin administration decreases plasma levels of amino acids at the same time increases tissue uptake. Interestingly, previous studies from our laboratory have demonstrated elevated tissue levels of Hcy in ischemic cardiomyopathic human hearts (Tyagi SC et al., 1998). In HHcy the levels of cystathione -lyase (CSE, an enzyme responsible for conversion of Hcy to H₂S, a potent antioxidant, vasorelaxing and anti-hypertensive agent) were decreased (Sen U et al., 2010), eliciting the mechanism by which tissue levels of Hcy were increased in HHcy. However, it is still unclear whether lowering Hcy has antiatherogenic effect (Toole JF et al., 2004).

Studies suggested that folic acid (FA), through its circulating form, 5-methyltetrahydrofolate (5-MTHF), may have antioxidant properties and exert biological effects that may or may not be directly related to changes in plasma Hcy (Doshi SN et al., 2002). Some studies suggested that folate may have direct effects on nitric oxide (NO)-mediated endothelial function, possibly through changes in endothelial NO synthase (eNOS) regulation (Hyndman ME et al., 2002).

NO has been shown to be of unequivocal important in regulating coronary blood flow (Tiefenbacher CP et al., 2000) and endothelium-dependent vasodilatory and vasoconstrictory mechanisms. In addition, NO plays determinant role in regulation of blood flow (Heitzer T et al., 2001), susceptible to various insults associated with hypertension (Boulanger CM et al., 1999), atherosclerosis (Shimokawa H. 1999), and heart failure (Wang J et al., 1994). Coronary blood flow is highly regulated to ensure an adequate matching of coronary perfusion to meet the metabolic demand imposed by a constantly beating heart (Muller JM et al., 1996). The main mechanisms controlling coronary artery tone are: metabolic, myogenic, neurohormonal, and endothelial (Westerhof N et al., 2006). Large coronary arteries have a greater dependency on endothelium-dependent mechanisms for maintenance of proper tone, while smaller arterioles depend more on metabolic and myogenic mechanisms (Feliciano L et al., 1999). Acetylcholine believed to dilate normal blood vessels by promoting the release of a vasorelaxant substance from the endothelium (Hyndman ME et al., 2002). Some clinical trials showed paradoxical vasoconstriction induced by acetylcholine, occurs early as well as late in the course of coronary atherosclerosis (Ludmer PL SA et al., 1986), implicating the pathogenesis of coronary vasospasm. Interestingly, we have shown elevated levels of Hcy in human coronary atherosclerotic plaques (Tyagi SC et al., 1998). This supports the hypothesis that Hcy increases vasoconstrictive tone of coronary artery, and FA supplementation ameliorates Hcy-induced vasoconstriction and endothelial dysfunction in CBS-/+ mice, via eNOS-mediated NO production.

Methods

Experimental Protocol

The wild type (CBS+/, CBS+/, C57BL/6J) and a breeding pair CBS-/+ mouse were obtained from Jackson Laboratories (Bar Harbor, Me., USA). The mice were bred and genotyped by PCR and phenotyped by Hcy levels, at the mouse breeding facility of the University of Louisville. The protocol was in accordance with the Animal Care and Use Committee, approved by the Institutional Review Committee. Adult male mice (20–25 g, CBS+/+ and CBS+/-) were randomly assigned into four groups, 10–12 weeks CBS+/+, CBS +/+ treated with FA ((CBS+/+)+FA), CBS-/+ and CBS-/+ treated with FA ((CBS-/+)+FA). Mice were given drinking water with or without FA (0.03 g/L) for 4 weeks.

Mouse coronary artery preparation

On the day of the experiment, mice were weighted and killed by opening thoracic cavity. The blood samples were collected from vena cava. The hearts were removed rapidly and placed in cold physiological salt solution (PSS, composition: 8.29g NaCl, 0.35g KCl, 0.42g MgSO₄, 0.15g EDTA, 0.41g CaCl₂, 2.38g HEPES, 0.16g KH₂PO₄, and 0.90g Glucose). Mouse coronary artery, 85–105 μ m in diameter and 1 mm in length, was isolated from the septum under a dissection microscope (Zeiss, Carl Zeiss Microimaging, Inc). Isolated coronary artery was placed in a microvascular chamber (Live-System, Inc) cannulated at one end with a glass micropipette, and secured with a 12–0 nylon monofilament suture. PSS solution was infused slowly through the cannula, until the vessel was completely filled. The other end of the vessel was then cannulated with a second micropipette filled with PSS solution. Both cannulas were connected to reservoirs filled with PSS solution. The system allowed monitoring the transmural pressure (P_{tm}). Inlet and outlet P_{tm} was measured continuously with two pressure transducers positioned at the level of the vascular lumen. In the microvascular chamber, the vessels were constantly superfused with recirculating PSS solution (total volume 5.1 ml) and maintained at 37°C. The chamber was placed on the stage of an inverted microscope (Olympus IX 51, Olympus, Japan) connected to a video camera. The vascular image was acquired and visualized on a computer screen, and intraluminal diameter (ID) was measured continuously by MyoCam system (IonOptix, Milton, MA).

Experimental protocol

Measurements of ID were started immediately after the vessel was mounted on cannulas and continued throughout the experiment. Initially, the isolated coronary artery was allowed to equilibrate in the microvascular chamber for 60 min at a P_{tm} of 10 mmHg. P_{tm} was increased to 60 mmHg in 10-mmHg increments every 10–15-min. To establish the spontaneous tone, isolated vessels were perfused for 60 minutes. After development of vascular spontaneous tone the vessel was stabilized for 30 min at the final P_{tm} of 60 mmHg. Then Ach dose-response protocol was performed. Ach doses were ranged from 10⁻⁸ to 10⁻⁵ M. Maximal responses were recorded during 10 min following of the each dose of Ach. After the last dose, the vascular preparation was washed with PSS for 3 times and was exposed to 10⁻⁵ M of sodium nitroprusside (SNP). Again maximal response during 10 min was recorded.

Histology

The hearts were collected from experimental animal. The septal part of whole heart was separated and frozen for further analyses. Frozen parts of heart were sectioned at 5 μ m thickness with cryostat (Leica Cryocut 1800, Leica Microsystems, Germany) and mounted on slides. Each slide was stained using a Masson's trichrome kit (Richard-Allan Scientific, Kalamazoo, MI) according to the manufacturer's recommendations. The heart muscle and vascular smooth muscles were stained in a pink color while the collagen was stained in blue.

Coronary arteries wall thickness and diameter were measured using IonOptix system, which measures vessel wall thickness and diameter during in vivo perfusion studies. In addition, with Masson's trichrome stained micrographs using image-pro software, the medial thickness and diameters were estimated.

Confocal microscopy

For immunohistochemical analysis, samples of coronary arteries from all experimental groups were immediately placed onto freezing media and stored at -70°C until they were used for the localization of endothelial cells using endothelial and eNOS markers. Briefly, transverse sections were post-fixed in 4% of paraformaldehyde, and labeling was performed

with primary antibodies to identify endothelial cells (with anti-mouse CD31 antibody from BD Biosciences - Franklin Lakes, NJ) and eNOS (with anti-rabbit eNOS antibody from Abcam Antibodies, Cambridge, MA). After an overnight incubation, sections were washed with PBS and incubated with appropriate secondary antibodies conjugated with Fluorescein isothiocyanate (FITC). After washing, sections were mounted with FluoroGel mounting medium (GeneTex, Inc) and visualized with confocal microscopy (Olympus, FluoView 1000, objective 60x). To enable the comparison in fluorescence intensity changes, the images were acquired under the identical set of microscope system settings. Fluorescence was imaged using excitation at 488 nm and 516 emission band pass filter. Coronary images from four animals in each group were analyzed to determine expression of eNOS and CD31. Total fluorescence (green) intensity in 5 random fields (for each experiment) was measured with image analysis software (Image-Pro Plus, Media Cybernetics) and expressed in fluorescence intensity units (FIU). The fluorescence intensity values were averaged for each experimental group.

To identify tissue level of Hcy and MTHFR, we labeled the sections with Hcy antibody (from Abcam Antibodies, Cambridge, MA) and MTHFR antibody (from Abcam Antibodies, Cambridge, MA). After an overnight incubation, sections were washed with PBS and incubated with appropriate secondary antibodies conjugated with Fluorescein isothiocyanate (FITC) and Texas Red. After washing, sections were mounted with FluoroGel mounting medium and visualized with confocal microscopy. The scan density was used as quantitative measure.

Production of NO in isolated vessels

Endothelial NO production was quantified in individually perfused coronary vessels imaging fluorescence of 4, 5-diaminofluorescein diacetate (DAF-2D), a fluorescent indicator of NO. In each experiment, after development of spontaneous tone a coronary vessel was perfused with PSS solution containing DAF-2 DA (5 μ M) for 45 min. Then the vessel was perfused with PSS solution for 10 min before acquiring the control images. The vessel was superfused with Ach, first 10^{-6} M then Ach 10^{-5} M. Responses to each dose were observed for 10 min. Then vessel was washed with PSS for 5-10 min and the NO donor, SNP (10^{-5} M) was applied to the vessel to examine the maximum production of NO. Images were analyzed using Image-Pro Plus (Media Cybernetics, Bethesda, MD) image analysis software. Selected regions of interest (ROIs) along the vessel wall were used to measure the fluorescence intensity (FI) of the DAF-2DA. Production of NO was defined by measuring maximum intensity of stimulated FI₁ and expressing as a percent of fluorescence intensity of the same ROI at the baseline FI₀ (FI₁/FI₀×100%). Data were averaged for each experimental group and compared to those from control experiments.

Hcy measurement

The 0.5 ml blood from each mouse was collected at the end of the experiment. The plasma was separated by centrifuged, to remove cell debris. To determine Hcy, 200 μ l of plasma were diluted with 100 μ l of water and then 300 μ l of 9 M urea (pH 9.0) were added. The 50 μ l of *n*-amyl alcohol was added to the solution as an antifoaming agent. Reduction of disulfides and cleavage of the protein-bound, sulfur-containing amino acids were performed by the addition of 50 μ l of NaBH₄ solution (10%, wt/vol) in 0.1 N NaOH. To perform the reaction, samples were incubated in a water bath at 50°C for 30 min. Samples were cooled down at room temperature and the reaction was stopped by the addition of 500 μ l of 20% trichloroacetic acid. The proteins were separated by centrifugation for 4 min at 12,000 g, and supernatants were filtered using a 0.45- μ m Millipore filter. High-pressure liquid chromatography (HPLC) analyses were performed in a Shimadzu Class-VP 5.0 chromatograph (Shimadzu) equipped with LC-10ADvp pump, SIL-10ADvp autoinjector,

CTO-10Avp column oven, and SPD-10Avp detector. We used a premier C18 amide 5- μm 150 $\times$ 2.1 mm column to detect Hcy, and during the sample run, the oven temperature was constantly maintained at 37°C. The chromatographic conditions were maintained as described (Sen U et al., 2010). Briefly, 0.1 M monochloroacetic acid and 1.8 mM octylsulfate mixed together, adjusted to pH 3.2, and was used as mobile phase. Before being used, this solvent was filtered through a Millipore filter (0.45 μm) and degassed under vacuum. The isocratic solvent was pumped and circulated through the column at a constant flow of 0.8 ml/min. Samples were injected through autoinjector, and an injection volume of 20 μl was used. During HPLC analysis, Hcy levels in the plasma were identified according to their retention time and co-chromatography with standards.

RNA extraction and quality assessment

The *mirVana*TM miRNA isolation kit (Ambion, Part number #AM1560) was used to isolate the total RNA heart following the protocol of kit. The quality of total RNA was assessed by NanoDrop ND-1000 and only highly pure quality RNA (260/280–2.00 and 260/230–2.0) was used for RT-PCR.

Semi-Quantitative Reverse Transcription-PCR (RT-PCR)

The RT-PCR was performed for mRNA expression of eNOS in experimental groups using ImProm-IITM Reverse Transcription system kit (Promega Corporation, Madison, WI, USA, cat # A3800). For gene amplification the RT-PCR program was 95 °C–7.00 min [95 °C–0.50 min, 55 °C–1.00 min, 72 °C–1.00 min] $\times$ 34, 72 °C–5.00 min, 4 °C– ∞ . The primers for RT-PCR were: reverse: AACATATGTCCTTGCTCAA; Forward: TTCCGGCTGCCACCTGATC.

Statistical analysis

Values are presented as mean $\pm$ SEM. Differences between groups were tested by two-way ANOVA. If ANOVA indicated a significant difference ($P < 0.05$), Tukey's multiple comparison test was used to compare group means and were considered significant if $P < 0.05$.

Results

Body weight and Hcy levels

Mice were weighted before sacrifice. CBS-/+ mice has low weight compare to the other groups. FA treatment helped to increase weight in CBS-/+ mice to the normal weight (Table 1). The plasma concentration of Hcy was measured in CBS+/+, (CBS+/+) +FA, CBS-/+, and (CBS-/+) +FA mice 4 weeks after the treatment with FA or water, respectively. The content of Hcy was greater in CBS-/+ mice compared to CBS+/+ mice. Treatment with FA mitigated the elevated levels of Hcy in (CBS-/+) +FA (Table 1).

Mouse septal coronary artery response to increase in Ptm

A typical example of pressurized mouse coronary artery mount was shown in Figure 1A. Increases of Ptm from 10 to 60 mmHg in 10 mmHg increments caused a progressive distention of coronary arteries (Figure 1). In CBS+/+, ID increased from 92 ± 1 to 136 ± 1 μm while in (CBS+/+) +FA ID increased from 92 ± 1 to 138 ± 0.3 μm . Step-wise increase of Ptm increased ID of coronary arteries from 58 ± 1 only to 73 ± 1 μm in CBS +/- mice (Figure 1). Treatment with FA improved vascular elasticity in CBS-/+ mice indicated by increased ID from 70 ± 1 to 106 ± 1 μm in response to Ptm increases by 10 mmHg. After Ptm was held constant at 60 mmHg, all vessels developed spontaneous constriction (ID change from 136 ± 1 μm to 116 ± 1 μm in CBS+/+ mice, from 138 ± 0.3 μm to 118 ± 0.5 μm in (CBS+/+) +FA

mice, from $73 \pm 1 \mu\text{m}$ to 63 ± 1 in CBS +/- mice and from $106 \pm 1 \mu\text{m}$ to $86 \pm 1 \mu\text{m}$ in (CBS +/-) +FA mice). These results suggest that Hcy causes rigidity in coronary vessel wall and folic acid mitigates this rigidity.

Coronary vascular responses to acetylcholine (Ach)

The Ach caused decrease in resting diameters, once pressurized to 60mmHg, in septal coronary vessels from CBS-/+ mice compared to those from CBS+/+ or (CBS+/+) +FA animals (Figure 2), suggesting vasoconstrictive response of Ach in CBS-/+ coronary. The maximal dose of Ach (10^{-5} M) dilated the CBS+/+ mice arteries from $116 \pm 1 \mu\text{m}$ to $138 \pm 1 \mu\text{m}$ and (CBS+/+) +FA mice arteries from $118 \pm 0.3 \mu\text{m}$ to $140 \pm 1 \mu\text{m}$ (Figure 2). Ach dose-dependent dilatations were observed in CBS+/+, (CBS+/+) +FA, and (CBS-/-) +FA mice (Figure 3a). However, the vascular responses to Ach were paradoxical in CBS-/+ mice (Figure 3a). At the maximal dose of Ach (10^{-5} M) coronary vessels from CBS-/+ mice developed vasoconstriction. The IDs decreased from $63 \pm 1 \mu\text{m}$ to $43 \pm 1 \mu\text{m}$ in CBS-/+ mice (Figure 3a). These paradoxical responses to Ach were abolished in CBS-/+ mice with FA supplementation (Figure 3a). At the maximal Ach dose in (CBS-/-) +FA mice ID of coronary vessels changed from $86 \pm 1 \mu\text{m}$ to $96 \pm 2 \mu\text{m}$ (Figure 3a). SNP, endothelium-independent vascular smooth muscle relaxant, induced dilation of coronary arteries from CBS-/+ to the similar level as of arteries from CBS+/+, (CBS+/+) +FA, and (CBS-/-) +FA animals (Figure 3a). To determine in vivo NO generation in CBS+/+ and CBS-/+ coronaries, we infused DAF-2DA (an NO indicator) into the coronary prior to adding Ach. The results revealed significant decrease in NO production in CBS-/+ coronary as compared to CBS+/+ mice (Figure 3b, c).

Mouse coronary artery wall thickness from CBS-/+ mice was significantly greater than that the CBS+/+ or (CBS+/+) +FA mice. Wall thickness was improved in CBS-/+ mice treated with FA. However, FA treatment did not affect the wall thickness of coronary arteries in CBS+/+ mice (Figure 4).

Endothelial integrity

To determine the integrity and levels of endothelium, we stained the coronaries with CD31, a marker of endothelium. The confocal image analyses indicated that in coronary arteries isolated from CBS-/+ mice, the expression of CD31 was reduced compared to coronary arteries from CBS+/+ and (CBS+/+) +FA mice (Figure 5). Supplementation with FA improved expression of CD31 in (CBS-/-) +FA mice (Figure 5).

eNOS

To determine whether the decrease in endothelium in CBS-/+ mice constitute to the decrease in NO production, we estimated the eNOS in coronary vascular wall of CBS-/+ mice. The results suggest reduced eNOS levels in CBS-/+ mouse coronaries in comparison to that in CBS+/+ or (CBS+/+) +FA mice (Figure 6). Treatment with FA improved expression of eNOS in coronary arteries from (CBS-/-) +FA mice (Figure 6). RT-PCR for MMP eNOS Expression:

We investigated the role of eNOS by measuring total protein by immuno-labeling, and mRNA levels by RT-PCR. The semi-quantitative RT-PCR among the CBS+/+, (CBS+/+) +FA, CBS-/+ and (CBS-/-) +FA mice septal coronary arteries revealed that eNOS was decreased in CBS+/+, (CBS+/+) +FA, CBS-/+ and (CBS-/-) +FA mice septal coronary arteries (Figure 6 C and D). There was no change in GAPDH (the loading control) expression (Figure 6 C and D).

MTHFR and Hcy

To determine whether the treatment with FA had effect on the levels of MTHFR and Hcy, we estimated the levels of MTHFR and Hcy in coronaries from CBS-/+ mice treated with and without FA. The results suggest that MTHFR levels were low in CBS-/+ coronary as compared to CBS+/+ or (CBS+/+) +FA mice, at the same time Hcy level was increased in CBS-/+ coronary as compared to CBS+/+ or (CBS+/+) +FA mice (Figure 7). Treatment with FA improved expression of MTHFR and mitigated Hcy level in coronaries from (CBS-/+)+FA mice (Figure 7).

Discussion

CBS-/+ mice are lower in body weight than that of CBS+/+ mice. It is known that CBS deficiency causes malformation and bone deformities (Robert K et al., 2005). This may attribute to decrease in body weight in CBS-/+ mice as compared to CBS+/+ mice. HHcy impairs Ach-induced (or methacholine) responses (including paradoxical vasoconstriction) (Dayal S et al., 2008. Eberhardt RT et al., 2000). Some labs have even discovered a similar impaired or paradoxical vasoconstriction to shear stress (Ungvari Z et al., 2003). Some of these studies have also nicely demonstrated that loss of NO bioavailability is the primary cause of the dysfunction. Although we did not measure the phosphorylation of eNOS, Looft-Wilson et al (Looft-Wilson RC et al., 2008) demonstrated similar findings but took the next important step by identifying the relevant phosphorylation states of eNOS under both control and Ach stimulated conditions. However, none of these studies were performed in mouse coronary. Our study is novel especially because we used CBS-/+ knockout mouse coronary. Although we did not perform analyses of the activation vs inactivation phosphorylation states of eNOS to advance understanding in this already well-studied area, we measured fundamentals of changes in coronary wall, diameters, NO-generation using DAF-2DA probe, levels of eNOS, MTHFR and endothelium in mouse coronary with respect to the HHcy. Previously we showed by Western blot analysis, the decrease in MTHFR levels in myocardial infarction, interestingly, FA treatment mitigated this decrease in MTHFR levels (Qipshidze N et al., 2010). Here we showed, by immunofluorescence stains, CD31, eNOS and MTHFR decreased in the coronaries of CBS-/+ HHcy mice. In addition, the DAF-2DA stain for eNOS is excellent representative for eNOS.

The vasoconstrictive response, in CBS-/+ mouse coronary, to Ach was endothelium-dependent, since this difference among groups was abolished upon Ach wash and treatment with nitroprusside, an endothelium-independent vasodilator. This suggests that the Ach-induced vasoconstriction in CBS-/+ mice was due to endothelial damage.

Although several studies have indicated that FA and/or its active metabolite 5-MTHF improve endothelial function (Nakano E HJ et al., 2001). Such effects may be mediated by the antioxidant properties of FA (Verhaar MC et al., 1998) and are likely to mediate a rapid reduction in arterial stiffness through a reduction in the catabolism of NO and an enhancement of endothelial-dependent vasodilatation (Wilkinson IB et al., 2002). Our study showed that the plasma level of Hcy in CBS-/+ were significantly higher than in CBS+/+ or in (CBS+/+) +FA mice, indicating that CBS-/+ mice had HHcy. FA supplementation reduced the level of plasma Hcy in CBS-/+ mice without affecting the body weight, suggesting FA has direct effect on blood Hcy levels. Although we showed that the folate (a cofactor for MTHFR activity) improved endothelial function in CBS-/+ mice, the vitamin B6 (a co-factor for CSE activity) can also improve the endothelial function, in part, by increasing CSE activity (Messika AH et al., 2010. Majtan T et al., 2010). However, in CBS heterozygous mice B6 may have minimal effect on Hcy level.

FA improves NO-mediated endothelial function and decreases superoxide production. These effects may occur through mechanisms independent of direct superoxide scavenging (Antoniades C et al., 2006). The effects of FA on the vascular endothelium appear to be mediated through restoration of the enzymatic function of eNOS (Antoniades C et al., 2006). Previously we have demonstrated the role of eNOS in endothelial dependent vasorelaxation by direct using eNOS knockout mice compares to CBS^{+/+} (Steed MM et al., 2010). Early clinical trials suggested that lowering Hcy with FA may retard progression of atherosclerosis (Schnyder G et al., 2001). The present study indicated that isolated mouse coronary arteries exhibited a progressive vasodilatation to increase of Ptm that was similar to that observed by others (Liu Q, 1999) in response to physiological (Ptm) and pharmacological (Ach) stimulation. Although we convincingly showed that CBS^{+/+} mice had endothelial dysfunction, in part, due to lack of eNOS activity and MTHFR, and increased coronary tissue Hcy levels. There are other mechanisms that can also cause endothelial dysfunction such as the use of anti-oxidant and/or over-expression of SOD can also mitigate the oxidative stress. Isolated mouse coronary arteries with endothelium, whereas responses to Ach for vessels from CBS^{+/+} mice differed markedly from the coronary arteries isolated from CBS^{-/+} mice, which developed myogenic response. Myogenic tone in coronary arteries in CBS^{-/+} mice, which may have damaged endothelium is more likely to result from damage endothelium than from vascular smooth muscle.

This study showed that there is a significant relation between coronary risk factors and the vasomotor response to Ach in CBS^{-/+} and (CBS^{-/+}) +FA mice coronary arteries. The results of the present study demonstrate that in coronary arteries from CBS^{-/+} mice may have impaired endothelium which may lead to changes in vascular function. This altered vascular function was improved by treatment with FA that restored level of endothelial layer to normal suggesting an effect of FA on vascular endothelium through lowering Hcy level. The oral supplementation with FA confirms and extends the findings of recent parallel group studies of folic acid alone or in combination with other B vitamins on endothelial function in coronary artery disease (Doshi SN et al., 2001).

In summary, the present study demonstrated that in isolated mouse coronary arteries with damaged endothelial cells, Ach caused endothelial-independent vasoconstriction, which was enhanced by FA treatment in CBS^{-/+} mice. FA improved endothelial dysfunction induced by HHcy. FA increased eNOS production by improving endothelial cell function in CBS^{-/+} mice. Thus, FA has a direct functional impact on endothelial cells in coronary artery during HHcy. This was indicated by improvement of vasomotor function, which occurred through improvement of endothelial cell function.

Limitations

A part of this study was to delineate the mechanism of coronary vasospasm. To this end we measured eNOS, via nitric oxide, which does not maintain tone, it reduces tone. Although the abnormal responses to Ach are not important in the pathogenesis of coronary vasospasm, however, they are important in condition of damaged endothelium. To that end, we showed coronary vasoconstriction to Ach in hyperhomocysteinemic arteries. It is known the Hcy damage endothelial unequivocally in every vascular bed. Although it would be complemented if we would use more physiologic vasodilators such as bradykinin, and moreover, use of vasoconstrictors would provide a more comprehensive analysis. In addition to the measurements of MTHFR, because FA has been shown to prevent eNOS uncoupling; and recently found via upregulation of the tetrahydrobiopterin salvage enzyme dihydrofolate reductase (Crabtree MJ et al., 2009. Crabtree MJ et al., 2009). It would be relevant to examine DHFR expression. To confirm if Ach-induced vasoconstriction in CBS^{-/+} mice are due to impaired eNOS activity, if folic acid-induced improvement in endothelial function will be abolished by NOS inhibitor incubation, it is important to incubate the vessels from

all groups of animals with nitric oxide synthase (NOS) inhibitor. The studies on eNOS activity should be performed in the different experimental settings. We reported the differential levels of NO in CBS-/+ and CBS+/+ mice treated with different doses of acetylcholine (an endothelial-dependent NO producer). These NO production was directly related to vessel diameter in different experimental settings.

References

- Albert CM, Cook NR, Gaziano JM, Zaharris E, MacFadyen J, Danielson E, Buring JE, Manson JE. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA*. 2008; 299:2027–2036. [PubMed: 18460663]
- Antoniades C, Shirodaria C, Warrick N, Cai S, de Bono J, Lee J, Leeson P, Neubauer S, Ratnatunga C, Pillai R, Refsum H, Channon KM. 5-Methyltetrahydrofolate Rapidly Improves Endothelial Function and Decreases Superoxide Production in Human Vessels: Effects on Vascular Tetrahydrobiopterin Availability and Endothelial Nitric Oxide Synthase Coupling. *Circulation*. 2006; 114:1193–1201. [PubMed: 16940192]
- Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, Rasmussen K. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med*. 2006; 354:1578–1588. [PubMed: 16531614]
- Boulanger CM. Secondary Endothelial Dysfunction: Hypertension and Heart Failure. *Journal of Molecular and Cellular Cardiology*. 1999; 31:39–49. [PubMed: 10072714]
- Crabtree MJ, Channon KM. Dihydrofolate reductase and biopterin recycling in cardiovascular disease. *J Mol Cell Cardiol*. 2009; 47:749–751. Editorial. [PubMed: 19799910]
- Crabtree MJ, Tatham AL, Hale AB, Alp NJ, Channon KM. Critical role of tetrahydrobiopterin recycling by dihydrofolate reductase in regulation of eNOS coupling: relative importance of the de novo biopterin synthesis versus salvage pathways. *J Biol Chem*. 2009; 284(41):28128–36. [PubMed: 19666465]
- Dayal S, Lentz SR. Murine models of hyperhomocysteinemia and their vascular phenotypes. *Arterioscler. Thromb. Vasc. Biol*. 2008; 28:1596–1605. [PubMed: 18556571]
- Den Heijer M, Lewington S, Clarke R. Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *J Thromb Haemost*. 2005; 3:292–299. [PubMed: 15670035]
- Doshi SN, McDowell IFW, Moat SJ, Lang D, Newcombe RG, Kredan MB, Lewis MJ, Goodfellow J. Folate Improves Endothelial Function in Coronary Artery Disease : An Effect Mediated by Reduction of Intracellular Superoxide? *Arterioscler Thromb Vasc Biol*. 2001; 21:1196–1202. [PubMed: 11451751]
- Doshi SN, McDowell IFW, Moat SJ, Payne N, Durrant HJ, Lewis MJ, Goodfellow J. Folic Acid Improves Endothelial Function in Coronary Artery Disease via Mechanisms Largely Independent of Homocysteine Lowering. *Circulation*. 2002; 105:22–26. [PubMed: 11772871]
- Eberhardt RT, et al. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest*. 2000; 106:483–491. [PubMed: 10953023]
- Feliciano L, Henning RJ. Coronary artery blood flow: Physiologic and pathophysiologic regulation. *Clinical Cardiology*. 1999; 22:775–786. [PubMed: 10626079]
- Fukagawa NK, Minaker KL, Young VR, Rowe JW. Insulin dose-dependent reductions in plasma amino acids in man. *Am J Physiol*. 1986; 250:E13–17. [PubMed: 3510558]
- Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial Dysfunction, Oxidative Stress, and Risk of Cardiovascular Events in Patients With Coronary Artery Disease. *Circulation*. 2001; 104:2673–2678. [PubMed: 11723017]
- Hyndman ME, Rosenfeld RJ, Anderson TJ, Parsons HG. Interaction of 5-methyltetrahydrofolate and tetrahydrobiopterin on endothelial function. *Am J Physiol Heart Circ Physiol*. 2002; 282:H2167–2172. [PubMed: 12003825]
- Liu Q. Constriction to hypoxia-reoxygenation in isolated mouse coronary arteries: role of endothelium and superoxide. *J Appl Physiol*. 1999; 87:1392–1396. [PubMed: 10517769]

- Looft-Wilson RC, Ashley BS, Billing JE, Wolfert MR, Ambrecht LA, Bearden SE. Chronic diet induced hyperhomocysteinemia impairs eNOS regulation in mouse mesenteric arteries. *Am J Physiol Regul Integr Comp Physiol*. 2008; 295:R59–R66.
- Ludmer PLSA, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med*. 1986; 315:1046–1051. [PubMed: 3093861]
- Majtan T, Liu L, Carpenter JF, Kraus JP. Rescue of cystathionine β -synthase (CBS) mutants with chemical chaperones. *JBC*. March 22.2010 DOI 10.1074/jbc.M110.107722.
- Messika AH, Kaluski DN, Lev E, Iakobishvili Z, Shohat M, Hasdai D, Mager A. Nutrigenetic impact of daily folate intake on plasma homocysteine and folate levels in patients with different methylenetetrahydrofolate reductase genotypes. *Eur J Cardiovasc Prev Rehabil*. Apr 23.2010
- Muller JM, Davis MJ, Chilian WM. Integrated regulation of pressure and flow in the coronary microcirculation. *Cardiovascular Research*. 1996; 32:668–678. [PubMed: 8915185]
- Nakano EHJ, Powers HJ. Folate protects against oxidative modification of human LDL. *Br J Nutr*. 2001; 86:637–639. [PubMed: 11749673]
- Qipshidze N, Tyagi N, Sen U, Givvimani S, Metreveli N, Lominadze D, Tyagi SC. Folic acid mitigated cardiac dysfunction by normalizing the levels of tissue inhibitor of metalloproteinase and homocysteine-metabolizing enzymes postmyocardial infarction in mice. *Am J Physiol Heart Circ Physiol*. Nov; 2010 299(5):H1484–93. [PubMed: 20802128]
- Robert K, Maurin N, Vayssettes C, Siauve N, Janel N. Cystathionine beta synthase deficiency affects mouse endochondral ossification. *Anat Rec A Discov Mol Cell Evol Biol*. Jan; 2005 282(1):1–7. [PubMed: 15622513]
- Schnyder G, Roffi M, Pin R, Flammer Y, Lange H, Eberli FR, Meier B, Turi ZG, Hess OM. Decreased Rate of Coronary Restenosis after Lowering of Plasma Homocysteine Levels. *N Engl J Med*. 2001; 345:1593–1600. [PubMed: 11757505]
- Sen U, Munjal C, Qipshidze N, Abe O, Gargoum R, Tyagi SC. Hydrogen sulfide regulates homocysteine-mediated glomerulosclerosis. *Am J Nephrol*. 2010; 31:442–455. [PubMed: 20395677]
- Shimokawa H. Primary Endothelial Dysfunction: Atherosclerosis. *Journal of Molecular and Cellular Cardiology*. 1999; 31:23–37. [PubMed: 10072713]
- Steed MM, Tyagi N, Sen U, Schuschke DA, Joshua IG, Tyagi SC. Functional consequences of the collagen/elastin switch in vascular remodeling in hyperhomocysteinemic wild-type, eNOS $^{-/-}$, and iNOS $^{-/-}$ mice. *Am J Physiol Lung Cell Mol Physiol*. Sep.2010 299(3)
- Tiefenbacher CP, Tina MB, Vahl C, Amann K, Vogt A, Kübler W. Endothelial Dysfunction of Coronary Resistance Arteries Is Improved by Tetrahydrobiopterin in Atherosclerosis. *Circulation*. 2000; 102(18):2172–2179. [PubMed: 11056088]
- Toole JF, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang C-H, Stampfer M. Lowering Homocysteine in Patients With Ischemic Stroke to Prevent Recurrent Stroke, Myocardial Infarction, and Death: The Vitamin Intervention for Stroke Prevention (VISP) Randomized Controlled Trial. *JAMA*. 2004; 291:565–575. [PubMed: 14762035]
- Tyagi SC, Smiley LM, Mujumdar VS, Clonts B, Parker JL. Reduction-oxidation (Redox) and vascular tissue level of homocyst(e)ine in human coronary atherosclerotic lesions and role in extracellular matrix remodeling and vascular tone. *Mol Cell Biochem*. 1998; 181:107–116. [PubMed: 9562247]
- Ungvari Z, Csiszar A, Huang A, Kaminski PM, Wolin MS, Koller A. High pressure induces superoxide production in isolated arteries via PKC-dependent activation of NADPHoxidase. *Circulation*. 2003; 108:1253–1258. [PubMed: 12874194]
- Verhaar MC, Wever RMF, Kastelein JJP, van Dam T, Koomans HA, Rabelink TJ. 5-Methyltetrahydrofolate, the Active Form of Folic Acid, Restores Endothelial Function in Familial Hypercholesterolemia. *Circulation*. 1998; 97:237–241. [PubMed: 9462523]
- Wang J, Xu XB, Wolin MS, Hintze TH. Defective endothelium-mediated control of coronary circulation in conscious dogs after heart failure. *Am J Physiol*. 1994; 226:H670–680. [PubMed: 8141368]
- Westerhof N, Boer C, Lamberts RR, Sipkema P. Cross-Talk Between Cardiac Muscle and Coronary Vasculature. *Physiol Rev*. 2006; 86:1263–1308. [PubMed: 17015490]

- Wilkinson IB, MacCallum H, Cockcroft JR, Webb DJ. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. *British Journal of Clinical Pharmacology*. 2002; 53:189–192. [PubMed: 11851643]
- Wilson PW. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA*. 2002; 288:2015–2022. [PubMed: 12387654]

Figure 1.

A: A typical example of mouse coronary perfusion preparation. B: Percent changes of responses to increase of transmural pressure in isolated mouse coronary arteries in CBS+/-, (CBS+/-) +FA, CBS+/+ and, (CBS+/+) +FA. The y axis indicates the intraluminal diameter expressed as % of baseline, where baseline is the intraluminal diameter at the spontaneous tone. * $P < 0.05$ vs. CBS+/-, (CBS+/-) +FA; $n=9$ for all groups.

Figure 2. Changes in intraluminal diameters of coronaries isolated from CBS+/+, (CBS+/+)+FA, CBS-/- and (CBS-/-)+FA mice. A. Example of images of isolated and cannulated coronaries. Arrows indicate intraluminal diameters. B. Bar graphs of intraluminal diameters. * $P < 0.05$ vs. CBS+/+, (CBS+/+)+FA; # $P < 0.05$ vs. CBS-/-; $n=8$ for all groups.

Figure 3.

A: Percent changes of responses to Ach and SNP in isolated mouse coronary arteries in CBS +/+, (CBS+/+) +FA, CBS -/+ and, (CBS-/-) +FA. * P < 0.05 vs. CBS+/+, (CBS+/+) +FA; # P < 0.05 vs. CBS-/-; n=8 for all groups. B: Percent changes of responses to Ach after DAF-2 infusion in isolated mouse coronary arteries in CBS+/+, (CBS+/+) +FA, CBS -/+ and, (CBS-/-) +FA. DAF-staining for NO showed clear differences in fluorescence, likely associated with the Ach-mediated NO-production and not the size of the vessels and condensed intensity. * P < 0.05 vs. CBS+/+, (CBS+/+) +FA; n=8 for all groups.

Figure 4.

A: Photomicrographs of CBS+/+, (CBS+/+) +F, CBS -/+ and, (CBS-/) +FA coronary arteries stained with Masson's trichrome. To avoid difference due to diameter and the wall

thickness of the vessels varying between proximal end and distal end, the blocks were prepared from septum containing coronary. Although no negative control images are shown, all quantification was performed using control negative stained images. B: Wall thickness of CBS+/, (CBS+/-) +FA, CBS-/+ and, (CBS-/-) +FA coronary arteries. * $P < 0.05$ vs. -/+ and, (CBS-/-) +FA; # $P < 0.05$ vs. CBS+/-; n=9 for all groups.

Figure 5.

a. Immunofluorescence micrographs of endothelial cells (CD31) in CBS+/+, (CBS+/+) +FA, CBS -/+ and (CBS-/-) +FA coronary artery. b. Changes in expression of CD31 in CBS+/+, (CBS+/+) +FA, CBS -/+ and (CBS-/-) +FA coronary. The micrographs were taken under the identical set of conditions for all groups. Although the apparent size of the vessel in the bottom-right corner may appear to be smaller than other 3 vessels, the vessels of the similar size were compared and quantified for data analysis. * $P < 0.05$ vs. CBS+/+, (CBS+/+) +FA; # $P < 0.05$ vs. CBS-/-; n=7 for all groups.

Figure 6.

a. Endothelial-dependent endothelial nitric oxide synthase (eNOS) immunostaining in CBS +/+, (CBS+/+) +FA, CBS -/+ and (CBS-/-) +FA coronary artery. b. Changes in expression of eNOS in CBS+/+, (CBS+/+) +FA, CBS -/+ and, (CBS-/-) +FA coronary. The micrographs were taken under the identical set of conditions for all groups. The image at the bottom-left corner, the auto-fluorescence from the cardiac muscle seems to be even stronger than the staining on the vessels. Although the eNOS staining is not completely convincing, the signal-noise ratio was not high and the background fluorescence from the cardiac tissue was subtracted in the quantification. C. Semi-quantitative RT-PCR analysis of eNOS. The mRNA from CBS+/+, (CBS+/+)+FA, CBS-/+ and (CBS-/-)+FA mice septal coronary artery was isolated and amplified for eNOS. GAPDH was used as loading control. D. The bar graph represents real-time PCR amplification of eNOS in CBS+/+, (CBS+/+) +FA, CBS-/+ and (CBS-/-)+FA mice septal coronary artery. *P < 0.05 vs. CBS+/+, (CBS+/+) +FA; # P < 0.05 vs. CBS-/-; n=7 for all groups.

Figure 7.

a. immunofluorescence micrographs of MTHFR (Green) and homocysteine (Hcy) (red) in CBS+/+, (CBS+/+) +FA, CBS -/+ and, (CBS-/-) +FA left ventricle (LV) heart tissue. b. Changes in expression of MTHFR and homocysteine in CBS+/+, (CBS+/+) +FA, CBS -/+ and, (CBS-/-) +FA LV heart tissue. The micrographs were taken under the identical set of conditions for all groups. * P < 0.05 vs. CBS+/+, (CBS+/+) +FA; # P < 0.05 vs. CBS-/-; n=7 for all groups.

Table 1

Comparison of plasma Hcy levels in CBS^{+/+}, CBS^{-/+} and CBS^{+/+}, CBS^{-/+} treated with folic acid.

Groups	Hcy, $\mu\text{mol/L}$	Body weight, g
CBS ^{+/+}	5 $\pm$ 2	32 $\pm$ 2
(CBS ^{+/+})+FA	4 $\pm$ 1	31 $\pm$ 1
CBS ^{-/+}	21 $\pm$ 4 *	27 $\pm$ 1 *
(CBS ^{-/+})+FA	12 $\pm$ 3 *#	31 $\pm$ 2

Although the CBS^{-/+} mouse model has been used by others (Dayal S et al., 2008; Eberhardt RT, et al., 2000), especially by feeding a high methionine/low folate diet, and measured the levels of Hcy with only a very slight increase in Hcy levels - in fact, subclinical levels as pertain to humans, i.e. levels of approximately 10 $\mu\text{mol/L}$ and there CBS^{+/+} littermates are in the low single digits. We measured the levels of Hcy in genotyped CBS^{-/+} mouse 21 $\pm$ 4 $\mu\text{mol/L}$ as compared to 5 $\pm$ 2 $\mu\text{mol/L}$ in CBS^{+/+} littermates, feeding with normal rodent diet. The Hcy levels were measured as described using HPLC C18 amide 5- μm 150 $\times$ 2.1 mm column (Sen U et al., 2010).

* P < 0.05 vs. CBS^{+/+}

P < 0.05 vs CBS^{-/+}. n=9 for all groups.