

Mesenteric vascular remodeling in hyperhomocysteinemia

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Abstract Remodeling by its very nature implies synthesis and degradation of extracellular matrix components (such as elastin, collagen, and connexins). Most of the vascular matrix metalloproteinase (MMP) are latent because of the presence of constitutive nitric oxide (NO). However, during oxidative stress peroxynitrite (ONOO⁻) activates the latent MMPs and instigates vascular remodeling. Interestingly, in mesenteric artery, homocysteine (Hcy) decreases the NO bio-availability, and folic acid (FA, an Hcy-lowering agent) mitigates the Hcy-mediated mesentery artery dysfunction. Dimethylarginine dimethylaminohydrolase-2 (DDAH-2) and endothelial nitric oxide synthase (eNOS) increases NO production. The hypothesis was that the Hcy decreased NO bio-availability, in part, activating MMP, decreasing elastin, DDAH-2, eNOS and increased vasomotor response by increasing connexin. To test this hypothesis, the authors used 12-week-old C57BL/6 wild type (WT) and hyperhomocysteinemic (HHcy)-cystathione beta synthase heterozygote knockout (CBS^{+/-}) mice. Blood pressure measurements were made by radio-telemetry. WT and MMP-9 knockout mice were administered with Hcy (0.67 mg/ml in drinking water). Superior mesenteric artery and mesenteric arcade were analyzed with light and confocal microscopy. The protein expressions were measured by western blot analysis. The mRNA levels for MMP-9 were measured by RT-PCR. The data showed decreased DDAH-2 and eNOS expressions in

mesentery in CBS^{-/+} mice compared with WT mice. Immuno-fluorescence and western blot results suggest increased MMP-9 and connexin-40 expression in mesenteric arcades of CBS^{-/+} mice compared with WT mice. The wall thickness of third-order mesenteric artery was increased in CBS^{-/+} mice compared to WT mice. Hcy treatment increased blood pressure in WT mice. Interestingly, in MMP-9 KO, Hcy did not increase blood pressure. These results may suggest that HHcy causes mesenteric artery remodeling and narrowing by activating MMP-9 and decreasing DDAH-2 and eNOS expressions, compromising the blood flow, instigating hypertension, and acute abdomen pain.

Keywords Endothelial · DDAH · Elastin · Resistance vessels · Hypertension

Introduction

Clinical studies has shown that the increased homocysteine (Hcy) levels are a significant risk factor for cardiovascular disease, stroke, and mesenteric thrombosis [1, 2]. Hcy is auto-oxidized to reactive oxygen species (ROS), causing inflammatory response leading to endothelial dysfunction. Hcy greatly affects NO bioproduction and NO bioavailability [3]. Hyperhomocysteinemia (HHcy) affects responsiveness of both vascular smooth muscle and endothelium to vasomotor agonists [4]. Vasomotor alterations and systemic redistribution of the blood flow, are known to have a crucial role in the development of progressive organ dysfunction [4, 5].

Connexins play a critical role in providing a conduit for the membrane structures, which permits direct intercellular passage of ions and small molecules, and which plays a

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role both in electrical coupling and intercellular communication during patterning and development. Also, connexins contribute to the maintenance and modulation of vasomotor tone [6]. Connexin-40 is essentially required for normal transmission of endothelium-dependent vasodilator response [7]. Interestingly, Connexin-40 promoter polymorphism has been associated with an increased risk of hypertension in men [8, 9].

High plasma Hcy levels induce a reduction in arterial distensibility and compliance [10]. Previous studies of our lab showed that Hcy causes constrictive vascular remodeling, and hence causes narrowing of arteriolar/artery lumen [11]. Hcy levels are independently associated with increased arterial wall thickness [12–14]. MMPs involved in Hcy induced vascular remodeling and further caused ECM proteolysis, and myoendothelial uncoupling [15, 16]. Hcy-induced activation of latent MMP has been previously shown, and it causes increased oxidative stress (ROS and nitrotyrosine) by differential expressions of Thioredoxin, Nox1, and NOS in MVEC [23]. The chronic exposure of blood vessels to homocysteine causes disruption elastic laminae, and hence causes elastinolysis [17]. Also, HHcy causes endothelial nitric oxide synthase dysfunction. This can further be explained by dimethylarginine dimethylaminohydrolase (DDAH), an enzyme involved in the regulation of nitric oxide synthase (NOS) by metabolizing the free endogenous arginine derivatives N(omega)-methyl-L-arginine (MMA) and N(omega),N(omega)-dimethyl-L-arginine (ADMA) [18]. Interestingly, DDAH expression in the vasculature was found to colocalize at all sites of expression isoform of NOS [19]. Furthermore, tissue-specific downregulation of DDAH in HHcy has been reported [18]. Hcy interferes with methylation modification of DDAH-2 gene resulting in elevation of ADMA [20]. Consequently, this ADMA further competitively inhibits eNOS, and hence decreases bio-availability of NO [21].

The authors hypothesize that Hcy decreases NO bio-availability, in part, by activating MMP, decreasing elastin, DDAH-2, eNOS, and increases vasomotor response by increasing connexins in mesenteric vasculature, HHcy causes thickening superior mesenteric artery which may affect the distensibility.

Methods

Antibodies and reagents

Rabbit polyclonal antibodies to MMP-2, MMP-9, and eNOS were purchased from Abcam (Cambridge, MA), rabbit polyclonal antibodies to DDAH-2, connexin-40, and 43 were purchased from Chemicon. GAPDH antibody and other analytical reagents were from Sigma-Aldrich

(St. Louis, MO). Horseradish peroxidase-linked anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). PVDF membrane was from Bio-Rad (Hercules, CA).

Animal model

Male C57BL/6 J wild-type mice (8–10-wk old) and heterozygous CBS (+/–) a model for HHcy, were obtained from Jackson Laboratory (Bar Harbor, ME) WT (C57BL/6 J), and kept in the animal care facility of University of Louisville where ambient environmental conditions (12:12-h light:dark cycle, 22–24°C) were maintained. The animals were fed standard chow and water ad libitum. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine in accordance with animal care and use program guidelines of the National Institutes of Health.

Blood pressure

For blood pressure measurements by radio-telemetry, animals were divided into four groups: WT group, Male C57BL/6 J wild-type mice (8–10-week old) were given drinking water; Hcy group, Male C57BL/6 J wild-type (4–5-week old) were given homocysteine in drinking water for 6–8-weeks; MMP-9 group, MMP-9 knock out mice given; MMP-9 knockout mice were administered Hcy (0.67 mg/ml) in drinking water. Direct radiotelemetric measurements of systolic/diastolic blood pressure were measured continuously during the experiment with a DSI (Data Sciences International; St. Paul, MN) telemetric system using a pressure transducer (PA-C20) surgically implanted into the aortic arch through the left common carotid artery as we described previously [22], starting after 1-week surgical recovery period. The data were analyzed with DSI Dataquest ART 3.1 software.

Video microscopy and digital image processing

A Nikon Diaphot 200 inverted microscope optimized was utilized. Observations were made with a Nikon fluorite $\times 20$ (numerical aperture = 0.75) objective and/or a Nikon fluorite oil $\times 40$ objective (numerical aperture = 1.30–0.80). A multi-image module (beam-splitting device) was connected to Hamamatsu intensified CCD camera system. Mesenteric third-order artery diameter and wall thickness were measured on-line with a video caliper device.

Cryosectioning

The mesenteric tissue was excised, cryopreserved in Peel-A-Way disposable plastic tissue-embedding molds

(Polysciences, Warrington, PA) containing tissue-freezing media (Triangle Biomedical Sciences, Durham, NC). These molds were kept frozen (-70°C) until serial 5- μm tissue sections were made in Cryocut 1800 (Reichert-Jung). Cryosections were placed on Superfrost/plus microscope slides and air dried.

Immunofluorescence

Immunofluorescence staining was performed on 5- μm thick frozen sections of the superior mesenteric artery. Anti-connxin-40, 43, eNOS, and anti-MMP-2, secondarily conjugated with Texas Red (Chemicon International, St. Charles, MO) were used for immunodetection of these two proteins, respectively.

Western blot analyses of MMP-2, & 9, DDAH-2, eNOS, Connexin-40

Western blot analyses were performed on mesenteric tissue homogenates using 10% SDS-PAGE. BCA method was used to estimate total protein, and 25 μg of protein was loaded in each well of electrophoresis gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (PVDF), blocked with 5% fat-free milk, and blotted with respective antibodies. As a loading control, GAPDH was used. The bands were normalized with GAPDH controls.

In situ Zymography

MMP activity in the media and intima of frozen mesenteric sections was measured by in situ zymography using a gelatinolytic fluorogenic substrate. Vessel sections were incubated in dark humidified chambers with 1.0 $\mu\text{g}/\text{ml}$ DQ gelatin in Tris–CaCl₂ Buffer for 1 h. The sections were examined using confocal microscope, and the image was captured at 60 \times magnification. The proteolytic activity was detected as bright green fluorescence, which indicates gelatin breakdown, which was quantified using the Image Pro software. The gelatinolytic activity was assessed by quantifying the intensity of green fluorescence from ten fields selected around the vessel circumference. Phenanthroline at 0.1 mmol l^{-1} inhibited MMPs activity in the mesenteric sections served as negative control.

RNA isolation and expression study

Total RNA from the mouse mesenteric arcade was isolated using Trizol Reagent (Gibco BRL) according to the instructions provided by the manufacturer. The cDNA was prepared using the Promega kit. The reverse transcription program was 25 $^{\circ}\text{C}$ for 10 min, 42 $^{\circ}\text{C}$ for 50 min, and then

70 $^{\circ}\text{C}$ for 15 min. PCR was performed using 2 μl of each RT product (cDNA), with a total reaction volume of 20 μl . The PCR thermal cycle was 94 $^{\circ}\text{C}$ for 6 min, 35 cycles at 94 $^{\circ}\text{C}$ for 50 s, 60 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min, finally 72 $^{\circ}\text{C}$ for 10 min, which gives a product of ~ 320 bp. Primers for GAPDH (116 bp product) were sense (5-GATG CAGGGATGATGTTCTG-3) and antisense (5-ACAAC TTT GGCATTGTGGAA-3). The PCR thermal cycle for GAPDH was 94 $^{\circ}\text{C}$ for 2 min, 30 cycles at 94 $^{\circ}\text{C}$ for 30 s, 57 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 1 min, finally 72 $^{\circ}\text{C}$ for 2 min. All of the primers were obtained from Invitrogen (Carlsbad, CA).

Nitrite assay

NO metabolites such as nitrite production was measured fluorimetrically using commercially available kit from Biovision. Results expressed as nanomole of nitrite produced per mg of protein.

Statistical analysis

All data are expressed as mean \pm SE. Data were analyzed using a one-way analysis of variance (ANOVA) to test for treatment effect. The level of significance was accepted at $P < 0.05$.

Results

Mesenteric remodeling

In Fig. 1, tissue elastin in WT mesenteric artery appeared to be more when compared to CBS+/- SMA. This suggested greater distensibility in the WT than CBS+/- mouse mesentery. Severe elastin breakdown and irregularities in the smooth muscle layer as observed in the histological section of SMA from CBS+/- . This suggested remodeling in mesenteric artery. Videomicroscopic data revealed significant increase ($P \leq 0.05$) in third-order mesenteric artery wall thickness in CBS-/+ mice than WT mice (Fig. 2b). This may suggest that CBS-/+ mesenteries are more prone to become stiffer and further priming these to ischemic events than the mesenteries from WT mice.

To determine whether the decrease in elastin in CBS-/+ mesentery is due to increase in metalloproteinases, the authors measured elastinolytic MMP-2 and -9. The results suggested that although there was the tendency to increase in MMP-2, MMP-9 was robust in CBS-/+ mesentery than WT mesentery (Fig. 3). These results support the concept that MMP-2 is constitutively expressed, and which plays a constitutive role by increase in during compensatory remodeling. However, MMP-9 is inducible and detrimental

Fig. 1 Histological analysis of frozen superior mesenteric artery (SMA) from wild type (a) and CBS^{+/-} mice (b), stained with van Gieson for elastin. Images were visualized using light microscopy and Q capture software. *Note:* the non uniform smooth muscle layer as well as thinning of the elastin lamina in CBS^{+/-} SMA. (c) Bar graph presentation of quantification of elastin using image-pro software. The y-axis represents arbitrary scan unit ($n = 4$ in each group); * $P < 0.05$ compared to WT control

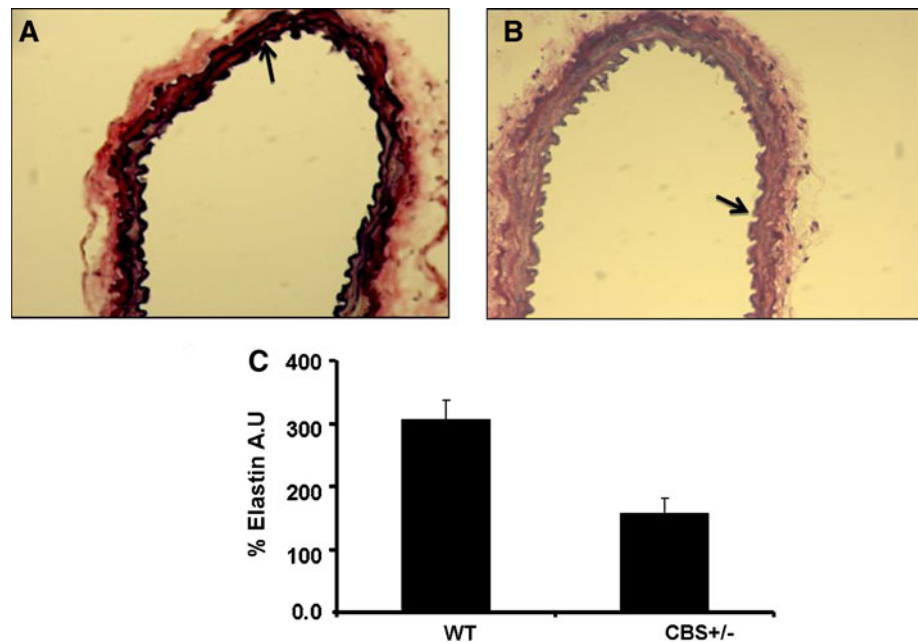
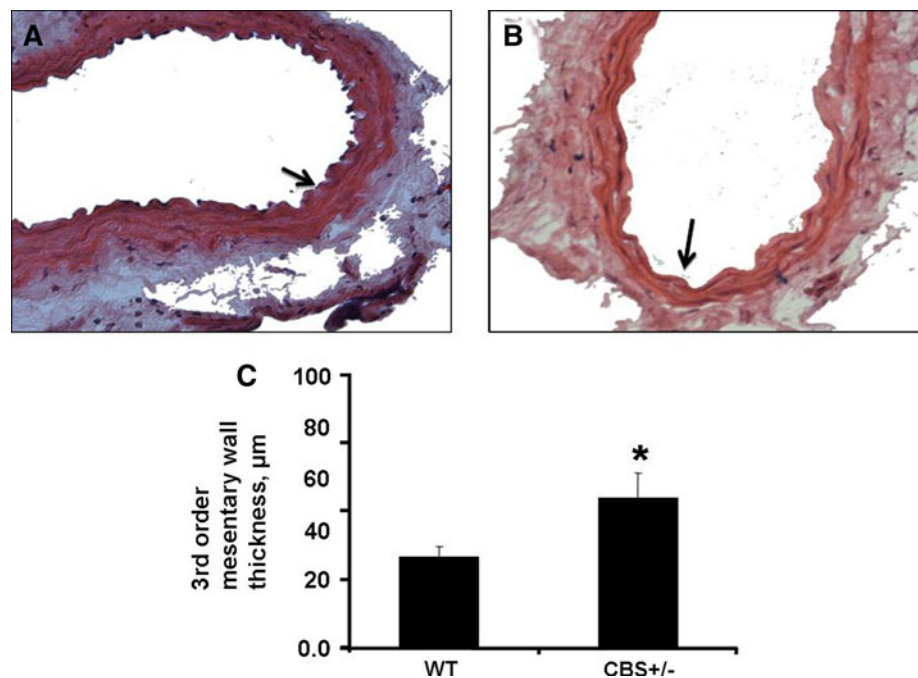


Fig. 2 Histological analysis with H and E staining of frozen superior mesenteric artery (SMA) from wild type (a) and CBS^{+/-} mice (b) mice, respectively, showing increased lamellar thickness of SMA in CBS^{+/-} mice compared to WT mice. (c) Graph showing Mesenteric 3rd order artery wall thickness, measured using video caliper Nikon Diaphot 200 inverted microscope from WT and CBS^{+/-} mice. Values are mean \pm SEM. $n = 4$ in each animal group; * $P < 0.05$ compared to WT control



and activated during pathological remodeling in HHcy. Furthermore, the authors checked gelatinolytic activity of MMPs using in situ zymography (Fig. 4). Micrograph from normal vessels (Fig. 4a) showed no gelatinolytic activity (green) (arrows). However, there was increased gelatinolytic activity (green) in CBS^{+/-} HHcy vessel (Fig. 4b). In addition, quantification using image pro software corroborated respective results (Fig. 4d).

The cell–cell connexins are involved in endothelial cells as well as smooth muscle cells. The authors measured the levels of connexin-40, connexin-43 in SMA sections using

immunofluorescence. The result suggests increase in connexin-40, 43 levels in CBS^{-/+} mesentery than WT (Figs. 5b–e, 6a–d). These results may indicate the increase in gap junction communication in CBS^{-/+} mesentery than WT. This may explain why HHcy arteries are more prone to become hypertrophied (Fig. 6).

Levels of DDAH-2 and eNOS

To examine the effect of HHcy on the production and bio-availability of NO in mesenteric arcade as well as SMA,

Fig. 3 Effect of Hcy on MMP-2 protein (a) MMP-9 protein (b) and MMP-9 mRNA (c) in mesenteric tissue. The protein was blotted with MMP-2, -9 and GAPDH antibodies. The mRNA was amplified using MMP-9 and GAPDH primers. The bands for MMP-2 and MMP-9 expression were quantified using densitometry and expressed fold change from wild type. The bar graphs represent MMP over GAPDH expression. Values are mean \pm SEM. $n = 4$ in each animal group; * $P < 0.05$ compared to WT control

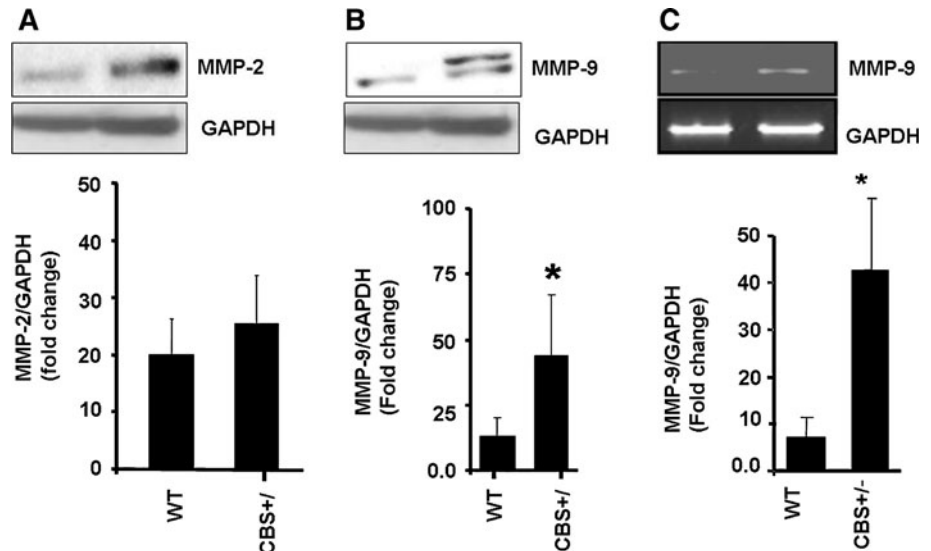
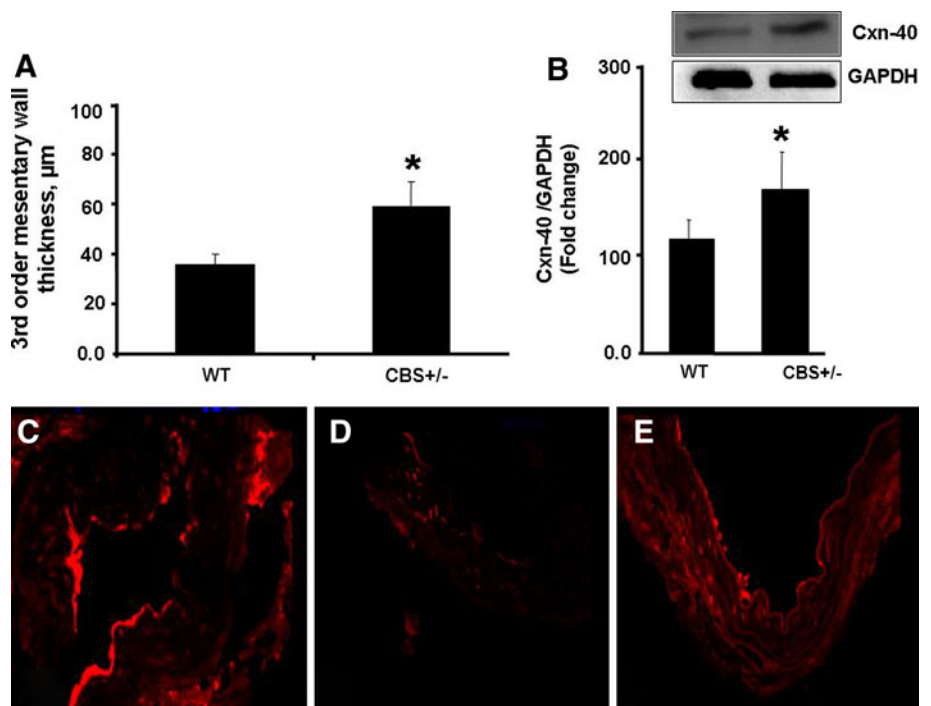


Fig. 4 a Western Blot analysis of connexin-40 in tissue lysate from mesenteric arcade. The bar graphs represent and connexin-40 expression over GAPDH. Values are mean \pm SEM. $n = 4$ in each animal group; * $P < 0.05$ compared to WT control. b Connexin-40 labeling in WT mesentery using immunofluorescence; c connexin-40 labeling in CBS-/+ mesentery; and d negative control using secondary antibody, without connexin-40 antibody



the authors measured DDAH-2 and eNOS levels. The immunohistochemistry data showed abundant expressions of DDAH-2 in SMA sections from C57BL/6 J mice when compared to CBS+/- mice (Fig. 7a, b). DDAH-2 expression was down regulated as can be seen in immunoblots from mesenteric tissue from CBS+/- mice (Fig. 7c). The similar results were obtained for eNOS expression (Fig. 8).

Arterial blood pressure, systolic and diastolic, in the Hcy-treated group was significantly greater than in WT controls. Also, pulse pressure, the difference between systolic and diastolic blood pressures was more in

comparison with WT controls. Interestingly, this increase was attenuated in MMP-9 knockout mice, when treated with Hcy (Fig. 9).

Levels of nitrite

To elucidate the role of homocysteine in NO bio-availability, the authors measured NO metabolite nitrite levels in mesenteric arcade as well as plasma. The graph (Fig. 10) shows the decreased NO metabolite levels in mesenteric tissue as well as plasma samples of CBS+/- mice when compared with control.

Fig. 5 Immunohistochemical staining using an antibody against connexin-43 in SMA sections from **a**: WT C57BL/6 J mice; **b** from CBS+/- mice; **c** negative control using secondary antibody, without connexin-40 antibody. **d** Bar graph presentation of quantification of connexin-43 per unit area using image-pro software. Values are mean \pm SEM. $n = 4$ in each animal group; * $P < 0.05$ compared to WT control

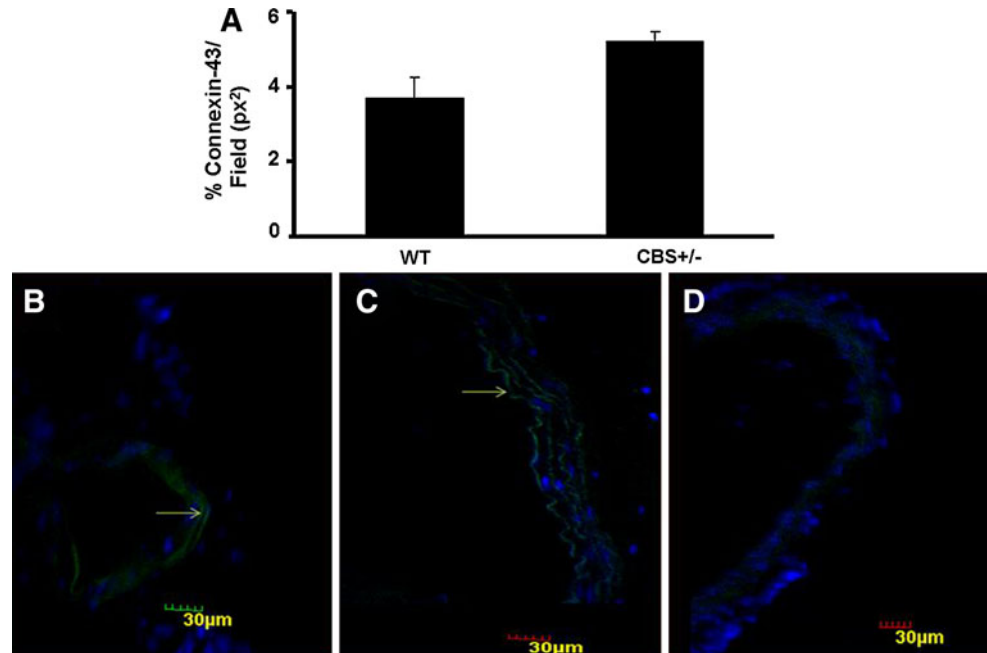
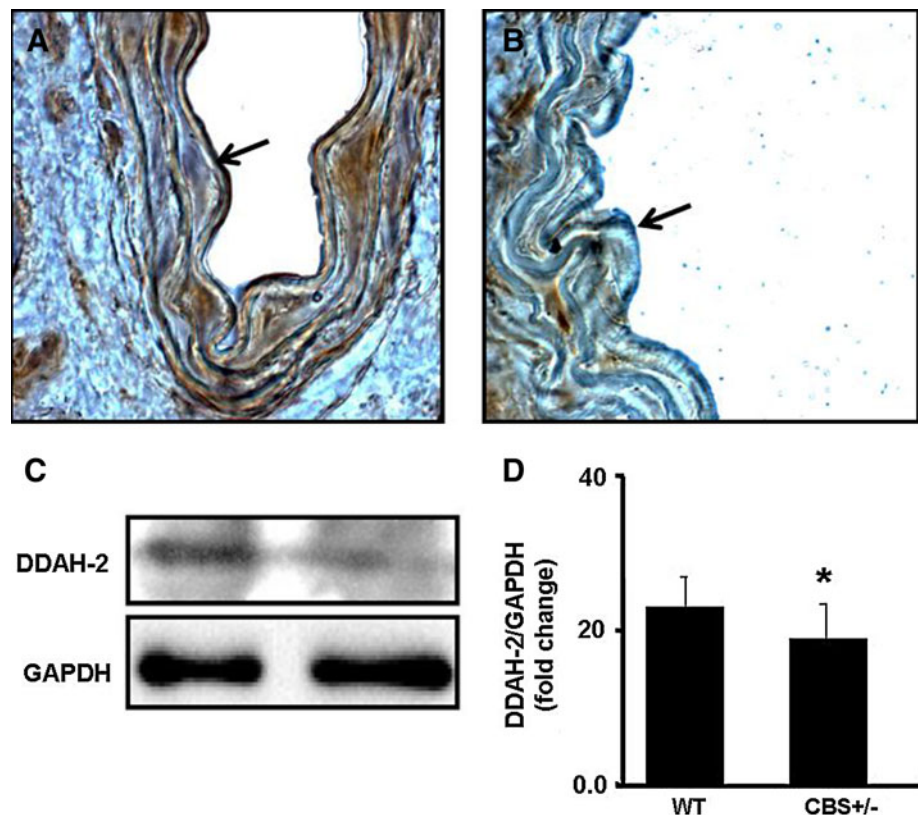


Fig. 6 Effects of Hcy on the expression of dimethylarginine dimethylaminohydrolase (DDAH-2) in the mesenteric tissue. Results are presented as the mean \pm SEM ($n = 4$). **a** Immunohistochemical staining with the use of an antibody against DDAH-2 protein showing SMA sections from WT C57BL/6 J mice; **b** from CBS+/- mice. **c** Immuno-blots of mesenteric arcade from WT and CBS-/+ mice. Note: abundant DDAH-2 positive cells in the medial layer of the SMA from C57BL/6 J WT mice than CBS-/+ mice



Discussion

Hcy depletes folic acid that leads to depletion of tetrahydrobiopterin, an essential co-factor of eNOS, thereby causing uncoupling of eNOS and decreasing bio-availability of NO, therefore, activates MMP-9 and connexin-

40, leading to vascular remodeling and hypertension [23]. To substantiate the hypothesis that Hcy decreases NO bio-availability by activating MMP, decreasing elastin, DDAH-2, eNOS and increases vasomotor response by increasing connexin, and to provide a cause-effect relationship between down regulation of eNOS and MMP-9 activation

Fig. 7 Effects of homocysteine on the expression of endothelial nitric oxide synthase (eNOS) in mesenteric tissue:

a Fluorescence immunohistochemical assays for eNOS in SMA sections from C57BL/6 J WT; **b** CBS+/- mice; and **c** negative control without primary antibody. All panels are representative of three independent experiments. **d** Western Blot analysis of eNOS in tissue lysate from mesenteric arcade of C57BL/6 J and CBS+/- mice. Bars are mean \pm SEM ($n = 4$)

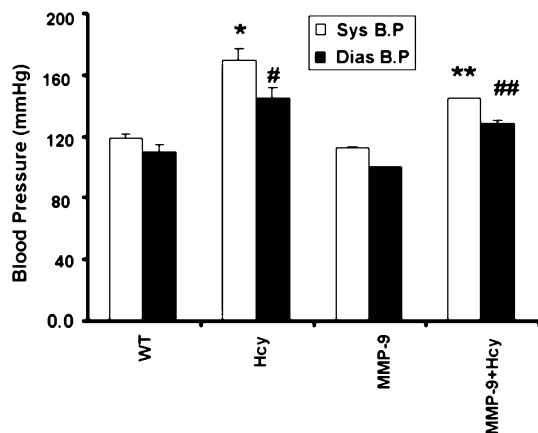
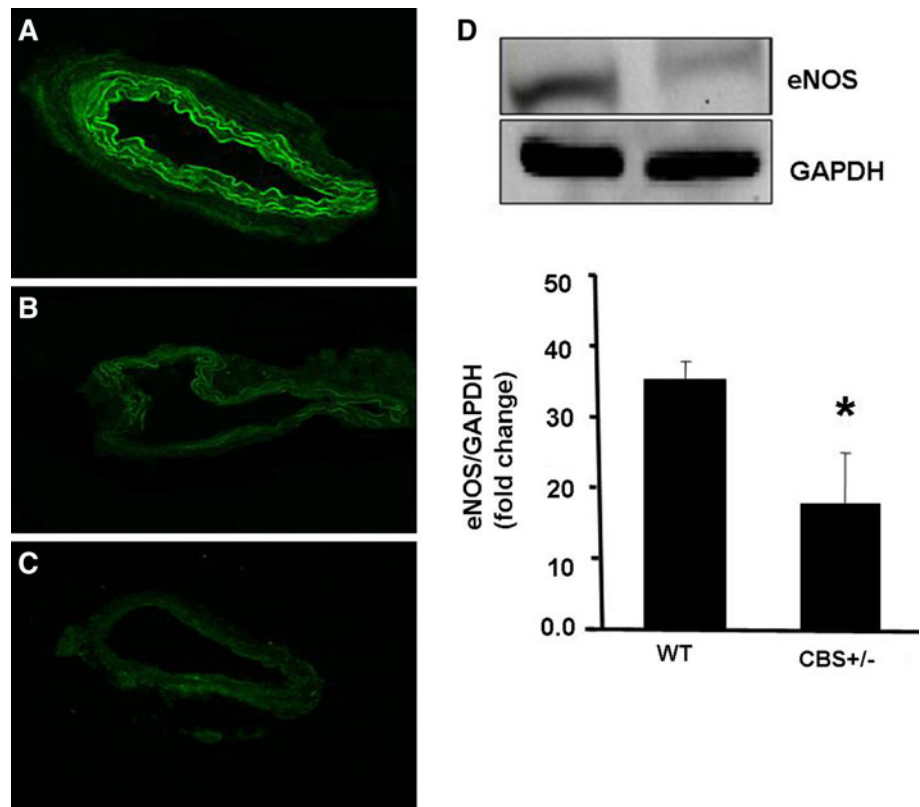


Fig. 8 Blood pressure (BP) measurement using telemetric DSI system in real time. WT, C57BL/6 J mice treated with vehicle; Hcy, C57BL/6 J mice treated with homocysteine; MMP-9, MMP-9 knock out mice treated with vehicle; MMP9 + Hcy, MMP-9 knock out mice treated with Homocysteine. Values are means \pm SE; $n = 4$ animals in each group. * $P < 0.05$ Systolic BP compared to WT control. # $P < 0.05$, Diastolic BP compared to WT control

in CBS+/- mice, the authors measured metabolites of NO (nitrite) in mesentery vasculature as well as in plasma (Fig. 10). This delineates the role of NO in modulating MMP activities and vascular remodeling and addresses the detrimental effect of Hcy in mesenteric vasculature of CBS-/+ mice.

Pathological vascular remodeling of mesenteric arteries leads to endothelial dysfunction and also wall thickening. Interestingly, blockade of angiotensin-2 receptor causes receptor decreased MMP activity and increased elastin content in small mesenteric arteries [23]. Furthermore, HHcy is associated with smooth muscle cell proliferation by activating cyclin-dependent kinases [24].

In this article showed elastin degradation in superior mesenteric arteries. Also, further evidences indicate altered properties of the elastin network in arteries cultured with physiological and pathological concentrations of homocysteine [25]. Moreover, long-term elevated blood pressure causes cellular and matrix “remodeling” in mesenteric vessels [26]. Mild hyperhomocysteinemia is associated with stiffer and smaller arteries with increased collagen deposition in the media [12]. Also, a linear correlation between Hcy levels and systolic hypertension was demonstrated previously [27]. There has been a direct connection between aortic wall thickness and plasma Hcy levels [28].

Symon et al. [29] showed HHcy produced dysfunction in mesenteric circulation, and also showed low folate diet associated with compromised in mesenteric vasculature function. Interestingly, folic acid and magnesium decrease Hcy-induced MMP secretion [30]. Previous studies have shown in CBS+/- (mild HHcy model) endothelium-dependent vasodilator function was decreased in mesenteric

Fig. 9 Mesenteric sections show MMP-2 and -9 activity using in situ zymography: Cryosections were incubated with DQ-gelatin-FITC substrate for 3 h. **a** Representative micrograph shows no gelatinolytic activity (green) in normal vessels (arrows) **b** Representative micrograph shows increase in gelatinolytic activity (green) in CBS+/- HHcy vessels (arrows) **c** Using MMPs inhibitor (phenanthroline) as a negative control **d** Quantification using image pro software revealed a two- to four-fold increase in MMP activity in CBS-/+ mouse vessels (* $P < 0.05$, $n = 5$ animals/group). (Color figure online)

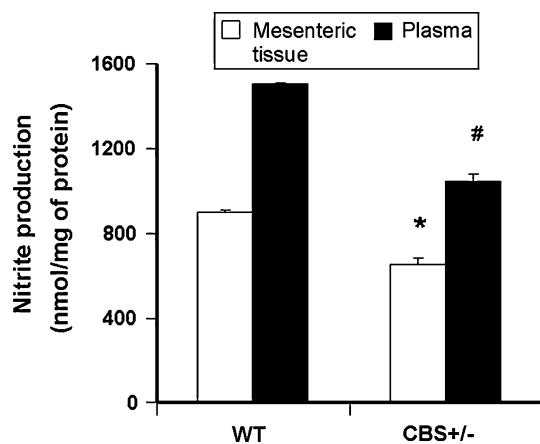
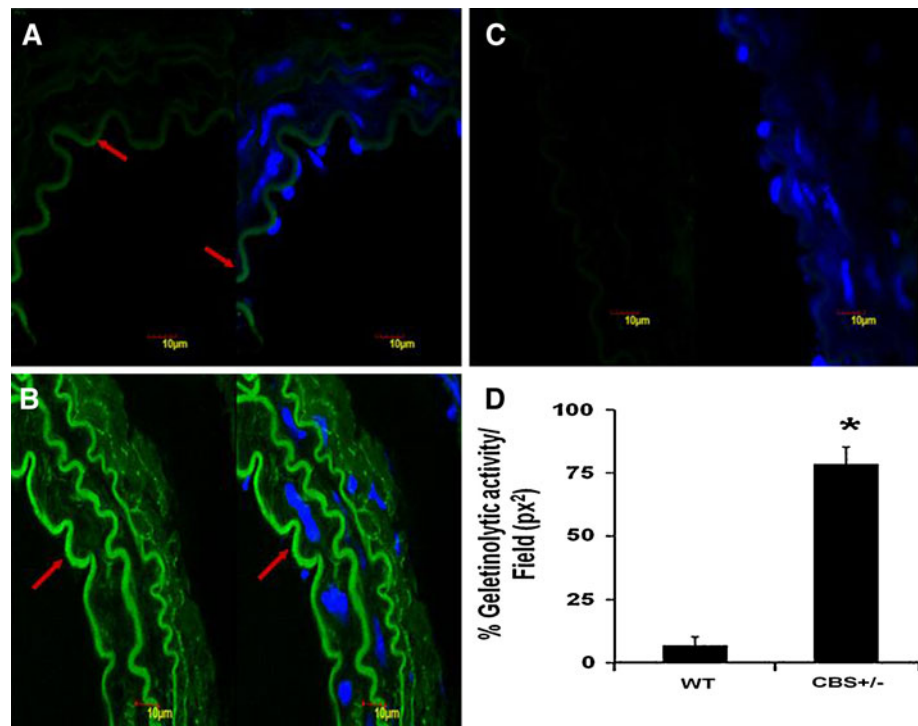


Fig. 10 Nitrite measurement using fluorometric assay (nmole/mg of protein): The graph shows decreased nitrite levels in mesenteric tissue as well as in plasma samples of CBS+/- mice with compared to control. * $P < 0.05$ compared to WT; # $P < 0.05$ compared to WT

arterioles and was associated with an increased in expression of P-selectin, indicating inflammation [31]. In addition, Hcy exposure resulted in impaired vascular reactivity of the superior mesenteric artery to the endothelium-dependent vasodilators acetylcholine, but not to the endothelium-independent vasodilator sodium nitroprusside [19].

The authors have shown Hcy induces a decline in eNOS and DDAH-2 expressions in mesenteric arcade as well as SMA. It is consistent with the previous report that DDAH-2 expression is required for EDRF/NO responses in mesenteric vessels [32]. DDAH may play an important role

in the regulation of extravillous trophoblast function via its effects on ADMA and NO productions [4]. DDAH plays a pivotal role in maintenance of endothelial function and is also a potential target for treating diseases associated with decreased NO bio-availability [33, 34].

A critical role of connexin-40 inhibition in EDHF (endothelium-derived hyperpolarizing factor)-mediated dilation of rat mesenteric arteries [35]. In addition, Cx40 is essentially required for normal transmission of endothelium-dependent vasodilator response [18]. Hence, Cx40 plays a crucial role in maintaining vasomotor tone. In this article, the authors showed elevated connexin-40, 43 expression in HHcy mesenteric tissue which is consistent with previous findings showing Hcy-induced upregulation of Cx43 transcript in HUVEC [36, 37]. On the contrary, some other authors have shown down regulation of connexin-40 in endothelial cells of the HHcy rat [12]. This suggests differential heterogeneity in connexins isoform in various vasculatures in HHcy. The results of this study show for the first time Hcy-induced MMP-9 activation in mesenteric vasculature. Altered vascular reactivity in mesenteric arteries from hypertensive mice due to vascular hypertrophy has been suggested [38]. Also, altered vasomotor function and systemic redistribution of the blood flow, have been suggested to play a crucial role in the development of progressive organ dysfunction [4, 5].

Reports from the authors lab showed Hcy associated thickening of the aortic wall, and hence causes constrictive remodeling in arteries [22, 39]. Interestingly, a marked

thickening of intima and media in the mesenteric arterioles of hypertensive rats was observed [40]. Also, one of the major findings in the present study, showed elevated blood pressure in HHcy mice which was decreased in MMP-9 KO mice treated with Hcy. Moreover, pressure alone stimulates wall stress as well as protooncogene expression in mesenteric arteries [41]. This further contributes toward stress, one of the signaling parameter and other cascade events in hypertension when arteries are tonically constricted. In previous reports showed distinct temporal and mechanistic responses to hypertensive injury in different vascular beds, which is indicative of differential susceptibility of organs to a hypertensive stimulus with mesentery and heart, exhibiting the greatest sensitivity to damage [42]. Furthermore, Hcy-mediated endothelial dysfunction can be ameliorated by peroxisome proliferator-activated receptor (PPAR) agonists [43].

In summary, the results of this study suggests that the Hcy-induced arterial hypertension leads to mesenteric arterial remodeling, mediated by MMPs and connexin's activation, elastin degradation, downregulation of DDAH-2, eNOS which may be attenuated in MMP-9 KO mice treated with homocysteine. Moreover, the authors previously reported the role of negative and positive remodelings in vascular diseases. Because the associated mechanism is yet obscure, further research is required to gain new insights into homocysteine induced negative remodeling in mesenteric arteries and the roles of different connexins isoforms.

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