Original Article
Remodeling in vein expresses arterial phenotype in hyperhomocysteinemia

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Abstract: Accumulating evidences suggest that homocysteine, a non-protein amino acid, is involved in vessel remodeling and blood flow at elevated level, although the exact mechanism is unclear. Here we hypothesized that homocysteine affects vein in such a way that vein develops arterial phenotype. We tested our hypothesis employing wild type (WT, C57BL/6J) and CBS+/- (cystathionine β-synthase heterozygote, a genetic model of hyperhomocysteinemia) supplemented with or without folic acid (FA, a homocysteine lowering agent). Vena cava blood flow was measured by ultrasound transonic flow probe. Tissue collagen and elastin were detected by histochemistry. Super oxide was detected by dihydroethidium (DHE) staining. Expressions of MMP-2, -9, -12, TIMP -2,- 4, were measured by Western blot. MMP-13, TIMP-1, -3, and vein and aortic markers, EphB4 and EphrinB2, respectively were measured by RT-PCR. The results indicated relatively low blood flow and significant increase of collagen/elastin ratio in the CBS+/- mice compared to WT. Although FA treatment did not alter blood flow in CBS+/- mice, the collagen/elastin ratio was normalized. A relatively increased content of super oxide and gelatinase activity was observed in CBS+/- vena cava vs WT and normalized by FA treatment. Western blot analyses showed significant increase in MMP-9,-12 and decrease in TIMP-2, -4 expressions. Expressions of MMP-13, TIMP-1 and -3, Ephrin B2 were increased, whereas EphB4 was decreased with reverse change in FA treatment, with no change in MMP-13 and TIMP-1. We conclude that chronic HHcy causes vascular remodeling that expresses arterial phenotype in vein.

Keywords: Homocysteine, vascular remodeling, matrix, metalloproteinase, collagen, elastin

Introduction

Evidences from in vitro and in vivo studies established that hyperhomocysteinemia, an elevated homocysteine level, is associated with vascular remodeling [1-3] and dysfunction [4-5]. Although the exact mechanism of homocysteine-induced vascular dysfunction is not clear and may be complex, one of the well documented mechanisms is oxidative stress [6]. Among the sources of oxidative radicals, NADPH oxidase plays a major role in hyperhomocysteinemia and causes changes in vascular phenotype including endothelial dysfunction [7], smooth muscle proliferation [8], and accumulation of collagen in the extracellular matrix (ECM) [9] leading to remodeling of the vessels [10].

Remodeling is a process of degradation and / or accumulation of matrix protein including collagen and elastin [11]. This is an active mecha-nism by which vessels are formed, maintain their integrity and repair in the event of injury [12]. While this mechanism is a part of normal physiological process in the healthy vessels, dysregulated remodeling causes vascular diseases, such as atherosclerosis and stroke [13].

Remodeling of the vessels is largely regulated by a family of calcium-dependent and zinc containing endopeptidases known as matrix metalloproteinases (MMPs) [14]. The MMPs are secreted in an inactive (latent) form called a zymogen or a pro-MMP [15]. These pro-MMPs require an activation step before they are able to cleave extracellular matrix component [14]. Reactive oxygen species (ROS) are a group of oxidative radicals which activates MMPs and initiates degradation of matrix protein in varieties of pathological conditions including arthritis, cancer and vascular diseases [16-18]. Of particular interests MMP-2 and -9 are activated by ROS

and reported to become involved in atherosclerotic vascular diseases associated with homocysteine-mediated generation of oxidative stress [9, 19].

Endogenously, these MMPs are primarily regulated by a class of inhibitory enzymes named tissue inhibitor of matrix metalloproteinase (TIMPs) [20]. TIMPs may be secreted by the cells or they may be associated with membrane bound MMPs [17]. The integrity of vessels is largely dependent on the balance between MMPs and TIMPs [15]. Dysregulated MMP/TIMP balance leads to degradation of matrix protein and is a characteristic of diverse pathological conditions including vascular hypertrophy and dysfunction [21-22].

Although MMPs are involved in tissue remodeling and cell migration, other intrinsic factors, such as Ephrin B2 and EphB4 are well known for their role in remodeling of vessels and proper vascular development [23-30]. While the roles of homocysteine in regulation of MMPs, particularly MMP-2 and -9 in vascular remodeling are well documented, the role of homocysteine in regulation of Ephrin B2 and Eph4 in mature vessels is not studied. We have previously reported that blood flow interplays with elastin/collagen and MMP/TIMP ratios to maintain normal vascular structure and function [31]. In this study we report that in addition to altered blood flow and MMP/TIMP imbalance, homocysteine induces arterial phenotype in vena cava as evidenced by EphB4 upregulation during remodeling process.

Materials and methods

Animal model

C57BL/6J (wild type, WT) and CBS+/- mice of C57 background of ages 14-18 weeks were used for this study. Mice were obtained from Jackson Laboratories Inc, Maine, USA, and housed in the animal care facility of the University of Louisville. CBS+/- and WT mice were divided into four groups and supplemented with or without folic acid (FA, 0.03 g / L) in the water for a period of 45 days. The groups were: 1) WT, 2) WT +FA, 3) CBS+/-, 4) CBS+/- + FA. The mice were fed with regular rodent chow and water supplied *ad libitum*. At the end of the experiments, animals were deeply anesthetized with Tribromoethanol (TBE, 240 mg/Kg body weight)

and sacrificed to harvest the inferior vena cava (VC). All animal procedures were in accordance with the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC # 09083) of the University of Louisville.

Blood flow measurement

The blood flow measurement was taken in anesthetized condition (TBE, 240 mg/kg b.w.). Abdomen was surgically opened, and a small window was made below the kidney, to isolate the vena cava. A transit time perivascular flow meter (Transonic System Inc, Ithaca, NY), equipped with transonic flow probe 0.5PSB630 was used to measure blood flow in the vena cava. The equipment was calibrated using a standard flow meter in ml/min. The waveform of the blood flow was recorded using DMSI-100 software.

Tissue sectioning and staining

The vena cava was isolated from each experimental groups of animal at the end of experiments. The tissues were cleaned off with phosphate-buffered saline (PBS) containing 20 unit/ml heparin. Heparin was used to remove blood clot. The tissues were then cut into approximately 2 mm pieces, placed vertically in tissue freezing media (Triangle Biomedical Sciences, Inc., Durham, NC)] and were frozen in liquid nitrogen. Frozen blocks with the molds were placed in a $-70°C$ freezer until serial sections were made. Cryosections (Leica CM1850) of 7 µm thickness were put on glass slides and stained with one of the following staining kits according to the supplied protocol: Masson Trichrome, van Gieson's or Hematoxylin and Eosin (Richard Allen Scientific, Kalamazoo, MI).

Measurement of elastin and collagen

Cryosections of vena cava were stained with Masson Trichrome, van Gieson or Hematoxylin Eosin and mounted. Slides were observed under light microscope and images were taken with an attached digital camera. To measure intensity of collagen and elastin staining in the vena cava sections, Un-ScanIt software was used to scan the images and densitometric data were collected. From the scanned densitometric data, elastin : collagen ratio was calculated. Medial thickness was measured in the hema-

toxylin eosin-stained vessels.

Detection of ROS

The oxidative fluorescent dye, dihydroethidium (DHE; Invitrogen, Carlsbad, CA) was used in frozen, 7 µm vena cava sections (40 µmol, 30 mins), and the fluorescence intensity was measured by the laser scanning confocal microscopy (Fluo View 1000, Olympus). Negative control sections were incubated for 25 min with 250 U/ml polyethylene glycol-superoxide dismutase (PEG-SOD; Sigma-Aldrich), before incubation with DHE. Fluorescent images were analyzed with ImagePro software (Media Cybernetics, Bethesda, MD).

Antibodies and reagents

MMP-2 antibody was obtained from Novas Biologicals, Littleton, CO. MMP-9 anti-body was purchased from Abcam Antibodies, Cambridge, MA, andTIMP-2 and TIMP-4 was obtained from Chemicon International, Tamecula, CA. MMP-12 and HRP conjugated secondary antibodies were bought from Santa Cruz Biotechnology, Santa Cruz, CA. All other reagents were used from commercially available highest grade.

Western blotting

Protein from isolated and finely copped vena cava was extracted using RIPA lysis buffer (Thermo Scientific Inc., Rockford, IL), containing protease inhibitors and PMSF. Protein content in the extracted samples was estimated by BCA assay, and 30 µg of total protein was loaded in each well of SDS-PAGE gels. Protein separated by electrophoresis, transferred to PVDF membrane and incubated with primary antibody followed by secondary HRP conjugated antibody. An ECL plus Western blotting detection reagent (GE Health Care, Little Chalfont, Buckinghamshire) was used to detect the protein of interests. For loading control, the membranes were stripped with membrane-stripping buffer (Boston BioProducts, Worcester, MA) and reprobed with GAPDH (Chemicon International). Intensity of bands was detected by Gel –Doc software, and was normalized with their corresponding GAPDH control.

In situ MMP activity

Seven µm frozen sections were incubated with

40 µmol DQ gelatin (Invitrogen) for 2 hours and the fluorescence was measured by the laser scanning confocal microscopy without washing. Fluorescent images were analyzed with ImagePro software.

Isolation of RNA and RT-PCR

Total RNA was isolated from the tissues by using Trizol reagent (Invitrogen) following manufacturer's instructions. Complementary total DNA was made by incubation of RNA with oligo dT at 70°C for 6 min. The RT cycle was 25°C for 2 min, 42°C for 50 min, 75°C for 5 min and 4°C forever as described previously [32]. All the primers were bought from Invitrogen and the primer sequences are shown in **Table 1**.

Table 1. Sequences of primers

TIMP-1	Sense, 5'-TCATCGGGCCCCAAGGGATCT
	Antisense, 5'-GCAGTGAAGAGTTTCTCATC
TIMP-3	Sense, 5'-GCCCTCCCATATGTATACCC
	Antisense, 5'-TAGGCCTCACCTCAAGTCTG
MMP-13	Forward, 5'-AGGCCTTCAGAAAAGCCTTC
	Reverse, 5'-CCCACCATAGTTTGGTCCAG
EphrinB2	Sense, 5'TCCAGGAGGGACTCTGTGTGGAAG
	Antisense,5'-CGGGGTATTCTCCTTCTTAATTGT
EphB4	Sense 5'-CCCAAATAGGAGACGAGTCC
	Antisense, 5'-CGGGGTATTCTCCTTCTTAATTGT
GAPDH	Sense, 5'-ATGGGAAGCTGGTCATCAAC
	Antisense, 5'-TGTGAGGGAGATGCTCAGTG

Vascular reactivity study

The vena cava was isolated from the animal and placed in ice cold physiological salt solution (PSS). The recipe of PSS solution was (in mM), NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KH_2 PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1, pH 7.4). The vessels were cut into 2.5-3 mm rings, fat and connective tissues were removed, and mounted with two tungsten wires. Wires were attached to the myobath and placed in a 25 ml organ bath filled with PSS at 37°C. The PSS in the myobath was constantly aerated with 20% O_2, 5% CO_2, and 75% N_2. Rings were stretched gradually to obtain an optimal resting tension of 0.1 g, and equilibrated for an hour. After equilibration dose (10^{-9} to 10^{-5} M) dependent response of phenylephrine (Phe) was detected. Acetylcholine (Ach) was added to the organ bath in the same manner as Phe to detect endothelial dependent response. Similarly, after washing with PSS, tissues were treated with sodium nitropruside (SNP) after a brief treatment of Phe (10^{-5} M) to measure endothelial-independent

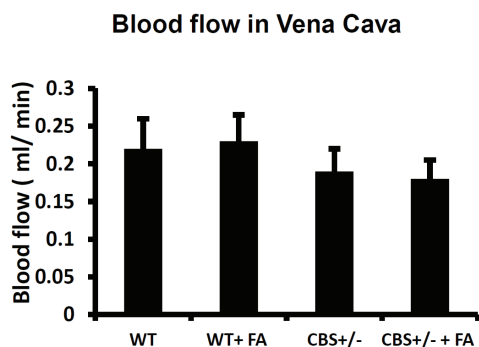**Blood flow in Vena Cava**

Figure 1. Blood flow in the vena cava. Blood flow was measured by Transonic perivascular flow meter. Data represent mean ± SD, n = 7.

response. The tissue responses as detected by Biopac System, Inc were recorded graphically using MP100 software loaded into a computer.

Statistical analysis

Each experiment was carried out using 4–6 specimens in each group. Paired T-Test was used to determine significance between groups; p < 0.05 was considered significant.

Values were taken as means ± standard deviation of measurement (SD).

Results

Blood flow and medial thickness

Blood flow (ml/min) in the vena cava of WT and WT + FA was similar (**Figure 1**). In the WT it was 0.22 ± 0.02 ml/ min and WT + FA was 0.23 ± 0.03 ml/min. Although in CBS+/- vena cava blood flow showed a tendency to decrease (0.19 ± 0.02 ml/min), the difference was not significant compared to WT. FA very slightly decreased the flow in CBS+/- (0.18 ± 0.02 ml/min) mice compared to CBs+/- alone (**Figure 1**). Again, no significant deference was observed.

Medial thickness of vena cava in WT was 2.1 X 10^{-2} ± 0.02 µm and in CBS +/- was 2 X 10^{-2} ± 0.02 µm. However, the difference was not significant. Similarly, although FA treatment slightly increased medial thickness in CBS+/- vena cava (2.1 X 10^{-2} ± 0.05 µm) compared to CBS+/-, the difference was not statistically significant (**Figure 2**).

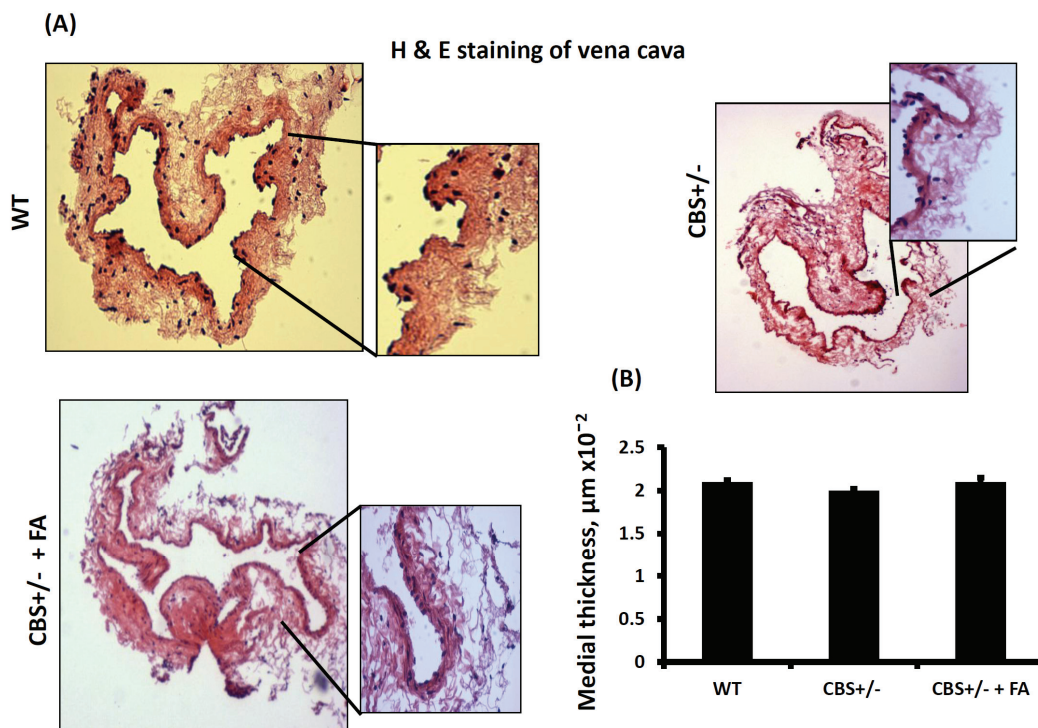**H & E staining of vena cava**

Figure 2. H&E staining of the vena cava. (A) The H&E staining is showing gross morphological structure. The images were taken in 6.4X magnification and the inset images were taken in 32X magnification to show the details of the structures. (B) The bar graph shows medial thickness. Data represent mean ± SD, n = 5.
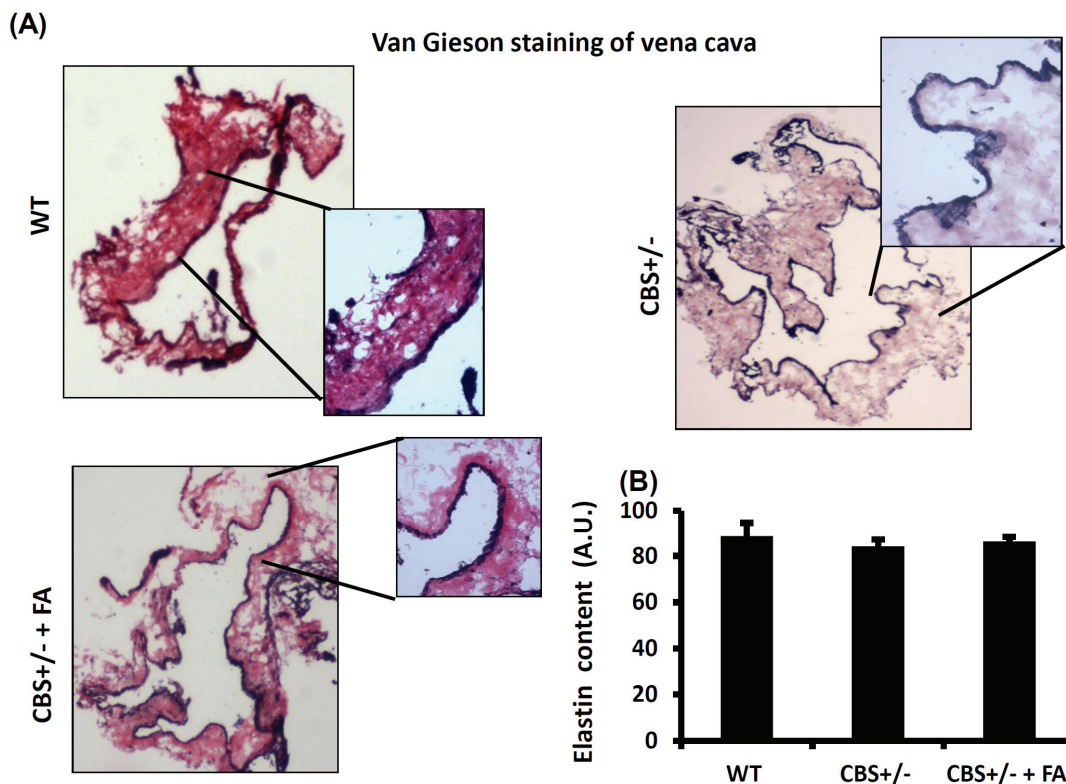
(A)

Van Gieson staining of vena cava

Figure 3. van Gieson staining of the vena cava. (A) The van Gieson staining of the vena cava is showing elastin content in the tissues. The images were taken in 6.4X magnification and the inset images were taken in 32X magnification to show details of the structures. (B) The bar graph represents relative elastin content among the groups. Data represent mean ± SD, n = 6.

Folic acid mitigated increased collagen/elastin ratio in CBS+/- vena cava

The elastin content of WT mice vena cava was 88.7± 8 A.U. (**Figure 3**). In CBS+/- mice treated without or with FA elastin content were 84.2 ± 4 A.U. and 86.3 ± 3 A.U., respectively. No significant changes of the elastin content were observed between the groups (**Figure 3**). Collagen content in the vena cava of WT was 72 ± 5 A.U.), and it was slightly increased in CBS+/- (80 ± 8 A.U.) group (**Figure 4**). This increase was mitigated in CBS+/- mice treated with FA (70 ± 5 A.U.). Collagen/ elastin ratio in the vena cava of WT was 0.81 ± 0.2 A.U. This ratio was significantly increased in CBS +/- mice (0.95 ± 0.4 A.U). Interestingly, FA treatment attenuated the increased collagen/elastin ratio in CBS+/- mice (0.81± 0.3 A.U.) (**Figure 5**).

Super oxide production

The intensity of dihydroethidium staining as an

indicator of ROS, particularly super oxide in CBS+/- vena cava was slightly increased, although it was not significant compared to WT (**Figure 6**). The ROS intensity in the vena cava of three groups were 0.8 ± 0.1 A.U. in WT, 0.9 ± 0.16 A.U. in CBS +/- and 0.89 ± 0.6 A.U. in FA treated CBS+/- mice (**Figure 6**). FA mitigated this increase of superoxide production in CBS+/- vena cava (**Figure 6**).

Expression of MMP/TIMP and vena cava remodeling in CBS+/- mice

The expression of MMP-2 in the vena cava did not change significantly among the groups (**Figure 7**). However, MMP-9 and -12 significantly increased in CBS+/- vena cava (**Figure 7**). Although FA treatment attenuated MMP-12 expression in CBS+/- vena cava, the increased expression of MMP-9 remained unchanged in CBS+/- vena cava after FA treatment (**Figure 7**). Both TIMP-2 and -4 expressions showed significant decrease in CBS+/- vena cava, compared
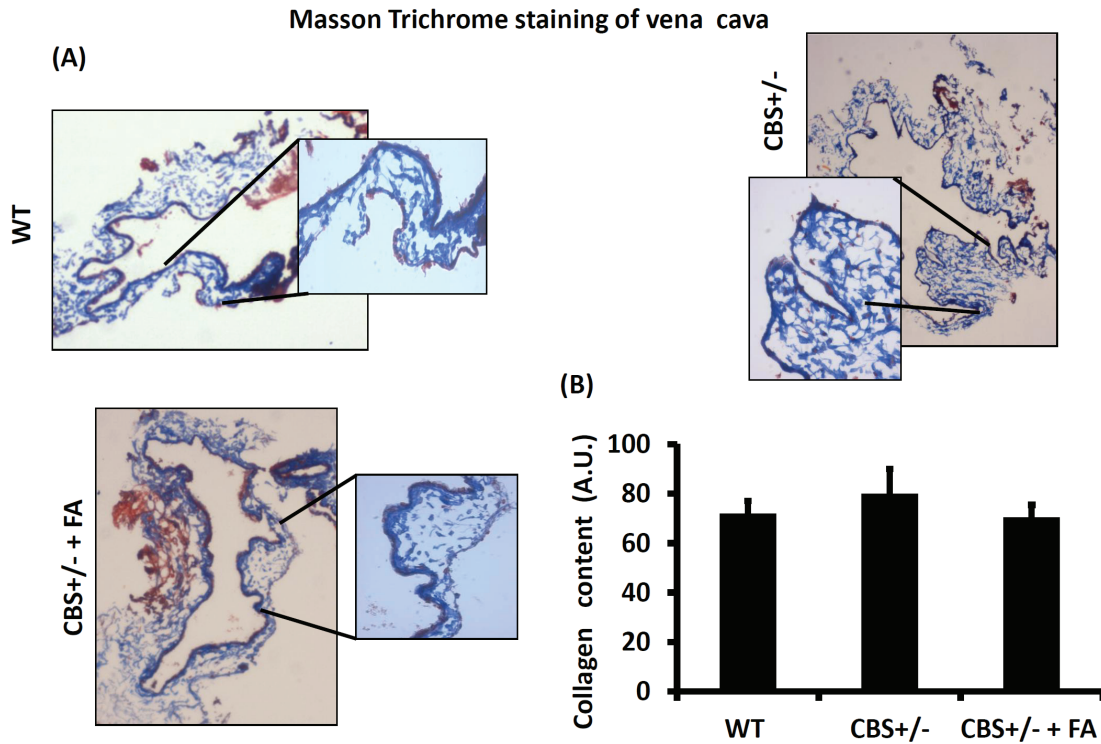
Masson Trichrome staining of vena cava

(A)

(B)

Figure 4. Masson-trichrome staining of the vena cava. (A) Masson-trichrome staining of the vena cava is showing collagen content in the tissue. The images were taken in 6.4X magnification and the inset images were taken in 32X magnification to show details of the structures. (B) The bar graph represents relative collagen content among the groups. Data represent mean ± SD, n = 6.
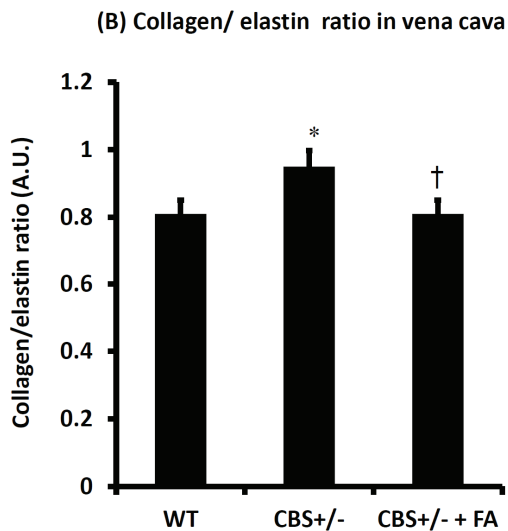
(B) Collagen/ elastin ratio in vena cava

Figure 5. Collagen/elastin ratio in the vena cava. Bar graphs indicate collagen/elastin ratio among the groups. Data represent mean ± SD, n = 5; *indicates $p < 0.05$ vs WT and † indicates $p<0.05$ vs CBS+/-.

to WT, and FA ameliorated expression of these two TIMPs in CBS+/- mice (**Figure 7**).

Vascular MMP activity

The MMP activity level as measured by *in situ* zymography, showed increased tendency in CBS +/- vena cava compared to WT (**Figure 8**); however, it was not significant. FA treatment normalized the activity of MMP (**Figure 8**).

MMP/TIMP mRNA and aorta marker in CBS+/- vena cava

TIMP-3 and mMMP-13 RNA expression showed significant increase in the CBS+/- mice compared to WT vena cava (**Figure 9**), whereas TIMP -1 expression did not change significantly (**Figures 9**). Treatment with FA mitigated TIMP-3 expressions in CBS+/- mice, whereas MMP-13 remained unchanged in vena cava (**Figure 9**).

The expression of aorta marker EphrinB2 signifi-

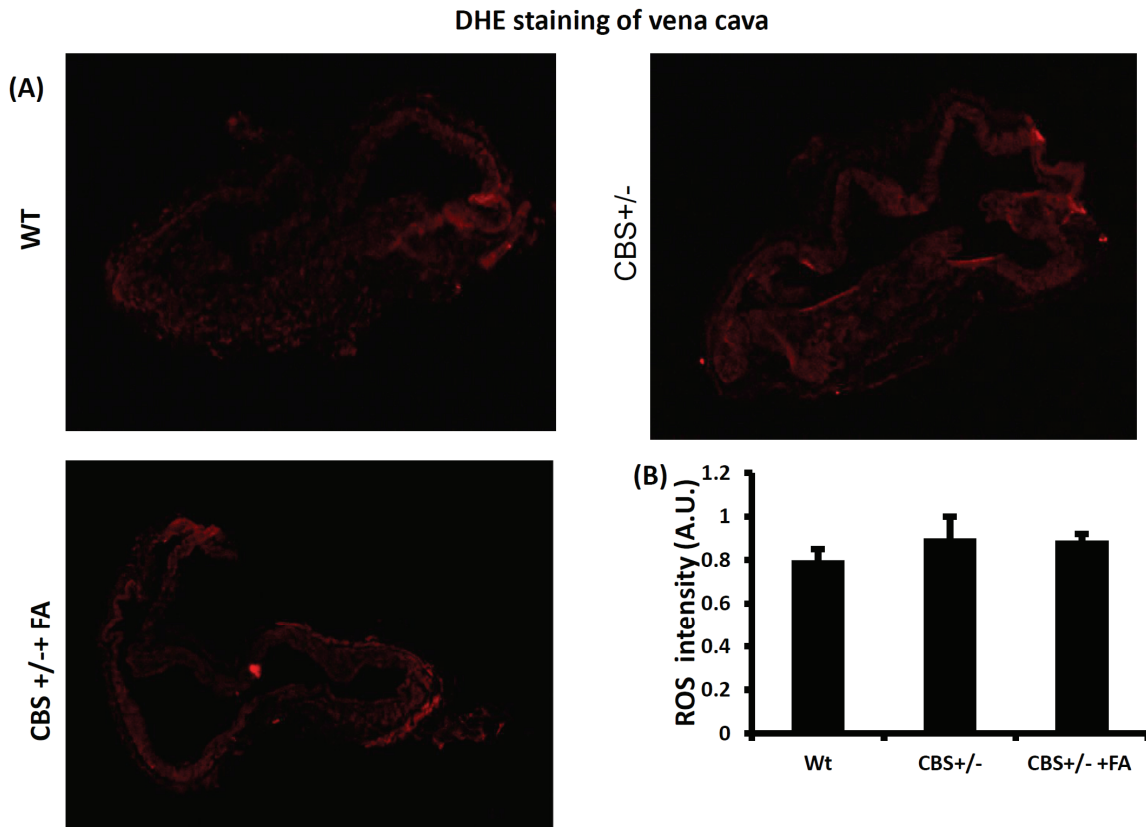**DHE staining of vena cava**

Figure 6: DHE staining of the vena cava. (A) The ROS content in the vena cava tissue were detected by dihydro-ethidium (DHE) staining. (B) The bar graph is showing relative intensity of fluorescence activities among the groups. Data represent mean ± SD, n = 5.

cantly increased in CBS+/- vena cava, whereas the expression of vena cava marker EphB4 showed significant decrease (**Figure 9**). FA treatment reversed the expressions of EphrinB2 and EphB4 in CBS+/- vena cava (**Figure 9**).

Vascular reactivity in CBS+/- vena cava

The dose-dependent vasoconstriction by Phe (phenylephrine) showed similar response in vena cava among the groups as shown in the figure 10. However, CBS+/- vena cava showed a slightly increased, although not significant, contraction at the highest dose of Phe (10^{-5} M) compared to WT (**Figures 10**). The dose response of Ach (acetylcholine) showed significant decreased relaxation in CBS+/- vena cava compared to WT. This decreased relaxation was not ameliorated by FA treatment (**Figures 10**). Tissue relaxation in response to SNP was greater in CBS+/- vena cava compared to WT (**Figure 10**). FA treatment normalized vascular relaxa-

tion in CBS+/- vena cava (**Figure 10**).

Discussion

Remodeling of vascular bed in pathophysiological hyperhomocysteinemia (HHcy) conditions have been well documented by various independent laboratories, including our own, in human as well as in vivo animal and in vitro cell culture models [6-7, 33-36]. To our knowledge none of these studies were done to investigate whether there is any shift of arterial phenotype in vena cava or vice versa during HHcy. We have previously reported that HHcy causes expression of venous phenotype in artery. Whether or not a reverse expression i.e. expression of arterial phenotype in vein during chronic HHcy occurs is unknown. Here we report that vena cava develops arterial phenotype in HHcy. This is a part of adaptive mechanism where vena cava accommodates with changing physiochemical environment in HHcy.
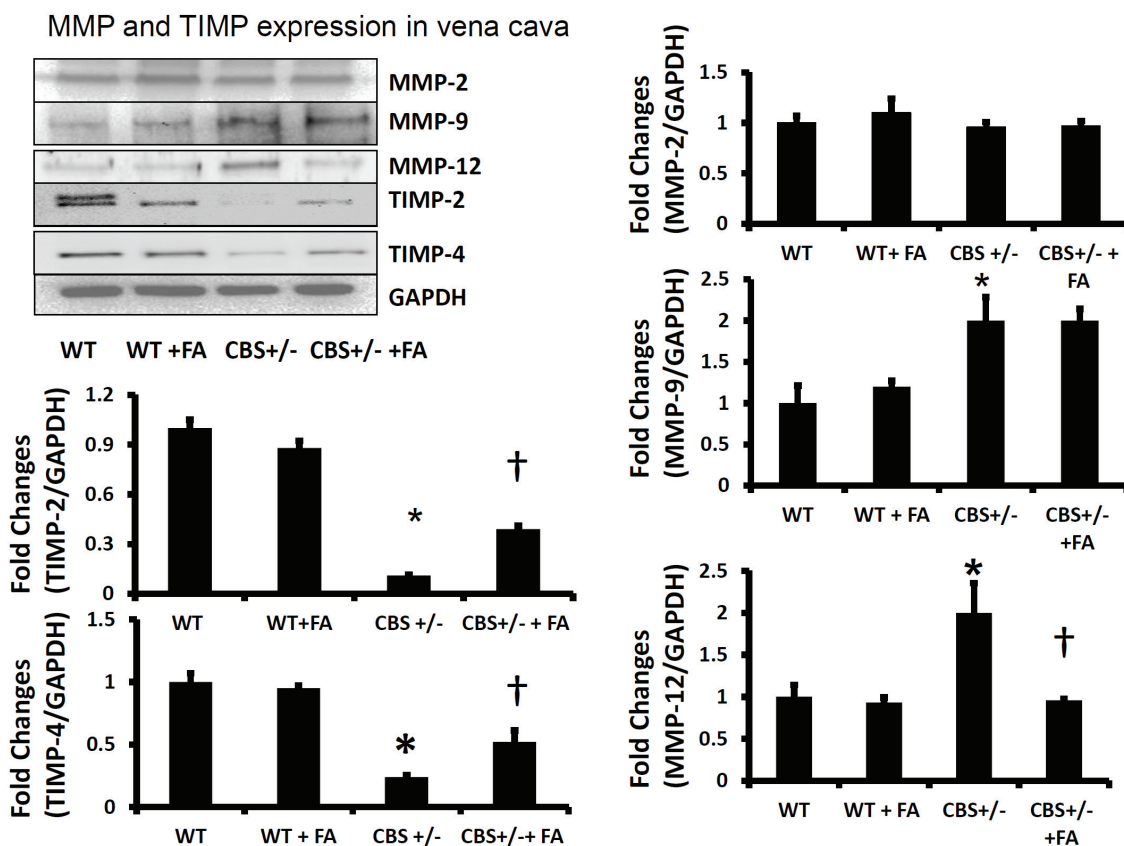
MMP and TIMP expression in vena cava

Figure 7. Western blot analyses of MMPs and TIMPs in the vena cava. (A) Tissue protein from the vena cava was extracted and analyzed for protein expression as shown in the figure. (B) Bar graph is showing relative expression of protein among groups. Data represents mean ± SD, n = 6. The *indicates $p < 0.01$ vs WT and †indicates $p < 0.05$ vs CBS+/-.

Homocysteine (Hcy) is formed during the metabolism of methionine and can be disposed out of the body by remethylation or transsulfuration pathway [37]. In transsulfuration pathway the CBS enzyme breaks down the Hcy to cysteine [37]. Individuals deficient of homozygous CBS (CBS-/-) develop severe HHcy and experience major clinical manifestations of premature vascular death, if untreated; whereas, CBS+/- individuals apparently live normal with cardiovascular abnormalities [38-39]. To mimic human disease processes we used CBS+/- mice, a well established model of HHcy, for our study because these animals live normal with vascular dysfunction due to moderate HHcy. Folic acid (FA) treatment was given to these mice to minimize the effect of Hcy, as the FA is an essential factor in remethylation pathway. Previously we reported that FA lowered Hcy level in CBS+/- animals (21 µmole/L in CBS+/- vs 12 µmole/L in CBS+/- + FA) [40]. Independent study have

also documented that FA reduced Hcy level in patients of HHcy [41-43]. In accordance with these previous studies, in the present study we have also recorded similar results with FA treatment (data not shown).

In our present study we observed an active remodeling process, such as alteration of collagen and collagen/elastin ratio, increased ROS production and altered expression of MMPs and TIMPs in the vena cava of CBS+/- mice. Among matrix protein, collagen is stiffer than elastin and deposition of collagen in the basement membrane of vascular smooth muscle cells and in the extracellular matrix makes the vessel rigid. Although we have not observed changes in the elastin protein in CBS+/- vena cava, the content of collagen was increased, and therefore collagen/elastin ratio was higher (Figure 5). We also observed increased expression (Figure 7) and activities (Figure 8) of MMP-2 and -9 in

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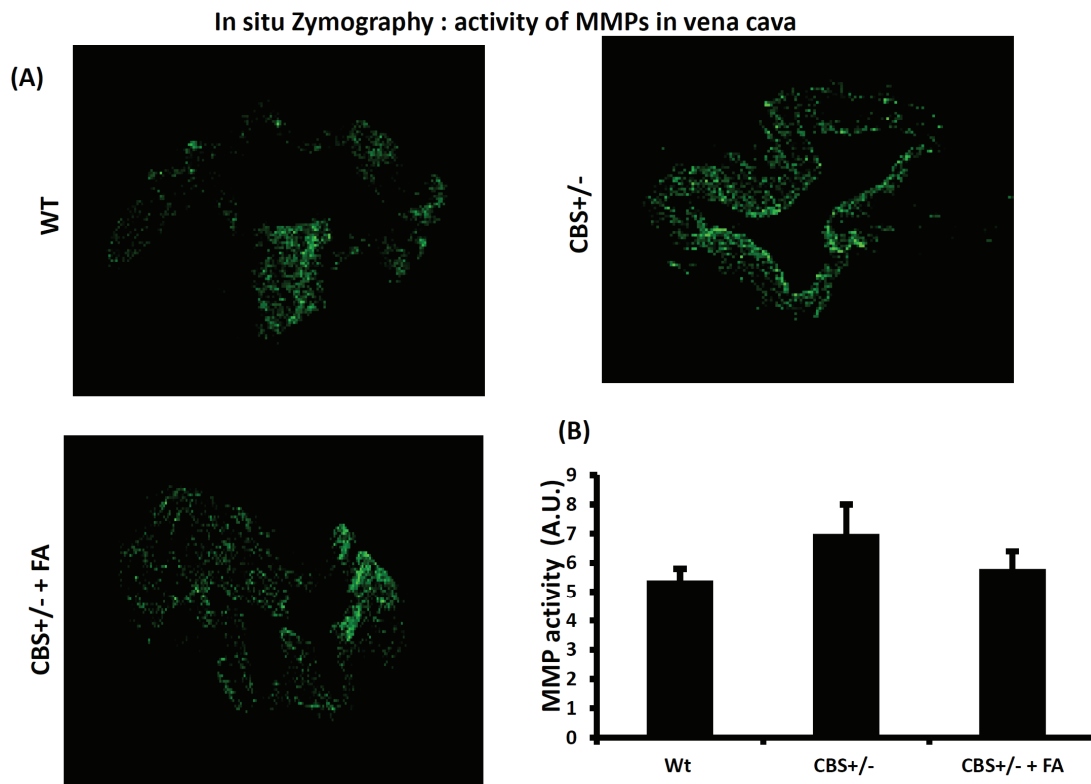**In situ Zymography : activity of MMPs in vena cava**

Figure 8: In situ zymography. (A) In situ zymography was performed using DQ gelatin as substrate and details were described in the methods. (B) Bar diagram indicates relative intensities of MMP activities among the groups.

our present study. Additionally, we measured upregulated mRNA expression of MMP-13 (Figure 9). Since one of the substrates of MMP-2, -9 and -13 are collagen [15], with increase expression and activities of these MMPs, collagen should have been digested and expressed in lower amount in HHcy vessels compared to WT control. Instead, we have detected paradoxical increase (Figure 5). The possible explanation is that due to increased oxidative radicals, especially superoxide in CBS+/- animal (Figure 6), matrix collagen oxidatively modified. When it is oxidized, MMPs can not cleave, and therefore accumulates in ECM [44-45]. Additionally, MMP-12 is a macrophage metalloelastage and increased expression of this MMP is related to elastin degradation [46]. Our present report in agreement with this previous finding suggest that increased MMP-12 is associated with lower, although not significant, elastin content in CBS+/- vena cava (Figure 3). FA treatment normalized MMP-12 expression with slightly increased elastin content in CBS+/- vena cava, suggesting a possible regulation of MMP-12 by Hcy.

Endogenously, MMPs are regulated by TIMPs. Although, by definition, TIMPs family inhibits MMPs activity, selective inhibition of MMPs has been reported [17, 47]. A classic example is TIMP-2, which in addition to inhibiting MMPs activities, interacts with membrane type-1(MT1)-MMP to facilitate activation of pro-MMP-2 [17, 47-48]. TIMP-1 is a prototypic inhibitor of MMPs and abundant in fibrotic tissues [49-50], whereas TIMP-3 primarily inhibits ADAM [51] and is apoptotic [52]. Compared to other TIMPs, relatively few studies are focused on TIMP-4 [51]. We previously reported that Hcy mitigates TIMP-4 in microvascular endothelial cells [53]. We have also shown that CBS+/- mice are deficient of TIMP-4 expression, both in mRNA and protein level, in neurovascular tissue [54]. Surprisingly, to our knowledge, none of the studies are focused to asses any involvement of TIMP-2 in vascular remodeling in HHcy. Since TIMP-2 and -4 are general MMP inhibitors, our present findings of increased MMP-2,-9 and -12 suggest that in HHcy TIMP-2 and -4 were actively participated in vena cava remodeling as evidenced with their mitigated expressions (Figure 7). Folic
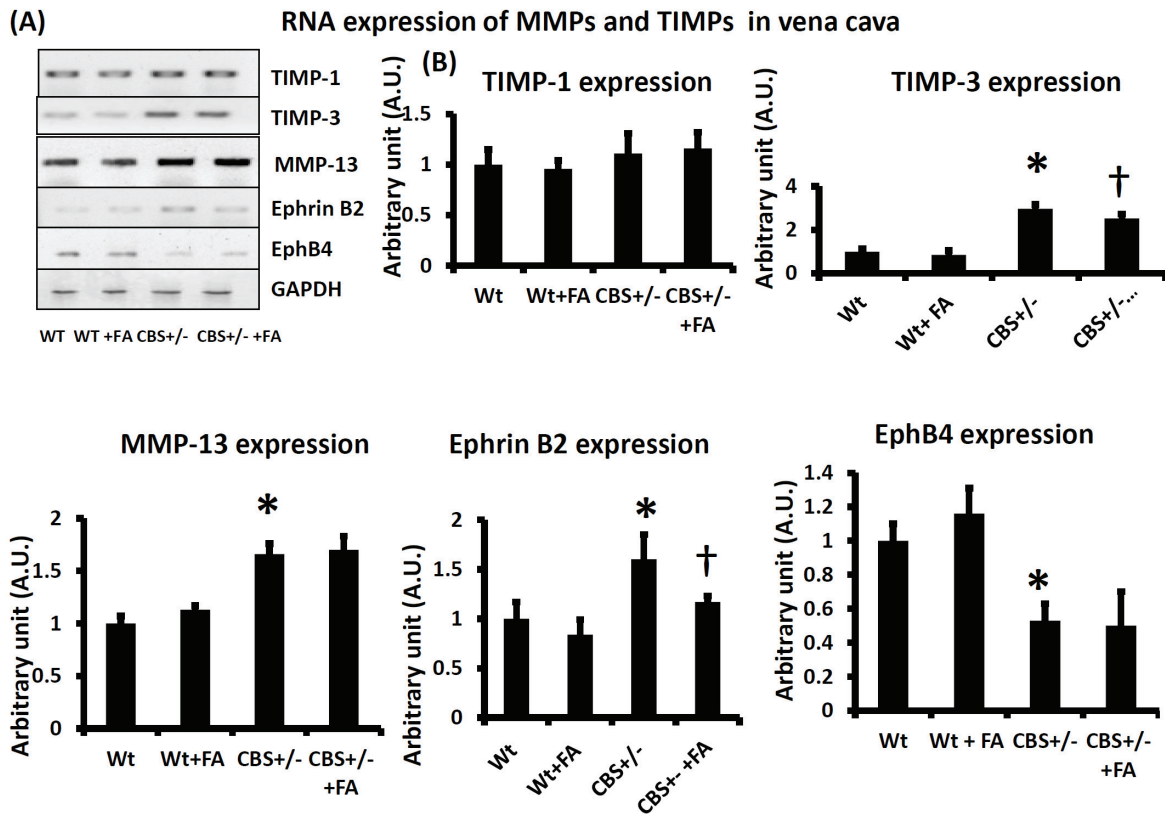
RNA expression of MMPs and TIMPs in vena cava

Figure 9. Expressions of mRNA in the vena cava. (A) Total RNA was isolated from vena cava as indicated in the methods and mRNA expressions were measured by RT-PCR. (B) The bar diagram indicated relative expression of mRNA among the samples. Data represents mean ± SD, n = 5. The * represents $p < 0.01$ vs WT and † represents $p < 0.05$ vs CBS+/-.

acid treatment partially ameliorated this mitigation by Hcy. This result suggests that Hcy played a crucial role in vena cava remodeling. Additionally, we have detected relatively higher mRNA expressions of TIMP-1 and -3 in CBS+/- vena cava; however, FA did not significantly altered expression of these TIMPs. From this result it appeared that reduction of Hcy level by FA did not have effect on TIMP-1 and -3 regulations, although high Hcy induced these two TIMPs. Whether the statistical power was enough to exclude the possibility or there may be transitional protein expressions of these TIMPs, which required FA treatment for a longer period of time needs to be investigated in future.

In addition to matrix protein, metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), a group of cell surface signaling molecules, known as ephrin family, actively participates in vascular cell assembly and proliferation of vas-

cular smooth muscle cells (VSMC); thus, contribute to the vessel remodeling. EphrinB2 and EphB4 belong to this family of cell surface signaling molecules, and known as receptor tyrosine kinase (RTK); play an important role in regulating vascular boundaries and integrity [55]. EprinnB2 is normally expressed on arterial wall, whereas its cognate receptor EphB4 dominantly expressed on venous endothelial cells [56]. Dysregulated EphrinB2 expression and signaling can result disrupted recruitment of VSMC in the vessel resulting in pathophysiological weak vessels [57-58]. Homocysteine is the only known molecule that induces apoptosis of endothelial cells and at the same time proliferate VSMC at higher levels [59-61]. In our study we have measured higher mRNA expression of EphrinB2 in CBS+/- compared to WT. In addition, venous EC marker EphB4 was low in CBS+/- vena cava (Figure 9). Although FA partially mitigated EphrinB2 in CBS+/- mice, the

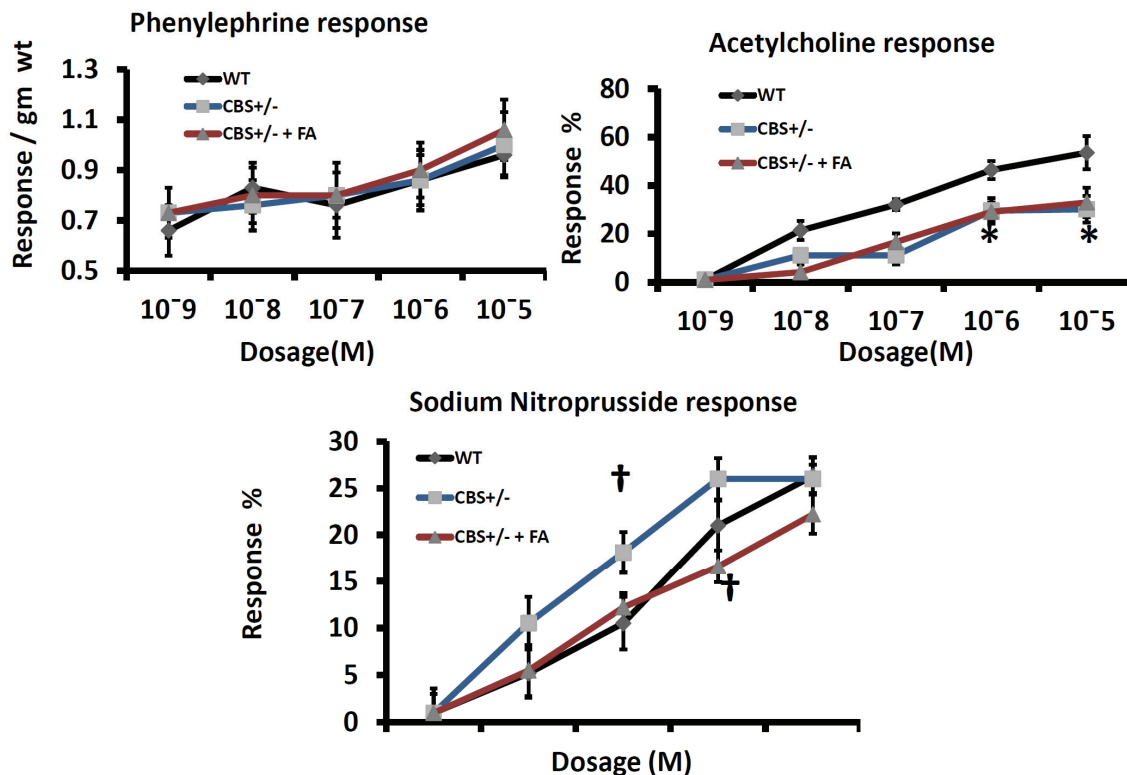## Dose response relationship of vena cava

Figure 10. Dose dependent vascular reactivity of the vena cava. The vena cava was isolated, and dose dependent responses against phenylephrine, acetylcholine and sodium nitropruside were performed as indicated in the method. Data represents mean ± SD, n = 4. The * represents $p<0.01$ vs WT and †represents $p<0.05$ vs CBS+/- mice.

expression of EphB4 did not augment to normal level (Figure 9). This result may suggest that while down regulating the venus endothelial marker EphB4, Hcy is mediating EphrinB2 expression and promoting VSMC in vena cava, which causing vascular phenotypic shift. Expression of protein levels of these two markers and detection of VSMC proliferation, if any, would have further strengthened and reinforced our hypothesis. Extracted protein from the vena cava of our animal model did not result detectable range of these two ephrin protein molecules in Western blot. Using greater amount of pooled vessels or larger animal may give further insights of our findings.

Results of vascular reactivity partially supported the hypothesis that Hcy mediates VSMC proliferation (Figure 10). In our study, CBS+/- vena cava relaxed at greater extent in endothelial-independent way (sodium nitropruside re-

sponse), which suggesting possible presence of more VSMC in CBS+/- compared to other two groups. This is a striking result, because, although FA treatment did not normalized impaired endothelial function, neither it did changed endothelial-independent vascular relaxation in CBS+/-, but increased relaxation was observed in CBS+/- mice with out FA treatment.

In conclusion, one important aspect of our study is that the expression of arterial endothelial markers, Ephrin B2 and venus endothelial marker EphB4 expressions in the vena cava is altered in the HHcy condition, which we correlate with a shift towards arterial phenotype. This is an interesting and novel finding, where HHcy contributes to vascular remodeling by phenotypic change, in addition to its well established role on imbalance of collagen/elastin and MMP/TIMP ratio.

References

[1] Munjal C, Givvimani S, Qipshidze N, Tyagi N, Falcone JC and Tyagi SC. Mesenteric vascular remodeling in hyperhomocysteinemia. Mol Cell Biochem 2011; 348: 99-108.

[2] Lim MH, Cho YI and Jeong SK. Homocysteine and pulsatility index of cerebral arteries. Stroke 2009; 40: 3216-3220.

[3] Steed MM, Tyagi N, Sen U, Schuschke DA, Joshua IG and 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 2010; 299: L301-311.

[4] He L, Zeng H, Li F, Feng J, Liu S, Liu J, Yu J, Mao J, Hong T, Chen AF, Wang X and Wang G. Homocysteine impairs coronary artery endothelial function by inhibiting tetrahydrobiopterin in patients with hyperhomocysteinemia. Am J Physiol Endocrinol Metab 2010; 299: E1061-1065.

[5] Bhalodia YS, Sheth NR, Vaghasiya JD and Jivani NP. Homocysteine-dependent endothelial dysfunction induced by renal ischemia/reperfusion injury. J Nephrol 2011;

[6] Faraci FM and Lentz SR. Hyperhomocysteinemia, oxidative stress, and cerebral vascular dysfunction. Stroke 2004; 35: 345-347.

[7] Edirimanne VE, Woo CW, Siow YL, Pierce GN, Xie JY and O K. Homocysteine stimulates NADPH oxidase-mediated superoxide production leading to endothelial dysfunction in rats. Can J Physiol Pharmacol 2007; 85: 1236-1247.

[8] Rodrigo R, Passalacqua W, Araya J, Orellana M and Rivera G. Homocysteine and essential hypertension. J Clin Pharmacol 2003; 43: 1299-1306.

[9] Sen U, Basu P, Abe OA, Givvimani S, Tyagi N, Metreveli N, Shah KS, Passmore JC and Tyagi SC. Hydrogen sulfide ameliorates hyperhomocysteinemia-associated chronic renal failure. Am J Physiol Renal Physiol 2009; 297: F410-419.

[10] Sen U, Rodriguez WE, Tyagi N, Kumar M, Kundu S and Tyagi SC. Ciglitazone, a PPARgamma agonist, ameliorates diabetic nephropathy in part through homocysteine clearance. Am J Physiol Endocrinol Metab 2008; 295: E1205-1212.

[11] Brasselet C, Durand E, Addad F, Al Haj Zen A, Smeets MB, Laurent-Maquin D, Bouthors S, Bellon G, de Kleijn D, Godeau G, Garnotel R, Gogly B and Lafont A. Collagen and elastin cross-linking: a mechanism of constrictive remodeling after arterial injury. Am J Physiol Heart Circ Physiol 2005; 289: H2228-2233.

[12] Pasterkamp G, de Kleijn DP and Borst C. Arterial remodeling in atherosclerosis, restenosis and after alteration of blood flow: potential mechanisms and clinical implications. Cardiovasc Res 2000; 45: 843-852.

[13] Adiguzel E, Ahmad PJ, Franco C and Bendeck MP. Collagens in the progression and complications of atherosclerosis. Vasc Med 2009; 14: 73-89.

[14] Bode W and Maskos K. Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases. Biol Chem 2003; 384: 863-872.

[15] Snoek-van Beurden PA and Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. Biotechniques 2005; 38: 73-83.

[16] Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J, Kinne RW, Santavirta S, Sorsa T, Lopez-Otin C and Takagi M. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis 1999; 58: 691-697.

[17] Baker AH, Edwards DR and Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002; 115: 3719-3727.

[18] Tetlow LC, Adlam DJ and Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001; 44: 585-594.

[19] Ke XD, Foucault-Bertaud A, Genovesio C, Dignat-George F, Lamy E and Charpiot P. Homocysteine modulates the proteolytic potential of human arterial smooth muscle cells through a reactive oxygen species dependant mechanism. Mol Cell Biochem 2010; 335: 203-210.

[20] Brew K, Dinakarpandian D and Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 2000; 1477: 267-283.

[21] Givvimani S, Tyagi N, Sen U, Mishra PK, Qipshidze N, Munjal C, Vacek JC, Abe OA and Tyagi SC. MMP-2/TIMP-2/TIMP-4 versus MMP-9/TIMP-3 in transition from compensatory

hypertrophy and angiogenesis to decompensatory heart failure. Arch Physiol Biochem 2010; 116: 63-72.

[22] Martinez ML, Castro MM, Rizzi E, Fernandes K, Demacq C, Bendhack LM, Gerlach RF and Tanus-Santos JE. Lercanidipine reduces matrix metalloproteinase-2 activity and reverses vascular dysfunction in renovascular hypertensive rats. Eur J Pharmacol 2008; 591: 224-230.

[23] Aitsebaomo J, Portbury AL, Schisler JC and Patterson C. Brothers and sisters: molecular insights into arterial-venous heterogeneity. Circ Res 2008; 103: 929-939.

[24] Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, Shokat KM and Stainier DY. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science 2009; 326: 294-298.

[25] Korff T, Dandekar G, Pfaff D, Fuller T, Goettsch W, Morawietz H, Schaffner F and Augustin HG. Endothelial ephrinB2 is controlled by microenvironmental determinants and associates context-dependently with CD31. Arterioscler Thromb Vasc Biol 2006; 26: 468-474.

[26] Pacilli A and Pasquinelli G. Vascular wall resident progenitor cells: a review. Exp Cell Res 2009; 315: 901-914.

[27] Rodriguez JA, Orbe J and Paramo JA. [Metalloproteases, vascular remodeling and atherothrombotic syndromes]. Rev Esp Cardiol 2007; 60: 959-967.

[28] Swift MR and Weinstein BM. Arterial-venous specification during development. Circ Res 2009; 104: 576-588.

[29] Taylor AC, Murfee WL and Peirce SM. EphB4 expression along adult rat microvascular networks: EphB4 is more than a venous specific marker. Microcirculation 2007; 14: 253-267.

[30] Obi S, Yamamoto K, Shimizu N, Kumagaya S, Masumura T, Sokabe T, Asahara T and Ando J. Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol 2009; 106: 203-211.

[31] Basu P, Sen U, Tyagi N and Tyagi SC. Blood flow interplays with elastin: collagen and MMP: TIMP ratios to maintain healthy vascular structure and function. Vasc Health Risk Manag 2010; 6: 215-228.

[32] Mishra PK, Tyagi N, Kundu S and Tyagi SC. MicroRNAs Are Involved in Homocysteine-Induced Cardiac Remodeling. Cell Biochemistry and Biophysics 2009; 55: 153-162.

[33] Chambers JC, McGregor A, Jean-Marie J, Obeid OA and Kooner JS. Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. Circulation 1999; 99: 1156-1160.

[34] Joseph J, Kennedy RH, Devi S, Wang J, Joseph L and Hauer-Jensen M. Protective role of mast cells in homocysteine-induced cardiac remodeling. Am J Physiol Heart Circ Physiol 2005; 288: H2541-2545.

[35] Demuth K, Drunat S, Girerd X, Moatti N, Paul JL, Safar M and Boutouyrie P. Homocysteine is the only plasma thiol associated with carotid artery remodeling. Atherosclerosis 2002; 165: 167-174.

[36] Sen U, Munjal C, Qipshidze N, Abe O, Gargoum R and Tyagi SC. Hydrogen sulfide regulates homocysteine-mediated glomerulosclerosis. Am J Nephrol 2010; 31: 442-455.

[37] Sen U, Mishra PK, Tyagi N and Tyagi SC. Homocysteine to hydrogen sulfide or hypertension. Cell Biochem Biophys 2010; 57: 49-58.

[38] Kluijtmans LA, Boers GH, Verbruggen B, Trijbels FJ, Novakova IR and Blom HJ. Homozygous cystathionine beta-synthase deficiency, combined with factor V Leiden or thermolabile methylenetetrahydrofolate reductase in the risk of venous thrombosis. Blood 1998; 91: 2015-2018.

[39] Beard RS, Jr. and Bearden SE. Vascular complications of cystathionine beta-synthase deficiency: future directions for homocysteine-to-hydrogen sulfide research. Am J Physiol Heart Circ Physiol 2011; 300: H13-26.

[40] Qipshidze N, Metreveli N, Lominadze D and Tyagi SC. Folic acid improves acetylcholine-induced vasoconstriction of coronary vessels isolated from hyperhomocysteinemic mice: An implication to coronary vasospasm. J Cell Physiol 2010;

[41] Toole JF, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang CH and 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.

[42] Armitage JM, Bowman L, Clarke RJ, Wallendszus K, Bulbulia R, Rahimi K, Haynes R, Parish S, Sleight P, Peto R and Collins R. Effects of homocysteine-lowering with folic acid plus vitamin B12 vs placebo on mortality and major morbidity in myocardial infarction survivors: a randomized trial. JAMA 2010; 303: 2486-2494.

[43] Ebbing M, Bleie O, Ueland PM, Nordrehaug JE, Nilsen DW, Vollset SE, Refsum H, Pedersen EK and Nygard O. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA 2008; 300: 795-804.

[44] Henderson BC and Tyagi SC. Oxidative mechanism and homeostasis of proteinase/antiproteinase in congestive heart failure. J Mol Cell Cardiol 2006; 41: 959-962.

[45] Lyons TJ. Glycation and oxidation: a role in the pathogenesis of atherosclerosis. Am J Cardiol 1993; 71: 26B-31B.

[46] Kerkela E, Ala-aho R, Klemi P, Grenman S, Shapiro SD, Kahari VM and Saarialho-Kere U. Metalloelastase (MMP-12) expression by tumour cells in squamous cell carcinoma of the vulva correlates with invasiveness, while that by macrophages predicts better outcome. J Pathol 2002; 198: 258-269.

[47] Lambert E, Dasse E, Haye B and Petitfrere E. TIMPs as multifacial proteins. Crit Rev Oncol Hematol 2004; 49: 187-198.

[48] Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest 1999; 103: 1237-1241.

[49] Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R and Pardo A. TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? Am J Physiol Lung Cell Mol Physiol 2000; 279: L562-574.

[50] Fattman CL, Gambelli F, Hoyle G, Pitt BR and Ortiz LA. Epithelial expression of TIMP-1 does not alter sensitivity to bleomycin-induced lung injury in C57BL/6 mice. Am J Physiol Lung Cell Mol Physiol 2008; 294: L572-581.

[51] Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Sci Signal 2008; 1: re6.

[52] Bond M, Murphy G, Bennett MR, Newby AC and Baker AH. Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. J Biol Chem 2002; 277: 13787-13795.

[53] Shastry S and Tyagi SC. Homocysteine induces metalloproteinase and shedding of beta -1 integrin in microvessel endothelial cells. J Cell Biochem 2004; 93: 207-213.

[54] Tyagi N, Givvimani S, Qipshidze N, Kundu S, Kapoor S, Vacek JC and Tyagi SC. Hydrogen sulfide mitigates matrix metalloproteinase-9 activity and neurovascular permeability in hyperhomocysteinemic mice. Neurochem Int 2010; 56: 301-307.

[55] Das A, Shergill U, Thakur L, Sinha S, Urrutia R, Mukhopadhyay D and Shah VH. Ephrin B2/ EphB4 pathway in hepatic stellate cells stimulates Erk-dependent VEGF production and sinusoidal endothelial cell recruitment. Am J Physiol Gastrointest Liver Physiol 2010; 298: G908-915.

[56] Huang J, Soffer SZ, Kim ES, McCrudden KW, New T, Manley CA, Middlesworth W, O'Toole K, Yamashiro DJ and Kandel JJ. Vascular remodeling marks tumors that recur during chronic suppression of angiogenesis. Mol Cancer Res 2004; 2: 36-42.

[57] Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W and Klein R. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 1999; 13: 295-306.

[58] Oike Y, Ito Y, Hamada K, Zhang XQ, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, Kisanuki YY, Yanagisawa M, Gale NW and Suda T. Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. Blood 2002; 100: 1326-1333.

[59] Tyagi SC. Homocysteine redox receptor and regulation of extracellular matrix components in vascular cells. Am J Physiol 1998; 274: C396-405.

[60] Suhara T, Fukuo K, Yasuda O, Tsubakimoto M, Takemura Y, Kawamoto H, Yokoi T, Mogi M, Kaimoto T and Ogihara T. Homocysteine enhances endothelial apoptosis via upregulation of Fas-mediated pathways. Hypertension 2004; 43: 1208-1213.

[61] Buemi M, Marino D, Di Pasquale G, Floccari F, Ruello A, Aloisi C, Corica F, Senatore M, Romeo A and Frisina N. Effects of homocysteine on proliferation, necrosis, and apoptosis of vascular smooth muscle cells in culture and influence of folic acid. Thromb Res 2001; 104: 207-213.