Introduction

Elevated levels of plasma homocysteine (Hcy) known as hyperhomocysteinemia (HHcy) cause endothelial dysfunction and vascular diseases. A connection of Hcy in vascular remodeling, structure and function, by defining their links to mitochondrial oxidative stress and nitric oxide metabolism is lacking. This study proposes a novel mechanism in which Hcy antagonizes the nuclear receptor which controls the mitochondrial oxidant and anti-oxidant balance of the cell. Also, the majority of hyperhomocysteinemia is due to the heterozygosity in CBS activity [1]. Therefore, to determine the role of Hcy in the hereditary hyperhomocysteinemia, it is innovative to use CBS-/+ and -/- mice as murine models of hyperhomocysteinemia.

Hcy induces aortic stenosis [2, 3]. Atheromas in the aortic arch and structural changes in systemic vessels contribute to hypertension. In the Hordaland Homocysteine Study of about 16,000 people 40-67 years old with no history diabetes or coronary vascular disease, the plasma Hcy levels were positively related to blood pressure [4]. Similarly, Malinow and co-workers [5] found that hypertensive men with no history of atherosclerotic disease had higher Hcy levels than non-hypertensive men. Sutton-
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Tyrrell and colleagues [6] also found a significant association of Hcy levels with systolic hypertension. Elevation of Hcy is associated with increased blood pressure in rats [7] and minipigs [8]. Hcy induces cardiac hypertrophy in rats [9]. Ventricular pressure overload studies in mice demonstrated reduced expression of PPAR during cardiac hypertrophy [9]. The administration of peroxisome proliferator reduced cardiac hypertrophy [10]. Over the past two decades, incidents related to coronary artery disease are prevented from increase, due to the fruits and vegetable diet that is low in methionine, a precursor of Hcy [11]. The administration of fruits and vegetable diet in hypertensive patients reduces systolic hypertension [12], and the agonist of PPAR decreases systolic blood pressure [13].

Hcy increases iNOS and renders eNOS ineffective and promotes formation of reactive nitrogen species (RNS) and nitrotyrosine in the vessel wall [14–17]. Previous studies from our laboratory demonstrated improved vascular function and decreased nitrotyrosine content in aortas of hyperhomocysteinemic mice treated with PPARα agonist [18]. Others showed that peroxiredoxin and thioredoxin are decreased, and NADPH oxidase is increased by oxidative stress [19–26]. Several lines of evidence suggest that SOD and catalase levels are decreased by Hcy [27–29]. PPAR agonists induce SOD and catalase [30, 31]. It is unclear, however, whether the Hcy-mediated increase in oxidative stress is, in part, due to decreases in mitochondrial peroxiredoxin, and thioredoxin and increase in NADPH oxidase, mtNOS activity, and ROS.

Hcy binds and antagonizes the peroxisome proliferators activated receptor (PPAR) α and γ. Induction of PPARα ameliorates oxidative nitrotyrosine formation, matrix metalloproteinase (MMP) activation, and endothelial dysfunction in a one copy, cystathionine β synthase (CBS-/+ ) genetic mouse model of HHCy [32]. However, the role of PPARγ in gene dose-dependent (i.e., CBS+/+, -/+ , and -/- ) HHCy-induced oxidative stress and vascular remodeling is unclear. The aim of this study was to determine the role of PPARγ in gene dose-dependent HHCy-induced oxidative stress and vascular remodeling. The hypothesis was that Hcy decreases thioredoxin, peroxiredoxin, increases NADH oxidase, mtNOS activity and reactive oxygen species (ROS) in mitochondria in a gene dose-dependent manner. ROS transduces metalloproteinase activation causing thickening (fibrosis) of the basement membrane, rendering ineffective endothelial nitric oxide synthase (eNOS) and promotes endothelial-smooth muscle disconnection/uncoupling by antagonizing PPARγ.

Materials and methods

Animals

The wild type (WT, C57BJ/L6) and a breeding pair of CBS-/+ mice (8–12 wks) were obtained from Jackson Laboratories (Bar Harbor, ME). Male and female CBS-/+ mice were cross-bred, yielding 10% CBS-/-, 60% CBS-/+ and 25% CBS+/. At 4 wks, mice were weaned, genotyped and phenotyped. Mice were grouped into WT CBS+/+, CBS-/+ , and CBS-/- mice and were also given CZ. The genotype and phenotype of offspring at the age of ~4 weeks was determined by collecting tail tissue and vein blood and analyzing: 1) genomic DNA by PCR using specific CBS primers, suggested by the supplier [18]; and 2) the levels of plasma Hcy. To avoid differences due to sex, only male mice were used and separated into 3 groups based on Hcy levels: 1) mice with Hcy levels between 3 and 6 μmole/L and one PCR product; 2) mice with Hcy levels between 8 and 15 μmole/L and two PCR products; 3) mice with Hcy levels between 28 and 30 μmole/L and one PCR product, with different electrophoretic mobility in agarose gels. Previously the levels of Hcy (<30 μM) was observed in CBS-/- mice [33]. The mice were split into six experimental groups; the control group (WT, CBS-/+ and CBS-/-) administered normal saline in drinking water and treatment group (WT, CBS-/+ and CBS-/- + CZ) administered CZ in drinking water. Each group of mice was fed normal rodent chow (Rodent Laboratory Chow) and had free access to the food and water. Each group was used to access a non-insult model (no direct injury to vascular endothelium or surgical alteration) of vascular remodeling. The genotype and phenotype information was collected from the JAX® Mice Database. The care and use of mice in the present study was done in accordance with the guidelines of the National Institute of Health Guidelines for animal research, the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine. Euthanasia was accomplished by giving an over-
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dose of anesthesia at the end of the experiment.

Ciglitazone (CZ) supplementation

To induce PPARγ, CZ (Chemicon Corp) was administered in food (50 µg/gram of rodent chow). The binding constant between CZ and PPARγ is in the micromole range [34]. Based on the assumption that mice eat 4 g/day, each will ingest ~200 µg/day of CZ. This produced a blood concentration of ~32µMole/L of CZ, which was enough to saturate most binding sites on PPARγ. To determine selectivity of CZ in the absence of Hcy, CZ was administered to control mice. To determine whether the CZ treatment causes any change in food intake, food intake was measured every 2 days during the treatment and found no difference. Because previous studies have demonstrated significant vascular dysfunction at 8 weeks of homocysteinaemia [7], we administered CZ for 12 wks to the 10-12 wks old mice. All mice were given standard mouse chow and Hcy water ad libitum. The plasma levels of Hcy were measured by HPLC [35]. At the end of the protocol, mice were deeply anesthetized with tribromoethanol (100 mg/kg i.p.). This drug has minimal effects on cardiovascular function in mice [36]. To determine plasma Hcy and to check mouse to mouse variation, 0.5 ml blood will be collected from each mouse by a catheter (PE-10) in the right common carotid artery. The heart was removed following euthanation. The thoracic aorta was excised.

Histopathology

Tissue for histology and immuno-staining was fixed in 10% zinc formalin. Mitochondria was identified using Mito-Tracker Red (Molecular Probes Corp). To determine whether changes in NOS/NO caused remodeling of vessel wall, descending thoracic aortas were embedded in tissue freezing medium (TBS, Durham, N.C.) and snap frozen in liquid nitrogen. The frozen aortas were sectioned transversely at 8-10 µm with a cryostat (Leica Cryostat 1800, Leica Microsystems, Germany), mounted onto Superfrost/Plus microscope slides (Fisher Scientific) and cover slipped. The unfixed tissue sections were stained with hematoxylin and eosin (H&E) for general tissue morphology, Trichrome for collagen and van Geisons’s stain for elastin. The images were taken with a "Q-color 3" digital camera on an IX81 inverted microscope with x100/1.35 UPlanApo objective (Olympus) and confocal microscope at different magnifications. The aortic medial thickness was measured by a micrometer. Levels of collagen and elastin were measured by colorimetric estimation of isodesmosine, desmosine, respectively as described [37].

In situ determination of ROS generation

Frozen aorta cross sections from control and Hcy treated WT were incubated with the nonfluorescent probe DCFH to determine ROS generation. To assess ROS formation in response to HHcy, oxidative stress was assessed by incubating aortic homogenates with a nonfluorescent probe 2,7'-dichlorofluorescein (DCFH). DCFH acquires fluorescence properties upon reaction with ROS and yields the fluorescent product dichlorofluorescein (DCF). This product can be detected at an emission wavelength of 530 nm (excitation wavelength of 485 nm). Briefly, aortic tissue samples were harvested and placed in cold PBS and embedded/snap frozen in tissue freezing medium (TBS, Durham, NC) for cryosectioning. Unfixed aortic segments were cut into 30µm thick sections using a Reicher-Jung cryocut 1800 cryostat and thaw mounted on Fisher Superfrost/Plus (Fisher Scientific) microscope slides. DCFH (10µmol/L) was topically applied and slides were incubated for 30 minutes in a light protected humidified chamber at 37°C. DCFH was removed and sections were cover-slipped. Images were obtained in a darkened microscopy room with a laser scanning confocal fluorescence microscope using a long pass filter (DCFH excitation at 485 nm and detection of fluorescence emission at 530 nm) Study groups and control were processed and imaged in parallel. All images were acquired using identical confocal settings for a given magnification (laser power, iris, gain, and black level). Fluorescence was detected by confocal microscopy (model FV-1000, Olympus). Images were analyzed using Matrox Inspector and presented as mean pixel intensity.

PPARγ expression in aortic nuclear extract was measured by electrophoretic mobility shift assay (EMSA) using promoter sequences that are specifically targeted to PPARγ [38] in WT (CBS+/+), CBS-/-, CBS-/+ mice treated with or without CZ. The PPARγ expression in CBS KO was decreased. The treatment with CZ normalized the
PPARγ expression.

To determine the levels of PPARγ, thioredoxin, peroxiredoxin, and NADH oxidase (NOX1), Western blot analysis on aortic tissue homogenates were performed using the respective antibodies. The specificity of antibody was established by immunoprecipitating the antigen by antibody conjugated-sepharose beads prior to loading onto the gel. This removed the bands in the gels specific for specific antigen, suggesting immunoprecipitating of antibody. The mRNA expression was measured by real time Q-RT-PCR analyses. The blots were scanned by a densitometer.

Vascular reactivity

Aortic rings were mounted in a 25 ml tissue myobath containing PSS maintained at 37°C and aerated with a 95% O₂-5% CO₂ gas mixture at pH 7.4. A total resting tension of 2.0g was applied stepwise and each ring was allowed to equilibrate for 60 minutes before dose response curves were generated by cumulative addition of α-adrenergic agonist PE. Aortas were then washed with several changes of PSS and constricted to ≈50% of maximum with PE. Relaxation dose response curves were generated by cumulative doses of endothelial-dependent bradykinin (BK) (10⁻⁹ to 10⁻³). Changes in isometric tension were recorded using a FORT 10 force transducer and an Acknowledge 3.7.3 MP 100 data acquisition system (World Precision Instruments, Inc.).

Tissue processing

At the end of the experiment, the anesthetized mice were prepared for the excision of heart, aorta, left and right kidneys and liver. Harvested tissue was weighed for comparison between groups. All harvested tissues were washed 3-4 times with cold phosphate buffered saline (PBS). The remaining supernatant fraction containing membrane and soluble proteins was used for further analysis. Protein content was determined by the Bradford method with BSA as the standard. Homogenates were stored at -80°C. A portion of the aortas were embedded in tissue freezing media for subsequent histological analysis and another portion processed for PCR. Whole blood from each animal was also collected in tubes containing a 1:10 ratio to sodium heparin, rapidly mixed, centrifuged at approximately 4000 x g and the supernatant transferred to a separate tube and frozen for further analysis.

Zymography and In situ zymography

To determine whether HHcy induces MMP-2 and -9 activity and subsequent vascular remodeling in the aorta, SDS-PAGE gelatin zymography was performed as previously described. Zymography is an electrophoretic technique used to identify proteolytic activity in enzymes separated in polyacrylamide gels under non-reducing conditions. To the aortic homogenates, 5-15 µl of non-reducing buffer was added and 15-30 µl of sample was loaded with identical amounts of total protein (30 µg) and separated in 8% polyacrylamide gels containing 2% gelatin (2 mg/ml) (Sigma). After electrophoresis, gels were washed 2-3 times for 20 minutes each in 2.5% Triton-X 100 for the removal of SDS and renaturation of MMPs. The gel was then incubated for 24 hours at 37°C in activation buffer (5mmol/L Tris HCl [pH 7.4], 0.005% (v/v) Brij-3S and 1mmol/L CaCl₂) to allow proteases to digest the surrounding substrate. Gels were stained with Coomassie blue stain (stain adheres to gelatin) until proteolytic activity was identified as clear bands on a blue background, sites of protease digestion of the gelatin substrate. The lytic bands were scanned using Gel Logic 200 image station. Band densities were normalized to control and presented as a fold change. Identity of MMPs is based on their molecular weight and confirmed by Western blot analysis. In situ zymography was performed as described [18].

RT-PCR

PCR is a method for generating unlimited copies of specific DNA or RNA fragments. Expression of glycerol aldehyde phosphate dehydrogenase (GAPDH) (housekeeping gene), NOX1, Prx-1, ET-1-A receptor, and Trx-1, mRNA were determined with reverse transcriptase-polymerase chain reaction (RT-PCR). DNA-free total RNA was isolated from thoracic aortas with TRIZol® reagent (Invitrogen) according to manufacturer’s instructions. Quantification and purity of the RNA was assessed by A260/A280 absorption (one A260 unit equals 50µg of double-stranded DNA/ml). Aliquots (2µg) of total RNA were reverse transcribed into cDNA for 5 minutes at 70°C using
1μl oligo (dT15) primers (Invitrogen) in a final volume of 5μl. To this mixture, 4μl 5× PCR reaction buffer, 4μl 5mM MgCl2, 1μl 10mM dNTPs, 0.5μl reaction Im-Prom-II™ RT and 20U of .5μl rRNasin were added for a total volume of 20μl sample. The mixture was incubated for 60 minutes at 42°C, heated to 70°C for 15 minutes, and cooled to 4°C. Sequence-specific oligonucleotide primers were prepared commercially (Invitrogen). Each PCR was performed in a final reaction volume of 20μl containing 2 μl of the cDNA, 10 μl master mix (Promega), 1.5μl forward primer, 1.5μl reverse primer and 5 μl water. PCR amplification reactions were performed with a DNA Engine peltier thermal cycler (Bio-Rad). Amplified products were resolved on a 1% agarose gel in the presence of ethidium bromide and detected under UV transillumination using the Gel Logic 200 imaging system (Kodak). The band intensities of the PCR product was analyzed with scanning densitometer software and normalized to GAPDH band intensity.

The mtNOS activity and mtNO

In fresh tissue isolated mitochondria, levels of NO was measured by an NO-electrode. Mitochondrial ROS was measured using 2, 7-dichlorofluorescein in continuous assay. Peroxides were measured by an H2O2-electrode (WPI Corp).

Statistical analysis

Statistical analysis was performed using GraphPad InStat to compare data collected from groups. Differences between groups and controls were determined by one-way analysis of variance (ANOVA). If a significant difference was indicated, the Tukey post-hoc test was used to identify groups that were significant. A probability level (p<0.05) is considered statistically significant. All values will be presented as mean ± SEM.

Results

The genotype and phenotype of CBS mice: Genotype data revealed two PCR products in heterozygote as compared to one product in WT or homozygote knockout CBS mice (Figure 1). The phenotype data revealed mild homocysteinemia in heterozygote however homozygote knockout demonstrated severe homocysteinemia. Interestingly, only CBS/+ mice revealed

![Figure 1. A. Genotype of CBS/+,-/+ and +/+ (wild type, WT) mice: Tail was clipped. The DNA was isolated and amplified using CBS mutant specific primers. A shift in mobility to lower molecular weight was observed in the disrupted DNA allele in both CSB/+- (heterozygote knockout) and CBS/-/- (homozygote knockout) mouse DNA with respective to their WT allele. PCR was performed using sequence specific primers for the WT and disrupted mutant allele. The 1.5 kb band indicates a wild type CBS gene while 0.7 kb band indicates a disrupted CBS gene. B. Phenotype of CBS knockout mice was performed by measuring plasma homocysteine levels by HPLC. Each bar represents mean±SEM from n=5. *, p<0.05 compared to WT; **, p<0.05 compared to WT or CBS/+.

Table 1. Gravimetric measurements of control wild type (WT), CBS/+,-/+, WT+CZ, CBS/++CZ and CBS/-+CZ. Body weight in grams and heart weight in mg are reported.

<table>
<thead>
<tr>
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<th>Body Weight, g</th>
<th>Heart Weight, mg</th>
<th>HW/BW, mg/g</th>
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<tbody>
<tr>
<td>WT</td>
<td>30±1</td>
<td>161±21</td>
<td>5.37±0.15</td>
</tr>
<tr>
<td>CBS/+</td>
<td>29±1</td>
<td>173±20</td>
<td>5.97±0.20*</td>
</tr>
<tr>
<td>CBS/-</td>
<td>28±1</td>
<td>140±21</td>
<td>5.00±0.15</td>
</tr>
<tr>
<td>WT+CZ</td>
<td>31±1</td>
<td>177±15</td>
<td>5.71±0.13</td>
</tr>
<tr>
<td>CBS/+ + CZ</td>
<td>30±1</td>
<td>168±15</td>
<td>5.60±0.25</td>
</tr>
<tr>
<td>CBS/- + CZ</td>
<td>28±1</td>
<td>146±15</td>
<td>5.21±0.20</td>
</tr>
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</table>

Values are mean±SEM of 10 animals for body weight and 6 animals for wet heart weight. * p <0.05 compared to WT. It is interesting to note that since CBS/- do not survive, cardiac hypertrophy was observed only in CBS/+ mice but not in CBS/- mice. The treatment with CZ mitigated this cardiac hypertrophy.
cardiac hypertrophy (Table 1).

The generation of oxygen radical in CBS-/+ mice aortas were co-labeled with mitochondria by mito-track-red and ROS by DHE-green. These data suggested that mitochondria from homocysteinemic vessels generated ROS (Figure 2).

To determine the mechanism of ROS generation we measured the levels of Nox1 (NADH oxidase subunit), thioredoxin (Trx) and peroxiredoxin (Prx) mRNA in aortas of CBS+/+, CBS-/-, and CBS-/+ mice. An increase in NADH oxidase, and decreases in thio- and peroxi-redoxins in gene-dose-dependent HHcy was observed (Figure 3A and B). In addition to determine the cellular mechanism of ROS generation the effects of Hcy on the induction of Nox1 (NADH oxidase subunit) and thioredoxin mRNA in rat cardiac microvascular endothelial cells (MVEC) were measured. The results suggested a dose-dependent induction in NOX1 gene and repression in thioredoxin genes by Hcy (Figure 3C).

To determine whether oxidative stress in CBS knockouts increases peroxinitrite generation in the mitochondria, we measured nitric oxide (NO) generation in the isolated mitochondria. The results suggested in gene-dose HHcy-dependent peroxinitrite generation in the mitochondria from WT, CBS+/+ and CBS-/- mice aortas (Figure 4).

To determine the MMP activity, we performed in situ zymography in aortic explants from CBS KO. The results suggested robust MMP activation in...
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CBS-/- mice. Interestingly, the treatment with CZ mitigated the MMP activation in CBS KO. The inner and outer elastic laminae were preserved in CZ treated mice (Figure 5).

To determine extent of vascular dysfunction in CBSKO, we measured aortic contractile function. Although aortas from WT revealed normal contractile response to endothelin-1 (ET-1) and relaxation to bradykinin (BK), aortas from CBS KO revealed paradoxic contraction to BK and decrease contractile response to ET-1 (Figure 6 A-C). Interestingly, the treatment with CZ mitigated the paradoxic contraction to BK and ET-1 response. To determine whether the decrease in ET-1 response was due in part to decrease in ET-1 receptor (ET-1 A), we measured ET-1A receptor expression in aortas from WT and CBS KO mice. The results revealed decrease in ET-1A receptor expression in CBS KO mice aortas (Figure 6D).

To determine whether the treatment with CZ has effect on survival of CBSKO mice, we estimated to survival of CBSKO mice treated with or without CZ. There was 90% morbidity in the CBS -/- mice by 12 wks. However, the treatment with CZ increased the survival of CBS-/- mice to 80% (Figure 7).

Discussion

The majority of genetic causes of homocysteinemia are due, primarily, to the heterozygosity (-/+ +) and homozygosity (-/-) in cystathionine β synthase (CBS), i.e. homocystinuria [1]. Our results show that CBS heterozygous and homozygous mice have a 3- and 8-fold increase in the levels of Hcy, respectively. Although betaine supplement increases the survival in CBS-/- [39-41], we show that PPARγ agonist (CZ) increases the survival rates in CBS-/- mice. Although it is known that PPARγ agonist increases longevity [42]. Here we demonstrated that PPARγ agonist rescues death in CBS-/- mice and other phenotype. This may support the notion that neonatal lethality in CBS-/- allele is beyond its role in Hcy metabolism [43]. Because mitochondria contribute significantly to oxidative stress, it is important to measure mitochondrial thioredoxin, peroxiredoxin and NADPH oxidase. A part of the hypothesis was that homocysteine (Hcy) de-
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Figure 4. Nitric oxide (NO) generation in the isolated mitochondria: Left panel: The mitochondria was activated with LPS. The L-arginine was added. The NO was detected using NO-sensitive electrode. To block Ca- dependent mtNOS EDTA was added. Right panel: The mitochondria without LPS activation. The bar graph represents the levels of mtNO generated by the mitochondria from WT, CBS-/+ and CBS-/- mouse aortas. Each bar represents the mean±SEM from three separate experiments. *, p <0.05 compared to WT; **, p<0.05 compared to WT or CBS-/+.

Figure 5. In situ zymography: aortic rings from CBS-/- (left panel) and CBS-/- treated with CZ (right panel, arrows indicate external and internal elastic membrane seen in CZ treated mice), WT and CBS-/+ mice treated with or without CZ were incubated on the gelatin-gel for 18 hrs, stained with commassie blue and image at 10x magnification were recoded by microscope. The bar graphs represent the lytic intensity (% MMP activity) in the gel. Each bar represents the mean±SEM from three separate experiments. *, p <0.05 compared to WT; **, p<0.05 compared to WT or CBS-/+; +, p<0.05 compared to CBS-/+ without CZ; ++, p<0.05 compared to CBS-/- without CZ. The data are representation of in situ total MMP zymography. The images for all groups were scanned and the quantification was achieved by Image-Pro software.

Increased thioredoxin (Trx), peroxiredoxin (Prx), increased NADH oxidase (NOX1), mitochondrial nitric oxide synthase (mtNOS) activity and reactive oxygen species (ROS) in mitochondria in a CBS gene dose-dependent manner. To test this hypothesis, we have modulated Hcy levels by varying CBS gene levels.

In vivo treatment with Hcy is associated with increased plasma metalloproteinase activity [7]. The treatment with an agonist of PPARα inhibited the MMP activity in aortas of CBS-/+ mice [18]. The induction of PPARγ decreases the metalloproteinase activity in macrophages [44, 45]. MMP-2 (72 kDa gelatinase a), is constitutively expressed across the species and MMP-2, as well as MMP (92 kDa gelatinase b), are induced during pathogenesis. These gelatinases are better elastases as compared to their interstitial collagenolytic activity [46]. Interestingly, 50% of the aortic protein is elastin [47] and is responsible for aortic compliance.

Treatment with the anti-oxidant nicotinamide and its derivatives alleviate Hcy-mediated endothelial-dependent vascular dysfunction [14] and hypertension [48]. The aortas of CBS-/+ mice demonstrated reduced vasodilatory response to acetylcholine [15]. Hcy increases the production of endothelin-1 (ET-1) at a pathophysiologic concentration [49], and it activates the latent MMP. MMP converts big-ET-1 to ET-1 [50]. The higher levels of Hcy synergize the ET-1-mediated contraction [17]. Normally, bradykinin (BK, EDHF-mediated) dilates the vessel via an increase in formation of EETs [51]. However, in CBS-/+ mice, bradykinin induces paradoxical vasoconstriction [15]. In addition, because prostaglandins (PGs, a precursor of EETs) activate PPARα, we hypothesize that BK-induced contraction in CBS-/+ mice is due to the increased levels of ET-1 and decreased levels of PGs, secondary to decreased PPARα activity. It is interesting that no relaxation to BK in CBS mice. We defined
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this phenomenon as paradoxic constriction and observed similar results by others [52].

To establish the cause and effect relationship of amelioration of aortic structural alteration by CZ, CBS-/- and CBS-/+ mice should be treated at birth with CZ. The longitudinal study is needed and the levels of MMP activation and elastinolysis should be measured at day 1, week 1, 2, 4, and 8 as compared to wild type control with and without CZ treatment. Treatment with ciprofibrate (an agonist to PPARα) decreases systolic blood pressure in humans [13]. Because blood pressure and vessel wall stress track together, it would be difficult to separate the alterations in blood pressure from changes in wall stress. The reduction in wall stress, vascular hypertrophy (wall thickness/diameter), and decrease in the oxidative stress level by the PPAR agonist may reduce intimal-medial thickness [53]. It is possible that CZ reduces vessel wall stress but has no effect on the levels of ROS. This may infer the direct effect of CZ on the Hcy-mediated vessel wall stress. Studies have demonstrated that acetylcholine does not generate relaxation in aortas of eNOS-/- mice [54], and that Hcy impairs endothelial-dependent vascular function [14, 15, 17]. Because CZ reverses the oxidative stress-mediated vascular dysfunction [13], we observed improved vascular function post CZ treatment in CBS mice (Figure 6). Due to an increase in ET-1 levels in HHcy, BK may induce a paradoxic contraction [55]. If PPAR and ET-A or ET-B receptors play a role in modulation of the BK effect, then the treatment with CZ or pretreatment with ET-A or ET-B blockers should reverse this paradoxic contractile effect of BK. In addition, Hcy has a direct role in smooth muscle cells contraction [56, 57].

Hcy increases production of ET-1 [49]. The results suggest that Hcy causes vascular endothelial dysfunction, and hypertension and the treatment with CZ alleviates vascular dysfunction in HHcy. Because chronic HHcy and MMP activation generate local tissue ET-1, this may desensitize the ET-1 receptor, which has an effect on contraction. Contractile response to ET-1 receptor in CBS mice is deceased in compared to WT mice. Gene expression of ET-1 receptor is also deceased in CBS mice as compared to WT shown by PCR analysis.

Hcy increases production of ET-1 [49]. The results suggest that Hcy causes vascular endothelial dysfunction, and hypertension and the treatment with CZ alleviates vascular dysfunction in HHcy. Because chronic HHcy and MMP activation generate local tissue ET-1, this may desensitize the ET-1 receptor, and the contractile response to ET-1 may be decreased in CBS mice.

Agonists of PPAR reduce oxidative stress by decreasing NADH oxidase [31, 58] and increasing SOD [30, 31]. Hcy decreases endothelial NO
and increases the production of nitrotyrosine [18]. We demonstrated a gene dose-dependent decrease in NO and increase in ROS levels in the CBS-/+ and -/- mice as compared to WT mice. Because Hcy antagonizes PPARγ, we observed a decrease in PPARγ expression in CBS-/+ and -/- mice. Although high levels of Hcy are negatively correlated with the levels of PPARγ expression, this does not necessarily suggest that the levels of Hcy will be reduced post CZ therapy. In fact, some studies suggest the contrary [59-61]. This may suggest that PPARγ activation improves Hcy-mediated vascular dysfunction, but does not affect Hcy metabolism. It is plausible, however, that the PPARγ agonist decreases oxidative stress by increasing mitochondrial redoxins and decreasing NADPH oxidase. It is known, however, that PPARγ induces SOD and cytosolic catalase [30, 31]. The reduced renal clearance in human and animals increases Hcy levels [62, 63]. Therefore, a decrease in Hcy-mediated oxidative stress by CZ may in part be due to increase in renal clearance by CZ.

Studies suggest that Hcy decreases eNOS and increases iNOS expression in MVEC [64]. CZ treatment increases NO formation and decrease ROS, which may also suggest that CZ increases eNOS and decreases iNOS expression. Furthermore, thioredoxin and peroxiredoxin will reduce oxidized-glutathione (GSSG) and neutralize ROS as well as reactive nitrogen species (ONOO-) as shown by the Equation 1 [65]:

The decrease in NADPH oxidase will reduce generation of ROS. These novel results suggest that the induction of PPARγ increases NO and decreases ROS in part by ameliorating the Hcy-effects, and increasing endothelial-smooth muscle disconnection/uncoupling by antagonizing PPARγ.
dependent models of hyperhomocysteinemia (Figure 8).

Limitations

Use of CZ to modify the phenotype of these mice is the novel component of this study. The major finding of the paper that is new in that CZ extended the lifespan of CBS-/− mice. The research using genetic manipulations (e.g., RNAi or knockout mice) to more completely define the role of PPARgamma in the context of hyperhomocysteinemia is in progress.

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