Toll-like receptor 4 mutation suppresses hyperhomocysteinemia-induced hypertension

Anastasia Familtseva, Pankaj Chaturvedi, Anuradha Kalani, Nevena Jeremic, Naira Metreveli, George H. Kunkel, and Suresh C. Tyagi

Department of Physiology, School of Medicine, University of Louisville, Louisville, Kentucky

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Familtseva A, Chaturvedi P, Kalani A, Jeremic N, Metreveli N, Kunkel GH, Tyagi SC. Toll-like receptor 4 mutation suppresses hyperhomocysteinemia-induced hypertension. Am J Physiol Cell Physiol 311: C596–C606, 2016. First published August 3, 2016; doi:10.1152/ajpcell.00088.2016.—Hyperhomocysteinemia (HHcy) has been observed to promote hypertension, but the mechanisms are unclear. Toll-like receptor 4 (TLR-4) is a cellular membrane protein that is ubiquitously expressed in all cell types of the vasculature. TLR-4 activation has been known to promote inflammation that has been associated with the pathogenesis of hypertension. In this study we hypothesize that HHcy induces hypertension by TLR-4 activation, which promotes inflammatory cytokine (IL-1β, IL-6, and TNF-α) upregulation and initiation of mitochondria-dependent apoptosis, leading to cell death and chronic vascular inflammation. To test this hypothesis, we used C57BL/6J (WT) mice, cystathionine β-synthase (CBS)-deficient (CBS<sup>−/−</sup>) mice with genetic mild HHcy, C3H/HeJ (C3H) mice with TLR-4 mutation, and mice with combined genetic HHcy and TLR-4 mutation (CBS<sup>−/−</sup>/C3H). Ultrasonography of the superior mesenteric artery (SMA) detected an increase in wall-to-lumen ratio, resistive index (RI), and pulsatility index (PI). Tail cuff blood pressure (BP) measurement revealed elevated BP in CBS<sup>−/−</sup>/C3H mice. RI, PI, and wall-to-lumen ratio of the SMA in CBS<sup>−/−</sup>/C3H mice were similar to the control group, and BP was significantly alleviated. TLR-4, IL-1β, IL-6, and TNF-α expression were upregulated in the SMA of CBS<sup>−/−</sup>/C3H mice. We conclude that HHcy promotes TLR-4-driven chronic vascular inflammation and mitochondria-mediated cell death, leading to hypertension. TLR-4 mutation attenuates vascular inflammation and cell death, which suppress hypertension.

hyperhomocysteinemia; vascular inflammation; mitochondria-mediated cell death; peripheral resistance; inward vascular remodeling

Primary, or essential, hypertension is the most common type of hypertension, with unclarified etiology that affects 95% of all hypertensive patients. It has been reported that 75 million adults in the United States and 1 billion people worldwide are affected by hypertension (48). Persistent elevation of blood pressure (BP) has been known to be a risk factor for development of stroke, myocardial infarction, and chronic kidney and vascular diseases (9, 44). A high level of homocysteine (Hcy), or hyperhomocysteinemia (HHcy), is an independent risk factor of hypertension (30, 40, 54). Hcy is a product of methionine metabolism that is cleared in the body by remethylation and transsulfuration pathways (55). Hcy remethylation is mediated by methionine synthase, where vitamin B<sub>12</sub> (cobalamin) is used as a cofactor and 5-methyltetrahydrofolate is utilized as the methyl donor (42). 5-Methyltetrahydrofolate, or the active form of folate (B<sub>9</sub>), is synthesized from 5,10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase. In transsulfuration, which occurs in the small intestine, liver, pancreas, and kidney, a cofactor, vitamin B<sub>6</sub> (pyridoxal phosphate), is required to convert Hcy to cystathionine by cystathionine β-synthase (CBS) (61). Cystathionine is hydrolyzed by vitamin B<sub>6</sub>-dependent cystathionine γ-lyase (CSE) to cysteine, which is used as a precursor for synthesis of the antioxidant glutathione or the vasodilator hydrogen sulfide (63). Nutritional deficiencies in vitamin cofactors (B<sub>12</sub>, B<sub>9</sub>, and B<sub>6</sub>) and mutations of methylenetetrahydrofolate reductase, CBS, and CSE enzymes are the common causes of HHcy (7, 20, 22, 38, 47).

HHcy-mediated vascular dysfunction and remodeling, as the hallmark of hypertension, developed as the result of a complex of mechanisms, including instigation of oxidative stress, mitochondrial apoptosis, and inflammation. Hcy undergoes autoxidation and has the ability to directly produce superoxide and hydrogen peroxide because of a highly reactive thiol group (46). The presence of nitric oxide in the oxidative environment leads to peroxynitrite formation, which further exacerbates oxidative stress, reducing nitric oxide bioavailability (59). In addition, our group and other studies have shown that, in HHcy, there is an increase in expression and activity of NADPH oxidase (NOX) that mediates superoxide production (17, 21). Elevation of the level of Hcy disrupts the oxidant-antioxidant balance by diminishing the activity and expression of the antioxidant enzyme glutathione peroxidase (3). Apart from oxidative stress, HHcy plays a role in the instigation of mitochondrial disorders.

Mitochondrial dysfunction and mitochondria-dependent apoptosis have been shown to promote endothelial cell loss, leading to endothelial dysfunction (1, 57), which contributes to the pathogenesis of hypertension (15, 18). The intrinsic pathway, or mitochondria-dependent apoptosis (27, 62), is triggered by various factors, reactive oxygen species (ROS), DNA damage, Ca<sup>2+</sup> overload, hypoxia, and oxidized LDL, and is regulated by a B cell lymphoma 2 (BCL-2) family of proteins, which are classified as proapoptotic (BAX) and antiapoptotic (BCL-2) proteins. BAX is expressed in the cytosol and, upon activation, is translocated to mitochondria, where it initiates mitochondria outer membrane permeabilization followed by cytochrome c release and apoptosome (cytochrome c-apoptotic protease-activating factor 1 complex) formation, which plays a role in caspase-9 and caspase-3 activation. The effector caspase-3 facilitates DNA and protein fragmentation, which promotes cell death. The third event mediated by HHcy, in addition to oxidative stress and mitochondrial dysfunction, is vascular inflammation.
Chronic vascular inflammation has been considered a mechanism of the initiation and exacerbation of hypertension (25, 35, 52). Inflammation is the immediate response of the immune system to the presence of pathogens, which is characterized by augmentation of proinflammatory cytokine (IL-1β, IL-6, and TNF-α) and chemokine [monocyte chemoattractant protein 1 (MCP-1)] secretion, which regulates the immune response and the migration of immune cells to target organs. Several in vitro studies have shown upregulation of inflammatory markers, including activation of NF-κB, an inflammatory cytokine transcription factor, in HHcy (11, 29). Zhang et al. reported that plasma IL-6, TNF-α, and MCP-1 levels are positively correlated with Hcy levels (66). Scherer et al. also observed mild HHcy-mediated augmentation of inflammatory cytokine (IL-1β, IL-6, TNF-α, and MCP-1) production in serum and different organs (51). Xia et al. showed HHcy-induced inflammation and mitochondria-dependent cell death. Although HHcy is known to promote proinflammatory cytokine elevation and attenuate hypertension (8, 13). Therefore, TLR-4 inhibition has been shown to reduce inflammatory response initiation remains unclarified. Pathogen recognition receptors and, in particular, Toll-like receptors (TLRs) are the antigen sensors that play a role in innate immune system activation and have recently gained significant attention in the field of hypertension. Thirteen TLRs have been described in mammals (TLR-1 to -10 in humans and TLR-11 to -13 in mice); cell surface TLRs that sense the presence of bacteria and fungi (TLR-1, -2, -4, and -5) and TLRs that are localized to intracellular membranes and recognize viral or microbial nucleic acid (TLR-3, -7, -8, and -9) (35). The role of TLR-4, which is ubiquitous within the vasculature (endothelial cells and vascular smooth muscle cells), has been recently highlighted in sterile inflammation. TLR-4 activation with downstream NF-κB-mediated cytokine upregulation has been implicated in the pathogenesis of hypertension (14, 36). Hence, TLR-4 inhibition has been shown to reduce inflammatory cytokine elevation and attenuate hypertension (8, 13). Therefore, we hypothesize that HHcy induces hypertension by TLR-4 activation, which promotes inflammatory cytokine (IL-1β, IL-6, and TNF-α) upregulation and initiation of mitochondria-dependent apoptosis, leading to cell death and chronic vascular inflammation. In addition, we aimed to elucidate the role of TLR-4 mutation in attenuation of HHcy-mediated vascular inflammation and mitochondria-dependent cell death.

**MATERIALS AND METHODS**

**Animal models.** C57BL/6J [wild-type (WT)] mice, CBS-deficient (CBS+/−) mice with genetic mild HHcy, mice with mutation of TLR-4 [C3H/HeJ (C3H)], and mice with combined genetic HHcy and TLR-4 mutation (CBS+/−/C3H) were used in the present study. The animals were 13–14 wk old; their body weight was 25–30 g. The mice were purchased from Jackson Laboratory (Bar Harbor, ME). All standard procedures and experiments involving animals conformed with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

**Genotyping.** The background of CBS+/− heterozygous mice was confirmed according to the Jackson Laboratory protocol for genotyping. Briefly, samples from the tails of the mice were collected for DNA extraction. DNA was used to amplify with CBS primer sets by PCR. The PCR product samples were run on 1.2% agarose gel (prepared in Tris base, acetic acid, and EDTA (TAE buffer), pH 8.4) with ethidium bromide. The images were recorded in a gel documentation system (Bio-Rad, Hercules, CA). To confirm TLR-4 mutation in C3H/HeJ mice, the respective protocol of restriction fragment length polymorphism (RFLP)-PCR was used (31). Briefly, DNA extracted from the tails of the animals was amplified with a specific TLR-4 set of primers (Table 1). PCR products were digested with NlaIII restriction enzyme (RE) overnight at 37°C. The RE digestion products were loaded on a 10% polyacrylamide gel and run at 80 V. The gel was incubated with 1× TAE buffer containing ethidium bromide. The images were made in a gel documentation system (Bio-Rad). The mutant TLR-4 gene includes the CATG sequence, which is cut by the RE, yielding 96- and 108-bp bands, while the absence of the TLR-4 mutation is confirmed by undigested products at 204 bp. To confirm the genetic background of CBS+/−/C3H mice, we first determined CBS gene deficiency in DNA samples that were selected for further detection of TLR-4 mutation.

**BP measurement.** BP was recorded using a noninvasive tail cuff method (CODA, Kent Scientific, Torrington, CT). Prior to BP measurements, the animals were placed in the restraining chambers on a warm platform for 30 min for 3 consecutive days to ensure that they adapted to the procedure. BP was recorded in a proper environment (room temperature, lighting, and noise-free atmosphere) for 4 groups, with 10 animals in each group.

**Vascular ultrasonography.** Ultrasound of the superior mesenteric artery (SMA) was performed with the Vevo 2100 system (Visual Sonics, Toronto, ON, Canada) under isoflurane anesthesia. Physiological parameters (heart rate and respiratory rate) were monitored during the procedure. The mouse was placed on a warmed (37°C) platform in the supine position, and the abdominal area was depilated. Imaging was performed using acousting gel (Other-Sonic, Pharmaceutical Innovations, Newark, NJ), which was applied to the skin in the abdominal area, and a 13- to 24-MHz transducer (model MS550D, Vevo). To calculate wall-to-lumen ratio, wall thickness and lumen diameter were measured using SMA images in B mode. The SMA resistive index (RI) was calculated as RI = (PSV − EDV)/PSV, with peak systolic velocities (PSVs) and end-diastolic velocities (EDVs) measured in pulsed-wave Doppler mode. The SMA pulsatility index (PI) was measured in pulsed-wave Doppler mode and calculated as PI = (PSV − EDV)/MV, where MV is mean velocity. Ultrasound of the SMA was performed for four groups, with five animals in each group.

<table>
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<th>Table 1. Sequence-specific oligonucleotide primers</th>
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<tr>
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<td>BAX</td>
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Quantitative RT-PCR. To assess mRNA expression of different genes in the SMA, RNA was isolated with TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. RNA quantification and purity were assessed by spectrophotometry (Nanodrop 1000, Thermo Scientific, Waltham, MA). Aliquots (2 μg) of total RNA were reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was performed for different genes (BAX, IL-1β, IL-6, and TNF-α) in a final reaction volume of 20 μL containing 10 μL of PerfeCTa SYBR Green supermix (Quanta Biosciences, Gaithersburg, MD), 6 μL of nuclease-free water, 2 μL of cDNA, and 40 pmol of forward and reverse primers. All sequence-specific oligonucleotide primers (Invitrogen, Carlsbad, CA) are presented in Table 1. Data are represented as fold expression, calculated as the cycle threshold difference between control and sample normalized with the housekeeping gene Rn18s.

Western blot analysis. SMA protein content was extracted using RIPA buffer (Boston BioProducts, Ashland, MA), PMSF (Calbiochem, La Jolla, CA), and protease inhibitor (Sigma Aldrich, St. Louis, MO). The protein extract was incubated overnight at 4°C with shaking and centrifuged at 13,000 rpm for 20 min. The supernatant was collected in another tube for protein estimation using the Bradford dye reagent (Bio-Rad, Hercules, CA). 6 μL of nuclease-free water, 2 μL of cDNA, and 40 pmol of forward and reverse primers. All sequence-specific oligonucleotide primers (Invitrogen, Carlsbad, CA) are presented in Table 1. Data are represented as fold expression, calculated as the cycle threshold difference between control and sample normalized with the housekeeping gene Rn18s.

Immunohistochemistry. SMA tissue was immersed in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) in a disposable plastic tissue-embedding mold (Polysciences, Warrington, PA). The tissue blocks were kept at −80°C until they were cut into 5-μm-thick sections (Cryocut, model CM 1850, Leica Microsystems, Buffalo Grove, IL). Tissue sections were placed on polyllysin-coated slides (Polysciences), incubated with permeabilization solution (0.2 g of bovine serum albumin and 3 μL of Triton X-100 in 10 mL of 1× PBS) for 1 h at room temperature, and then washed with 1× PBS. The sections were incubated with a 1:250 dilution of primary antibody (Table 2) overnight at 4°C. After the slides were washed with 1× PBS, they were incubated with a 1:500 dilution of fluorescently labeled secondary antibodies (goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Texas Red, Invitrogen, Waltham, MA) for 1 h at room temperature and then stained with a 1:10,000 dilution of 4’,6-diamino-2-phenylindole (DAPI) for 20 min at room temperature. After they were washed, the slides were mounted with mounting medium and visualized using a laser scanning confocal microscope (Fluo View 1000, Olympus, Center Valley, PA). The images were analyzed by measurement of fluorescence intensity with Image-Pro Plus software (Media Cybernetics, Rockville, MD).

TUNEL assay. The DeadEnd fluorometric terminal deoxynucleotidyl transferase dUTP nick-end-labeling (TUNEL) system (Promega, Madison, WI) measures nuclear DNA fragmentation, which is an important biochemical hallmark of apoptosis. The TUNEL system detects fragmented DNA by catalytically binding fluorescein-12-dUTP to 3’-OH DNA ends using recombinant terminal deoxynucleotidyl transferase. The TUNEL assay was performed on frozen SMA tissue sections using a commercially available kit (DeadEnd fluorometric TUNEL system). The assay was done according to the manufacturer’s protocol for tissue staining, including a positive control preparation and nuclear staining with DAPI. The slides were visualized with a confocal microscope (Fluo View 1000, Olympus) using a green fluorescence filter to detect DNA fragmentation and a blue DAPI filter to detect the nucleus. The images were analyzed with Image-Pro Plus software (Media Cybernetics).

Statistical analysis. Statistical analysis was performed with Primer of Biostatistics 7.0 (McGraw-Hill). Significance was determined by one-way analysis of variance followed by Holm’s multiple-comparison test between the groups. The difference was considered significant when P < 0.05. Values are means ± SE (n ≥ 4).

RESULTS

Genotyping for WT, CBS+/−, C3H, and CBS+/−/C3H mice. For genotype analysis, the Jackson Laboratory protocol was followed. CBS+/− and CBS+/−/C3H mice had two bands at 450 and 308 bp, while CBS+/+ mice had a single band at 308 bp, when primers specific for the CBS gene were used (Fig. 1A). TLR-4 mutant (C3H) and CBS+/−/C3H mice had bands at 96 and 108 bp, whereas nonmutant (WT and CBS+/−) mice had a single band at 204 bp (Fig. 1B), when RFLP-PCR was used with TLR-4 primers.

TLR-4 mutation suppresses HHcy-mediated hypertension. Systolic, diastolic, and mean BP were significantly higher in HHcy (CBS+/−) than WT mice (Fig. 2). Systolic, diastolic, and mean BP were significantly lower in TLR-4-mutant (C3H) than

Table 2. Primary antibodies

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<tr>
<td>Caspase-3 (cleaved)</td>
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<tr>
<td>BAX</td>
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<td>TLR-4</td>
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<td>β-Actin</td>
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Fig. 1. A: genotyping for the cystathionine β-synthase (CBS) gene. CBS+/− and CBS+/−/C3H mice had 2 bands, at 450 and 308 bp; wild-type (WT) mice had 1 band, at 308 bp. B: restriction fragment length polymorphism-PCR for the Toll-like receptor 4 (TLR-4) gene. TLR-4 mutant (C3H and CBS+/−/C3H) mice had 2 bands, at 96 and 108 bp; nonmutant (WT and CBS+/−) mice had 1 band, at 204 bp.
expression were elevated in the SMA of CBS (Fig. 5). IHC showed increased intensity of TLR-4 and TNF-α in the SMA of mice with combined genetic HHcy and TLR-4 mutation (C3H) compared with WT and C3H mice (Fig. 6). These results suggest that induction of mitochondria-dependent apoptosis in HHcy and TLR-4 mutation alleviates mitochondria-mediated apoptosis.

**TLR-4 mutation mitigates HHcy-induced DNA fragmentation.** TUNEL assay was used to evaluate DNA fragmentation in the SMA of different groups (Fig. 7). DNA fragmentation was significantly augmented in the SMA of CBS +/- mice compared with WT and C3H mice (Fig. 7). TUNEL-positive cell count was reduced in the SMA of CBS +/-/C3H mice compared with CBS +/- mice (Fig. 7).

**DISCUSSION**

Elevated plasma Hcy has been shown to be a risk factor for peripheral arterial disease and hypertension (30, 33, 38, 65). In our previous work we showed that HHcy induced endothelial cell injury and peripheral vascular remodeling with collagen deposition in the SMA (21). Consistent with our findings, several studies reported that HHcy promotes endothelial cell injury, vascular endothelial dysfunction, and vascular remodeling, which contribute to the pathogenesis of hypertension (34, 41, 50). The elevation of total peripheral resistance that is attributed to alterations in structural and physical properties of the resistance arteries is the hallmark of primary, or essential, hypertension. In the present study, using ultrasonography, we detected an increase in SMA wall-to-lumen ratio, RI, and PI in CBS +/-/C3H mice, which indicates inward vascular remodeling and an increase in peripheral resistance due to HHcy. The increase in peripheral vascular resistance has been associated with raised systolic, diastolic, and mean arterial BP in CBS +/- mice. RI, PI, and wall-to-lumen ratio of the SMA in mice with combined genetic HHcy and TLR-4 mutation (CBS +/-/C3H) were similar to the control group, which could explain attenuation of HHcy-mediated high BP.

A significant number of studies have described inflammation as one of the toxic effects of Hcy. Zhang et al. reported that plasma Hcy was positively correlated with plasma proinflammatory cytokine and chemokine (IL-6, TNF-α, and MCP-1) levels and promoted inflammatory monocyte differentiation (66). Zanin et al. showed that HHcy is involved in the synthesis and secretion of IL-1β via NF-kB in murine macrophages (64). Han et al. observed endothelial cell inflammatory injury through activation of NF-kB and cytokine IL-6 upregulation in HHcy (23). In agreement with previous findings, we found an upregulation of proinflammatory cytokine (IL-1β, IL-6, and TNF-α) expression in the SMA of CBS +/- mice, confirming chronic vascular inflammation induced by genetic mild HHcy. Interestingly, we observed an active presence of IL-1β in the SMA tissue of mice with the TLR-4 mutation (C3H) that could be explained by potential involvement of other Toll-like/IL-1 receptors. However, we have found that other cytokines (IL-6 and TNF-α) are reduced in the SMA tissue of C3H mice, suggesting that TLR-4 mutation attenuates vascular inflammation. In addition, we observed that mice with combined genetic HHcy and TLR-4 mutation exhibit reduced levels of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in the SMA compared with CBS +/- mice, further indicating that TLR-4 mutation prevents HHcy-induced chronic vascular inflammation.
tion. The role of the TLR-4-mediated signaling pathway has been recently highlighted in the pathophysiology of several cardiovascular diseases, including hypertension (5, 16, 19, 24). Pryshchep et al. observed the abundant expression of TLR-4 in all cell types of six different vascular beds (aorta, carotid, temporal, subclavian, iliac, and mesenteric arteries). It was confirmed that TLR-4 is expressed in endothelial and vascular smooth muscle cells in atherosclerosis, while in normal, non-inflamed arteries, adventitial dendritic cells are the major sensors of pathogen-related motifs (45). McCarthy and Webb described the role of the interaction of musculoskeletal injury-induced endothelial TLR-4 and damage-associated molecular pattern molecules (high-mobility group box 1 and mitochondrial DNA) in the development of hypertension in football players (36). Dange et al. reported increased blood and brain TLR-4, TNF-α, and IL-1β expression in angiotensin II-induced hypertension and also showed that central blockade of TLR-4 delayed the progression of hypertension and improved cardiac function in hypertensive rats (12). Bobek et al. observed that TNF-α-infused mice developed proteinuric hypertension similar to human preeclampsia that was accompanied by upregulation of TLR-4 protein expression in the placenta (6). Li et al. reported TLR-4-induced matrix metalloproteinase (MMP)-9 elevation in human aortic smooth muscle cells and confirmed that TLR-4 siRNA silencing regulated MMP-9 expression, indicating the role of TLR-4-mediated signaling pathway in vascular remodeling (28). Balistreri et al., for the first time, described a rs4986790 TLR-4 polymorphism that confers

Fig. 3. A: wall-to-lumen ratio. To assess structural changes in the superior mesenteric artery (SMA), wall thickness and lumen diameter were measured and the SMA wall-to-lumen ratio was calculated. The SMA wall-to-lumen ratio was increased in CBS+/−/C3H mice compared with WT and C3H mice. The SMA wall-to-lumen ratio of CBS+/−/C3H mice was similar to the control group. Values are means ± SE (n = 5). *P < 0.05 vs. WT. #P < 0.05 vs. CBS+/−.

B: pulsatility index (PI) and resistive index (RI) of the SMA. PI and RI are calculated from blood flow velocities in the SMA during the cardiac cycle and used to determine a peripheral resistance. PI and RI of the SMA were increased in CBS+/− mice compared with WT and C3H mice. SMA PI and RI were similar to controls in mice with combined genetic HHcy and TLR-4 mutation. Values are means ± SE (n = 5). *P < 0.05 vs. WT. #P < 0.05 vs. CBS+/−.
a higher susceptibility for sporadic thoracic aorta aneurism and, together with rs1799752 angiotensin-converting enzyme, rs3918242 MMP-9, and rs2285053 MMP-2 single-nucleotide polymorphisms, is an independent risk factor for sporadic thoracic aorta aneurism. In addition, they observed that the cases with combined risk genotype showed higher levels of inflammatory mediators and plasma MMP-9 and MMP-2 levels that were accompanied by elastic fragmentation in tissue aorta samples (4, 49). In previous work we reported TLR-4, IL-1β, IL-6, and TNF-α upregulation in heart tissues of CBS+/−/C3H mice with genetic mild HHcy. The inflammatory markers were further increased when mice were fed a high-methionine diet, which exacerbated HHcy (10). In agreement with previous studies, we found upregulation of TLR-4 expression in the SMA of CBS+/−/C3H mice that connects the downstream inflammatory cytokine signaling pathway activation in mild HHcy. Mice with combined genetic HHcy and TLR-4 mutation exhibit less TLR-4 expression than CBS+/−/C3H mice, preventing downstream inflammatory cytokine elevation. Mitochondria-dependent apoptosis has been implicated in the induction of vascular remodeling that contributes to pathogenesis of hypertension (37, 56, 67). A significant number of studies have described the role of HHcy in the initiation of mitochondria-dependent apoptosis through activation of ROS. Kim et al. showed that Hcy treatment of primary human bone marrow stromal cells led to activation of ROS, caspase-9, caspase-3, and cytochrome c release into the cytosol from mitochondria, suggesting that a mitochondria-initiated cell death pathway is a predominant mechanism of apoptosis in HHcy (26). Sipkens et al. observed a NOX2, NOX4, and Hcy concentration-dependent increase in caspase-3 in human umbilical vein endothelial cells (53). Moreira et al. reported that Hcy increases superoxide levels and cell death in human aortic endothelial cells, but vitamin B12 supplementation prevents oxidative stress-induced cell death (39). However, some studies have been stressing an inflammatory component as the critical mechanism of mitochondria-mediated cell death. Pan et al. reported that the transcription factor NF-κB regulates the expression of both proinflammatory genes and genes that contribute to mitochondria-dependent apoptosis (the BCL-2 gene family) (43). It has been implied that NF-κB activation of NF-κB occurs prior to DNA fragmentation and is accompanied by upregulation of proapoptotic proteins (BAX) (32, 58). Aoki et al. reported that NF-κB promotes endothelial cell death
Fig. 6. A: quantitative RT-PCR for the BAX gene. BAX mRNA expression was significantly upregulated in CBS+/+ mice compared with other groups. BAX mRNA expression was reduced in CBS-deficient mice with TLR-4 mutation (CBS+-/C3H) compared with CBS+/+ mice. Values are means ± SE (n = 4). *P < 0.05 vs. WT. #P < 0.05 vs. CBS+/+.

B: Western blotting for BAX protein expression. BAX protein expression was increased in CBS+/+ mice compared with the other groups. BAX protein expression was reduced in CBS-deficient mice with TLR-4 mutation (CBS+-/C3H) compared with CBS+/+ mice.

C: Western blot for caspase-9 protein expression. Caspase-9 protein expression was increased in HHcy mice (CBS+/+) and CBS+-/C3H mice compared with WT and TLR-4 mutant (C3H) mice. Values are means ± SE (n = 6). *P < 0.05, CBS+/+ vs. C3H. #P < 0.05, C3H vs. CBS+-/C3H.

D: IHC showed elevated levels of cleaved caspase-3 in the SMA of CBS+/+ mice. Intensity of cleaved caspase-3 was reduced in the SMA of CBS+-/C3H mice compared with CBS+/+ mice. Values are means ± SE (n = 4). *P < 0.05 vs. WT. #P < 0.05 vs. CBS+/+. 
through translocation of BAX to mitochondria and downregulation of Bcl-2 (2). In the present study we found an upregulation of BAX, caspase-9, and caspase-3 expression in the SMA of CBS/H11001/H11002 mice with mild HHcy compared with controls. Mice with TLR-4 mutation alone expressed less BAX, caspase-9, and cleaved caspase-3 in the SMA. Mice with combined genetic HHcy and TLR-4 mutation exhibited downregulation of BAX and cleaved caspase-3 expression in the SMA. In addition, DNA fragmentation in the SMA was increased in the mouse model of mild HHcy, but mice with TLR-4 mutation (C3H and CBS/H11001/H11002/C3H) showed decreased DNA fragmentation in the SMA. These findings

![Fig. 7. TLR-4 mutation mitigates HHcy-induced DNA fragmentation. TUNEL assay was used to evaluate DNA fragmentation (arrows) in the SMA. DNA fragmentation was significantly augmented in the SMA of CBS/+ mice compared with WT and C3H mice. TUNEL-positive cell count was reduced in the SMA of CBS/+ /C3H mice compared with CBS/+ mice. ctr, Control. Values are means ± SE (n = 4). *P < 0.05 vs. WT. #P < 0.05 vs. CBS/+.

Fig. 8. Schematic representation of the hypothesis. HHcy induces hypertension by TLR-4 activation followed by inflammatory cytokine (IL-1β, IL-6, and TNF-α) elevation and initiation of mitochondria-dependent apoptosis, which lead to cell death and chronic vascular inflammation. TLR-4 mutation attenuates vascular inflammation and cell death, which suppress hypertension. ΔΨm, mitochondrial membrane potential; Apaf-1, apoptotic protease-activating factor 1.
suggest that the TLR-4-driven inflammatory signaling pathway contributes to initiation of mitochondria-mediated cell death; thus TLR-4 mutation plays a role in alleviation of mitochondrial apoptosis.

**Summary.** Our study demonstrates that HHcy induces hypertension by TLR-4 activation followed by inflammatory cytokine elevation and initiation of mitochondrial apoptosis, which lead to cell death and chronic vascular inflammation (Fig. 8). TLR-4 mutation attenuates vascular inflammation and cell death, which suppress hypertension.

**Conclusions and perspectives.** Experimental studies have demonstrated that elevated plasma Hcy is a risk factor for peripheral artery disease and hypertension. However, the precise mechanism of detrimental Hcy interaction with the vascular wall is incompletely understood. In the present study we have described different aspects of Hcy action in the development of peripheral vascular remodeling, which is a hallmark of hypertension. Our study, for the first time, has illustrated that Hcy acts through TLR-4, which is highly expressed in all cell types of the vascular bed. HHcy-mediated TLR-4 activation promotes chronic inflammatory signaling with proinflammatory cytokine elevation, facilitating mitochondria-mediated cell death, which favors inward vascular remodeling. We have also demonstrated that TLR-4 mutation attenuated chronic vascular inflammation and mitochondria-induced cell injury, which prevented peripheral vascular remodeling and suppressed hypertension. Several experimental studies have illustrated the promising effect of TLR-4-targeted therapy in prevention of hypertension. However, further studies are required for detailed investigation of the role of the TLR-4-mediated signaling pathway in the pathogenesis of hypertension and other cardiovascular diseases. Future studies that will elucidate the mechanisms that underlie the TLR-4-mediated inflammatory pathway may offer novel concepts for hypertension therapy.

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**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

A.F. and S.C.T. developed the concept and designed the research; A.F., N.J., and N.M. performed the experiments; A.F. analyzed the data; A.F., P.C., A.K., S.C.T. edited and revised the manuscript; S.C.T. approved the final version of the manuscript.

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