Cystathionine β-synthase and cystathionine γ-lyase double gene transfer ameliorate homocysteine-mediated mesangial inflammation through hydrogen sulfide generation

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Homocysteine (Hcy) is a potent antioxidant, vasorelaxant, and antihypertensive agent, was decreased in the kidney of another model of HHcy, the uninephrectomized cystathionine β-synthase heterozygous (CBS+/–) mice (36). In these animals, decreased endogenous H2S production associated with decreased CSE activity. Supplementation with H2S resulted in increased CSE activity.

Several laboratories, including our own, have shown that Hcy induces glomerular injury (34, 56), in part mediated through the induction of inflammatory molecules (36). In glomerular injury, similar to other injuries, circulating monocytes through upregulated inflammatory molecules adhere to the glomerular vessel wall, roll and migrate to the site of injury. This is a physiological defense mechanism and migrated monocytes through a sequence of events modulate extracellular matrix, resulting in matrix deposition and glomerulosclerosis in the glomerulus. This ultimately causes chronic kidney disease (CKD) and leads to declined renal function (4). Cytokine- and chemokine-mediated inflammation is a major contributory factor in the development and progression of CKD. Clinical evidence show that elevated Hcy levels in CKD patients have been linked to the inflammatory state associated with CKD (31, 53). Specifically, Hcy induces the expression of cytokines, such as monocyte chemotactrant protein-1 (MCP-1) and chemokines, such as macrophage inflammatory protein-2 (MIP-2), in cultured mesangial cells (4, 40). The mechanism of such induction, however, is not well understood.

Multiple other factors are also involved in the inflammation associated with CKD, such as oxidative stress (32), chronic kidney disease; monocyte chemoattractant protein-1; macrophage inflammatory protein-2; extracellular signal-regulated kinase1/2; c-Jun NH2-terminal kinases/stress-activated protein kinase 1/2.

CARDIOVASCULAR DISEASE is the major cause of morbidity and mortality in patients with chronic kidney disease and end-stage renal disease. A number of prospective observational studies have shown that hyperhomocysteinemia (HHcy), an elevated homocysteine (Hcy) level, is associated with vascular morbidity and mortality (6, 54). However, randomized clinical trials designed to lower Hcy levels in patients with cardiovascular disease have failed to show any clinical benefit despite a demonstrable reduction in serum Hcy levels (15, 27, 46). While the reasons for the failure of the trials are not known, one major limitation in these trials was the failure to demonstrate reduced tissue levels of Hcy. Folic acid, the treatment used to reduce Hcy in most trials, may in fact promote tissue uptake of Hcy. This seemingly paradoxical effect has been demonstrated with other hormones and growth factors, such as insulin (11), which decreases plasma levels of amino acids by increasing tissue uptake. Previously, we demonstrated elevated tissue levels of Hcy in the kidney (37) and heart tissue (33) of hyperhomocysteinemic mice with alloxan-induced diabetes mellitus. We also demonstrated that the level of cystathione γ-lyase (CSE), an enzyme responsible for conversion of Hcy to H2S, a potent antioxidant, vasorelaxant, and antihypertensive agent, was decreased in the kidney of another model of HHcy, the uninephrectomized cystathionine β-synthase heterozygous (CBS+/–) mice (36). In these animals, decreased endogenous H2S production associated with decreased CSE activity. Supplementation with H2S resulted in increased CSE activity.

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hyperleptinemia (28), C-reactive protein (CRP) (44), nitric oxide (NO) (23), and carbon monoxide (CO) (13) among others. Recently, hydrogen sulfide (H₂S), an endogenous gaseous molecule, has been identified as a major regulator of inflammatory responses (22, 26). We have previously shown that H₂S prevents HHcy-associated renal damage (34) and regulates glomerular matrix remodeling and inflammation during HHcy (36). Others have reported protective effects of H₂S on renal function and inflammation (2). Under normal physiological conditions Hcy is metabolized by two pyridoxal-5'-phosphate-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (1, 17), and one pyridoxal-5'-phosphate-independent enzyme 3-mercaptopropionate sulfurtransferase (3-MST) (41–42) to produce H₂S. Although Hcy is one of the precursors of H₂S and high levels of Hcy seem to promote H₂S generation, Chang et al. (3) have reported that HHcy inhibited CSE in myocardial tissue resulting in decreased H₂S production. In accordance with their finding, our recent report (36) suggests that CSE expression was also attenuated in the renal cortical tissue of HHcy mice. These previous findings led us to hypothesize that CBS and CSE gene therapy may protect renal tissue from Hcy-mediated injury and inflammation through promoting H₂S generation during HHcy. Also, signaling mechanism of this pathway may provide further insight to modulate HHcy-associated renal inflammatory disease processes. Our in vitro data suggest that HHCy causes upregulation of inflammatory molecules MCP-1 and MIP-2 in kidney mesangial cells (MCs) through attenuated H₂S generation. Overexpression of CBS/CSE gene mitigates these inflammatory molecules in MCs by increasing H₂S generation through extracellular signal-regulated kinase 1/2 (ERK1/2)- and c-Jun NH²-terminal kinase (JNK1/2)-dependent pathways.

MATERIALS AND METHODS

Cell culture. Mouse kidney glomerular MCs of 7- to 10-wk-old mice were purchased from ATCC (Manassas, VA). We used mouse cells, instead of human cells, to maintain consistency of our experimental model (34, 36). These cells were cultured and maintained in DMEM/F-12 (50/50) medium containing 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics (Mediatech, Herndon, VA). T-25 flasks were kept in a humid chamber at 37°C in an atmosphere of 5% CO₂ and 95% air and allowed to grow at 80% confluency. Flasks were then trypsinized (0.25% trypsin, 0.1% EDTA in HBSS without Ca²⁺, Mg²⁺, and sodium bicarbonate; Mediatech), and cells were washed with DMEM and plated onto 12-well TPP (Techno Plastic Products, Trasadingen, Switzerland) cell culture plate. Cells were allowed to grow about 80% confluent before experiments. Termination of our experiments was based on our previous reports (36, 38).

Antibodies and reagents. Mouse monoclonal CBS and CSE antibodies were from Novus Biologicals (Littleton, CO). Phospho-SAPK/JNK1/2, phospho-ERK1/2, and p47phox antibodies were purchased from Cell Signaling (Danvers, MA). Inhibitors of MEK1 (PD98059) and SAPK/JNK1/2 (SP600125) were purchased from Calbiochem (San Diego, CA). l-Hcy was from Chem-Impex International, Wood Dale, IL. L-lysophosphatidylcholine for 30 min. Cells were washed with PBS (3 ×, 5 min each) and blocked with 1% BSA for 1 h. After two washes of 5 min each, primary antibody (p47phox, 1:100 dilutions in 1% BSA) was added and incubated for overnight at 4°C with gentle agitation. Excess antibody washed by PBS (3 ×, 5 min each) and secondary antibody removed by PBS wash (3 ×, 5 min each) and fluorescence visualization was visualized in a laser scanning confocal microscope (Olympus Fluoview 1000) with appropriate filter.

Western blot analysis. The cells were lysed in RIPA lysis buffer (Boston BioProducts, Worcester, MA), protein content was measured using BCA method, and equal amount of protein was separated onto 10% SDS-PAGE. Protein was transferred to nitrocellulose membrane and probed with appropriate antibodies following our earlier adopted method (38).

HPLC analysis. Hcy and Cys were extracted from cell culture supernatant and analyzed as described previously (38). The method was adopted from Malinow et al. (29).

Statistical analysis. Values are given as means ± SE from “n” numbers of experiments in each group as mentioned in each of the figure legends. The difference between mean values of multiple experiments was analyzed by one-way ANOVA followed by Scheffé’s post hoc analysis. Comparisons between groups were made with the use of Student’s independent t-test. A value of P < 0.05 was accepted significance.

RESULTS

Hcy induced MCP-1 and MIP-2 by attenuating H₂S production. To determine whether Hcy stimulates the production of inflammatory molecules in mouse mesangial cells (MCs), we treated MCs with increasing concentrations of Hcy as demonstrated in Fig. 1A. At a concentration of 50 μM, Hcy significantly stimulated MCP-1, but not MIP-2, when compared with control. Both of these molecules, however, robustly increased at the 75 μM dose of Hcy, with little increase thereafter (Fig. 1A). Two amino acids in the Hcy synthesis and metabolism pathways, methionine and cysteine, respectively, had no effect in the expressions of these two molecules at the 75 μM dose (Fig. 1A, inset).

To determine whether Hcy decreases generation of H₂S in MCs, we incubated MCs with 75 μM of Hcy for 48 h and measured H₂S generation in the cell homogenate. Hcy diminished H₂S generation, whereas methionine and cysteine had no effect (Fig. 1B). To confirm that the decrease in H₂S generation was attributable to Hcy, we repeated the experiment using MCs
transfected with the genes encoding for the enzymes responsible for Hcy metabolism. As shown in Fig. 1B, MCs transfected with CBS, CSE, or both genes significantly enhanced MCs ability to produce H2S.

**CBS and CSE genes transfected governed cysteine production during HHcy.** Hcy is an intermediate product in the pathway whereby methionine is metabolized to cysteine. This pathway is regulated by the activities of CBS and CSE. To determine whether CBS and CSE increase Cys production from Hcy in MCs, we measured Hcy and Cys in the cell culture medium before and after addition of Hcy. As shown in Table 1, Hcy was not detectable in the medium at baseline or at 48 h in either control or doubly transfected MCs. When exogenous Hcy was added to the medium of nontransfected cells, the concentration of Hcy in the medium remained the same at 48 h as at baseline; however, when Hcy was added to the medium of doubly transfected MCs, the concentration of Hcy significantly decreased in the medium after 48 h. The concentration of cysteine in the medium of nontransfected cells decreased by a similar amount over 48 h in the presence or absence of exogenous Hcy. In doubly transfected MCs, in contrast, the concentration of Cys decreased by 89% in the absence of exogenous Hcy but only by 65% in the presence of Hcy. These findings suggest that the overexpression of CBS and CSE increase Hcy metabolism, resulting in increased cysteine production.

**Table 1. Overexpression of CBS and CSE metabolized Hcy and increased Cys concentration in Hcy-treated MCs**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hcy Concentration, μM</th>
<th>Cysteine Concentration, μM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>At 0 h</td>
<td>After 48 h</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control + L-Hcy (75 μM)</td>
<td>75.0</td>
<td>77.4 ± 3.62</td>
</tr>
<tr>
<td>CBS+CSE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CBS+CSE + L-Hcy (75 μM)</td>
<td>75.0</td>
<td>13.7 ± 1.14†</td>
</tr>
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Mesangial cells (MCs) were transfected with cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) double genes as stated in MATERIALS AND METHODS. Appropriate controls were taken as shown in the table. Data are presented as means ± SE, n = 5 independent experiments, *Significant difference (P < 0.05); †P < 0.05 vs. 0 h. ND, not detectable.
Hcy-induced MCP-1 and MIP-2 were attenuated by CBS and CSE overexpression. Multiple reports demonstrate that endogenous H2S is protective against inflammatory induction (18, 57). To determine whether overexpression of CBS and CSE prevent the appearance of markers of inflammation in MCs, we treated MCs doubly transfected with CBS and CSE with Hcy for 48 h and measured MCP-1 and MIP-2. Control cells received either vehicle, Hcy, H2S, or H2S + Hcy (Fig. 2). Hcy induced MCP-1 and MIP-2 in MCs, whereas simultaneous exogenous supplementation with H2S significantly reduced the expression of these inflammatory molecules (Fig. 2). Interestingly, overexpression of either CBS or CSE also significantly diminished MCP-1 and MIP-2 expression induced by Hcy (Fig. 2). MCP-1 and MIP-2 expression was even further reduced in the cells overexpressing both genes (Fig. 2). Vehicle or H2S alone had no effect on these two molecules expression.

CBS and CSE gene therapy regulated p47phox expression in HHcy through H2S generation. Hcy induces NAD(P)H oxidase, which is a predominant source of O2·− generation and plays a vital role in Hcy-mediated oxidative damage (36, 51). To determine whether endogenous H2S has a regulatory role in NAD(P)H oxidase p47phox expression in HHcy, we incubated MCs transfected with CBS and CSE alone or in combination (double transfection), and with Hcy for 48 h followed by immunostaining with p47phox antibody. As shown in Fig. 3A, p47phox was upregulated in the MCs treated with Hcy. This induction was attenuated in both CBS and CSE transfected MCs. Interestingly, the expression of p47phox was completely abolished in the MCs transfected with these genes together (double transfection) (Fig. 3A). This result was confirmed by Western blot analysis (Fig. 3B).

Hcy-induced phosphorylation of ERK1/2 and JNK1/2 was diminished by CBS and CSE. Several laboratories have reported that Hcy exerts its effects through activation of ERK1/2 (30, 47) and JNK1/2 (25, 47) pathways. To determine whether H2S has any regulatory role in Hcy-mediated ERK1/2 and JNK1/2 induction, we compared the effect of H2S on Hcy-stimulated ERK1/2 and JNK1/2 in MCs, in the presence or absence of a pharmacological inhibitor of MEK1 (PD98059), a pharmacological inhibitor of JNK1/2 (SP600125), or in cells where expression of ERK1/2 or JNK1/2 was reduced by small interfering RNA (siRNA). As shown in Fig. 4, A and B, Hcy stimulated ERK1/2 and JNK1/2 activation in MCs as evidenced by increased expression of the phosphorylated forms. Hcy-stimulated ERK1/2 and JNK1/2 activation were blocked by H2S treatment (Fig. 4, A and B). Similar results were seen with pharmacological and siRNA blockers of these two signaling molecules (Fig. 4, A and B). Interestingly, CBS and CSE genes alone or double gene transfer inhibited phosphorylation of both ERK1/2 and JNK1/2 molecules induced by Hcy (Fig. 5).

Hcy-induced MCP-1 and MIP-2 induction were through ERK1/2 and JNK1/2 pathways. To determine whether Hcy stimulates production of inflammatory molecules through ERK1/2- and JNK1/2-dependent pathways, we measured expression of MCP-1 and MIP-2 in MCs treated with or without Hcy and H2S (Fig. 6). To compare the effect of H2S supplementation and CBS and/or CSE overexpression on MCP-1 and MIP-2 expression, we overexpressed CBS, CSE, and doubly in MCs and treated with Hcy. To determine the involvement of ERK1/2 and JNK1/2 in Hcy-mediated MCP-1 and MIP-2 induction, we used pharmacological and siRNA blockers of ERK and JNK as indicated in Fig. 6. As demonstrated in Fig. 6, Hcy-treated cells exhibited significantly decreased expression of MCP-1 and MIP-2 in the presence of H2S compared with MCs treated with Hcy alone (Fig. 6). The expression of these two molecules were further diminished in cells overexpressing either CBS or CSE. Interestingly, double-gene transfer completely inhibited and normalized expressions of MCP-1 and MIP-2 treated with Hcy (Fig. 6). Inhibition of ERK1/2 by siRNA or pharmacological MEK1 inhibitor PD98059 also significantly diminished expressions of these two molecules (Fig. 6). Similar effects were obtained by blocking JNK1/2 by siRNA or pharmacological blocker SP600125 (Fig. 6).

DISCUSSION

The present study demonstrates that HHcy decreases endogenous generation of H2S, which is a potent anti-inflammatory molecule, resulting in induction of MCP-1 and MIP-2 in MCs. Our study also demonstrates that overexpression of CBS and CSE accelerates Hcy metabolism, normalizes H2S generation, and attenuates MCP-1 and MIP-2 expression in MCs mediated by Hcy. This study thus highlights the importance of CBS and CSE in the regulation of HHcy and Hcy-mediated inflammatory induction.

There are multiple ways by which HHcy develops. These include: 1) methionine-rich diet; 2) vitamin B12/folate deficiency; 3) mutation or impairment of transsulfuration enzymes; and 4) renal impairment (35). None of these pathways are related to protein malnutrition and/or of cytokine-induced stressful disorders. A fifth mechanism of HHcy development has been reported in patients suffering from protein malnutrition and/or intestinal malabsorption characterized by insufficient N intake or assimilation, which also reduces body S accretion rates (19). This is an adaptive mechanism depressing the activity of CBS and allows accumulation of Hcy in biological fluids. Accumulated Hcy during this process in turn through remethylation maintain methionine homeostasis. HHcy in this regard considered as...
a consequence of cystathionase impairment and not as a causal factor (19, 49). However, if HHcy is due to pathways involving 1–4 reasons, as stated above, it could be both as a consequence and/or causal factor depending on the source. Our in vitro experiments were performed in a condition that does not mimic protein malnutrition or malabsorption, rather HHcy was created with excess Hcy supplementation. Therefore, we believe that HHcy in our model was a causal factor of cystathionase (CBS and/or CSE) impairment, as reported earlier (3, 36), resulting in H2S deficiency in the culture milieu.

HHcy has been reported in a number of CKD (10, 16) and implicated in the high frequency of vascular events in patients with CKD (52). This finding is somewhat paradoxical as Hcy undergoes metabolism to produce H2S, a well-known anti-inflammatory agent that would be expected to ameliorate vascular damage. The three enzymes responsible for the metabolism of Hcy to H2S are CBS, CSE, and 3-MST (12, 41–42, 48). Under pathological conditions, elevated levels of Hcy alter the transsulfuration pathway by inhibiting the CSE enzyme activity (3), which may further elevate Hcy in the body. This results in protein homocysteinyllation and damage (21) manifested as loss of function. For example, Jakubowski (21) reported that homocysteinylation of methionyl-tRNA synthetase and trypsin causes inactivation of these protein molecules. We speculate that high Hcy may inactivate H2S-generating enzymes in the body, including CBS, CSE, and/or 3-MST, which metabolize Hcy. Together these may reduce endogenous production of H2S. In the present study, we have demonstrated reduced production of endogenous H2S in MCs exposed to high ambient concentrations of Hcy (Fig. 1B), which can be overcome by overexpression of CBS and CSE (Fig. 1B).

Our observation that CBS/CSE overexpression results in higher production of H2S, faster clearance of Hcy, and improvement or even restoration of HHcy-mediated molecular lesions suggest new avenues for therapy of HHcy and associated vascular complications. Although it is reported that at elevated level, Hcy competes with cysteine (Cys) in binding to

**Fig. 3.** CBS and CSE double gene therapy attenuated Hcy-induced p47\(^{\text{phox}}\) upregulation in MCs. A: MCs were cultured in 8-well chamber slide and transiently transfected with CBS, CSE, or both the cDNAs and treated with Hcy (75 μM) for 48 h. Cells were fixed, permeabilized, blocked with BSA in PBS, and immunostained with anti-p47\(^{\text{phox}}\) antibody secondarily conjugated with Texas Red. Cells were also counterstained with DAPI. Fluorescence images were taken under laser scanning confocal microscope (Fluoview 1000, Olympus) and merged. Red fluorescence indicates p47\(^{\text{phox}}\) expression. Representative images from four independent experiments were shown here. B: similar experiment was performed in 12-well plastic plates and expression of p47\(^{\text{phox}}\) protein was determined by Western blot analysis. Blots reprobed with β-actin for loading control. C: bar diagram showing relative expression of p47\(^{\text{phox}}\) normalized with β-actin loading control. Data represent means ± SE; n = 4. *P < 0.01 vs. control; ♂P < 0.05 vs. Hcy.
Hcy (75 μM) for 30 min with appropriate controls. Phosphorylation of ERK1/2 was detected by Western blot analysis. The blot reprobed with β-actin as loading control. The blot was blocked with pharmacological inhibitor (PD98059, 50 μM) or supplemented with H2S (30 μM, in the form of NaHS) and treated with Hcy (75 μM) for 30 min. Appropriate controls were taken. Cells were lysed in RIPA lysis buffer and phosphorylation of ERK1/2 was detected by Western blot analysis. The blot reprobed with β-actin as loading control.

It is reported that high circulating concentrations of the inflammatory markers IL-1ra and IL-6 were significantly correlated with HHcy and associated with atherosclerosis in older populations (14). Recent studies have demonstrated that Hcy induces MCP-1 (4) and MIP-2 (40) in MCs and that the induction of these inflammatory markers is linked to adverse outcomes in patients with cardiovascular disease and CKD. The current study confirms Hcy induction of MCP-1 and MIP-2 in mouse MCs (Fig. 1A) and shows that this induction is due, in part, to downregulation of endogenous H2S production (Fig. 1B). Although H2S supplementation alone reduced Hcy-mediated induction of inflammatory molecules, further reduction occurred when CBS or CSE were overexpressed (Fig. 2) due to increased cellular ability to generate H2S (Fig. 1B).

These results suggest an alternative approach to combat Hcy-associated inflammation in CKD.

Multiple laboratories including our own have reported that Hcy stimulates O2•− generation through a NAD(P)H-mediated mechanism (8, 9, 34, 55). We have previously reported that mice with HHcy expressed higher level of p47phox subunit of NADPH oxidase (AJP-Cell Physiol. 2011; 300: C160–C171).

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NAD(P)H oxidase in kidney cortical tissue compared with mouse with normal Hcy level, and H2S supplementation normalized p47\textsuperscript{phox} expression \((36)\). Similarly, upregulated p47\textsuperscript{phox} expression in Hcy-treated mouse MCs was diminished by H2S treatment \((36)\). Others have reported that inhibition of p22\textsuperscript{phox} by siRNA transfection normalized Hcy-induced \(O_2^\cdot\) production in human endothelial and vascular smooth muscle cells \((9)\). The present study supports our previous findings and suggests the possibility of gene-based therapy as a mechanism to enhance endogenous H2S production and reduce \(O_2^\cdot\) production (through reduced oxidative p47\textsuperscript{phox}, Fig. 3, \(A\) and \(B\)) induced by HHcy.

Mitogen-activated protein kinase (MAPK) cascades are reported to regulate several biological responses, including mesangial proliferation \((5, 20)\), migration \((7)\), and inflammation \((24)\). Of particular interests, the involvement of two important MAPKs ERK1/2 and JNK1/2 pathways in mediating inflammation was reported by several laboratories. Recent reports also suggest that Hcy stimulated MCP-1 expression in rat MCs via nuclear factor-\(\kappa\)B \((4)\), and MIP-2 production via phosphoinositol trisphosphate kinase- and p38MAPK-dependent pathways \((40)\); however, none of these studies were focused to investigate whether ERK-1/2 and JNK-1/2 are involved in Hcy-mediated MCP-1 and MIP-2 expression in MCs. Therefore, we determined whether these two signaling pathways are involved in Hcy-induced MCP-1 and MIP-2 induction and possible regulatory role of H2S in these pathways. Our results suggested that Hcy induced phosphorylation of ERK1/2 and JNK1/2, and H2S supplementation attenuated these activations \((Fig. 4)\). Interestingly, CBS or CSE overexpression also inhibited ERK1/2 and JNK1/2 phosphorylation in MCs induced by Hcy \((Fig. 5)\), which confirms the regulatory role of H2S in these signaling cascades. Furthermore, the expressions of MCP-1 and MIP-2 were, at least in part, mediated by ERK1/2 and JNK1/2 pathways \((Fig. 6)\). In a report, although Ingram et al. \((20)\) demonstrated that Hcy-induced ERK1/2 signaling mechanism was involved in MCs proliferation and endoplasmic reticulum stress, to our knowledge, our study is the first to report the involvement of these two cascades in Hcy-mediated MCP-1 and MIP-2 expression in MCs. The novelty of the present study is that CBS and CSE overexpression blocks these two pathways through generation of H2S in HHcy and ameliorate inflammation. An overall hypothesis and possible mechanism of Hcy-mediated MCP-1 and MIP-2 induction is depicted in Fig. 7.

In summary, we have demonstrated that Hcy induced inflammatory molecules MCP-1 and MIP-2, in part, through upregulation of NAD(P)H p47\textsuperscript{phox} subunit and downregulation of MCs’ capability to generate H2S through ERK1/2- and JNK1/2-dependent pathways. Supplementation of H2S or either CBS or CSE gene delivery normalized p47\textsuperscript{phox} expression and enhanced cellular capability to generate endogenous H2S, which partially inhibited MCP-1 and MIP-2 expression induced by Hcy. CBS and CSE double gene delivery was, however, more effective than H2S supplementation or single gene delivery.
gene transfer. Together, these results may partially explain inflammatory mechanisms associated with HHcy in CKD, and reveals that CBS/CSE-dependent H$_2$S generation play major role in this process.

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**DISCLOSURES**

The views articulated in this paper do not necessarily reflect the views of the Department of Veterans Affairs.

No conflicts of interest, financial or otherwise, are declared by the author(s).

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