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Contribution of Guanine Nucleotide Exchange Factor Vav2 to Hyperhomocysteinemic Glomerulosclerosis in Rats

Fan Yi, Min Xia, Ningjun Li, Chun Zhang, Lin Tang, Pin-Lan Li

Abstract—We currently reported that Vav2, a member of the guanine nucleotide exchange factor-Vav subfamily, participates in homocysteine-induced increases in Rac1 activity and consequent activation of NADPH oxidase in rat mesangial cells. However, the physiological relevance of this cellular action of Vav2 remains unknown. The present study tested a hypothesis that Vav2 importantly mediates the injurious action of homocysteine on glomeruli and thereby contributes to the development of glomerulosclerosis during hyperhomocysteinemia. We found that, among Vav members, Vav2 was abundantly expressed in glomeruli. When Vav2 short hairpin RNA was transfected into the kidneys of Sprague-Dawley rats, hyperhomocysteinemia induced by folate-free diet failed to significantly enhance Rac1 activity and increase NADPH-dependent superoxide production. In these rats with silenced renal Vav2 gene, glomerular injury during hyperhomocysteinemia was markedly attenuated compared with those rats only receiving mock vector transfection, as shown by ameliorated albuminuria and extracellular matrix metabolism. In the rat kidneys with transfection of a dominant-active Vav2 variant (onco-Vav2), we found that overexpression of Vav2 led to significant increases in Rac1 activity, superoxide production, and glomerular injury, which was similar to that induced by hyperhomocysteinemia. However, this Vav2 overexpression was unable to further enhance homocysteine-induced glomerular injury. We concluded that Vav2-mediated activation of NADPH oxidase is an important initiating mechanism resulting in hyperhomocysteinemic glomerular injury through enhanced local oxidative stress. (*Hypertension*. 2009;53:90-96.)

Key Words: end-stage renal disease ■ homocystinemia ■ redox signaling ■ kidney glomerulus

Hyperhomocysteinemia (hHcys) is known as a critical pathogenic factor in the progression of end-stage renal disease and in the development of cardiovascular complications related to end-stage renal disease.^{1,2} We and others have demonstrated that oxidative stress mediated by NADPH oxidase is importantly involved in progressive glomerular injury associated with hHcys.³⁻⁵ However, it remains unknown how NADPH oxidase is activated during hHcys. Many studies have demonstrated that NADPH oxidase is a multiple protein complex in which cytosolic subunits (p47^{phox}, p40^{phox}, p67^{phox}, and Rac GTPase) assemble with membrane-associated subunits (NOX and p22^{phox}) to generate superoxide (O₂⁻). During complex assembly, p47^{phox} translocation and Rac-mediated GTP binding play a critical role in the activation of the complex as a functioning enzyme. Recent studies have indicated that enhanced Rac activity is even able to activate NADPH oxidase independent of p47^{phox} translocation.⁶ On cell activation, GDP-bound Rac under resting condition may be converted into GTP-Rac through the action of a guanine nucleotide exchange factor.⁷ This GTP form of Rac interacts with NADPH oxidase via a tetrapeptide repeat motif in the N-terminal part of p67^{phox}, leading to O₂⁻ production via this oxidase.

Among >100 guanine nucleotide exchange factors, Vav subfamily exhibits the high specificity to Rac-mediated NADPH oxidase activation.^{8,9} We demonstrated recently that Vav2 contributes to homocysteine (Hcys)-induced increase in Rac1 activity and consequent activation of NADPH oxidase in rat renal mesangial cells.¹⁰ Chen et al¹¹ have also reported that constitutive upregulation of Rac1 because of activation of Vav2 and resulting enhancement of reactive oxygen species production are a hallmark of renal diseases characterized by irreversible fibrosis and sclerosis. These results led to a hypothesis that Vav2 may importantly mediate the injurious action of Hcys on glomeruli and thereby contribute to the development of glomerulosclerosis during hHcys.

To test this hypothesis, experiments with *in vivo* gene silencing and gene overexpression in the rat kidney were performed to observe the role of Vav2 in mediating glomerular injury during chronic hHcys induced by folate-free (FF) diet. Our results indicate that Vav2 importantly mediates activation of NADPH oxidase in the glomeruli of rats on the FF diet, leading to initiation and development of glomerulosclerosis. We also demonstrated that this Vav2-mediated damaging mechanism upregulates tissue inhibi-

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tor of metalloproteinase-1 (TIMP-1) via redox regulatory pathway, thereby decreasing matrix metalloproteinase (MMP) activities and resulting in the disturbance of extracellular matrix metabolism.

Materials and Methods

Isolation of rat glomeruli, Western blot analysis, real time RT-PCR, immunohistochemistry, and morphological examinations were performed as we described previously.⁴ A brief section about some specifics of these methods used in this study was presented as online supplemental materials. Some new and special methods were presented below.

Mammalian Expression Vectors

The N-terminally truncated (constitutively active) form of Vav2 (pEGFPN1-oncoVav2) was the generous gift from Dr Keith Burrige (University of North Carolina at Chapel Hill), which was used in other studies on the regulation of Rac-GTPase.¹² The sequence of Vav2-small-interfering RNA used was as follows: 5'-AA GGAGAGGTTCCCTGTTTAT-3',¹³ which was inserted into a small-interfering RNA vector with cytomegalovirus promoter. Specifically, the Vav2-small-interfering RNA was engineered into the *Bam*I and *Xho*I sites of the vector pRNAT-CMV3.2 by Genescript, which we called shRNA-Vav2 in the present study. Luciferase expression plasmid for in vivo monitoring of gene transfection efficiency was obtained from Promega Corporation.

Animals and Gene Transfection of the Kidney by Ultrasound-Microbubble Technique

Experiments were performed using Sprague-Dawley rats (200 g; 6 weeks old) from Harlan Inc (Madison, Wis), and all of the rats were uninephrectomized. After a 1-week recovery period from uninephrectomy, shRNA-Vav2 or a dominant-active Vav2 variant (onco-Vav2) plasmid with a luciferase expression vector was cotransfected into the kidneys via intrarenal artery injection using the ultrasound-microbubble system. Plasmid containing scrambled small RNA was used as a control. A full description of the procedures for the ultrasound-microbubble gene transfer technique can be found in the online supplemental section available at <http://hyper.ahajournals.org>. After introduction of plasmid into the kidney, these uninephrectomized rats were maintained on a normal or a FF diet (Dyets Inc) for 4 weeks. All of the protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Over the experimental days, blood and a 24-hour urine sample were collected. Plasma total Hcys (tHcys) was measured by fluorescence high-performance liquid chromatography analysis, and urinary albumin excretion was measured using a rat albumin ELISA quantitation kit (Bethyl Laboratories).⁴ Glomeruli from the rat kidneys were prepared by a graded or series sieving as described previously.¹⁴

In Vivo Imaging of Gene Expression

To monitor the efficiency of gene expression through somatic plasmid transfection daily, rats were anesthetized with ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP), and an aqueous solution of luciferin (150 mg/kg IP) was injected 5 minutes before imaging, as others described.¹⁵ The anesthetized rats were imaged using the IVIS200 in vivo imaging system (Xenogen). Photons emitted from luciferase-expressing cells within the animal body and transmitted through tissue layers were quantified over a defined period of time ranging up to 5 minutes using the software program Living Image (Xenogen) as an overlay on an Igor program (Wavemetrics).

Rac GTPase Activation Assay

A pull-down experiment was performed to determine Rac GTPase activity using a Rac activation assay kit (Upstate), as we described previously.⁴

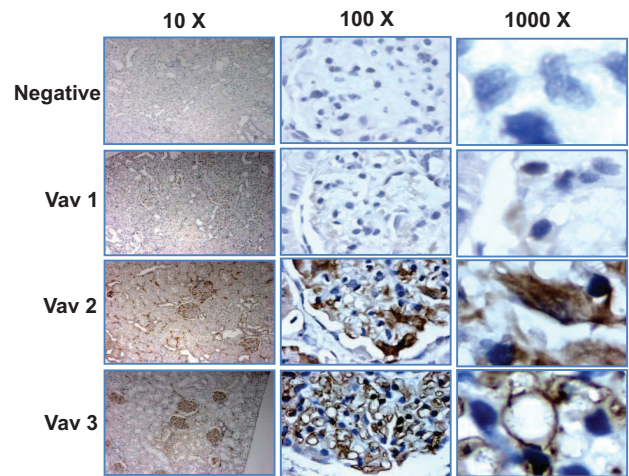


Figure 1. Immunohistochemical staining of Vav1, Vav2, and Vav3 proteins in the rat kidney. Results were representative of the Vav staining in the kidneys from 8 rats.

Fluorescence Resonance Energy Transfer Assay for MMP Activities

MMP activities were measured using EnzoLyte 520 MMP assay kits from AnaSpec, Inc. These kits contain different synthetic fluorescence resonance energy transfer peptide substrates of MMPs for use as fluorogenic indicators in the assay. In addition to control and experimental assays, for each tissue sample 1 specificity test was added, which included a preincubation of the sample with 10 mmol/L of EDTA for 30 minutes and then measurement of MMP activities. The MMP activities were presented as percentages of change in relative fluorescence resonance energy transfer efficiency during experimental treatments compared with the value obtained from control rats on a normal diet.

O₂^{•-} Detection by Electronic Spin Resonance

The measurement of O₂^{•-} by electronic spin resonance was performed according to the methods in our previous studies.^{16,17}

Statistics

Data are expressed as means ± SEs. The significance of differences in mean values between and within multiple groups was examined by 1-way ANOVA followed by a Duncan's multiple range test. *P* < 0.05 was considered statistically significant.

Results

Immunohistochemical Analysis of Vav Expression in Rat Glomeruli

By immunohistochemical analysis, we found that, among the Vav family, Vav2 and Vav3 but not Vav1 were detected in renal glomeruli. Under high magnification it was shown that Vav2 was enriched in the mesangial area and glomerular capillaries, whereas Vav3 was mainly present in glomerular capillaries (Figure 1). This is consistent with previous reports that Vav1 is predominantly expressed in hematopoietic cells, whereas Vav2 and Vav3 are ubiquitously expressed.

In Vivo Imaging of Vav2 Gene or shRNA Transfection

As shown in Figure 2A, using an in vivo imaging system, gene expression of the cotransfected luciferase gene could be monitored daily. Even on the second day after the kidney was transfected by this ultrasound-microbubble plasmid introduc-

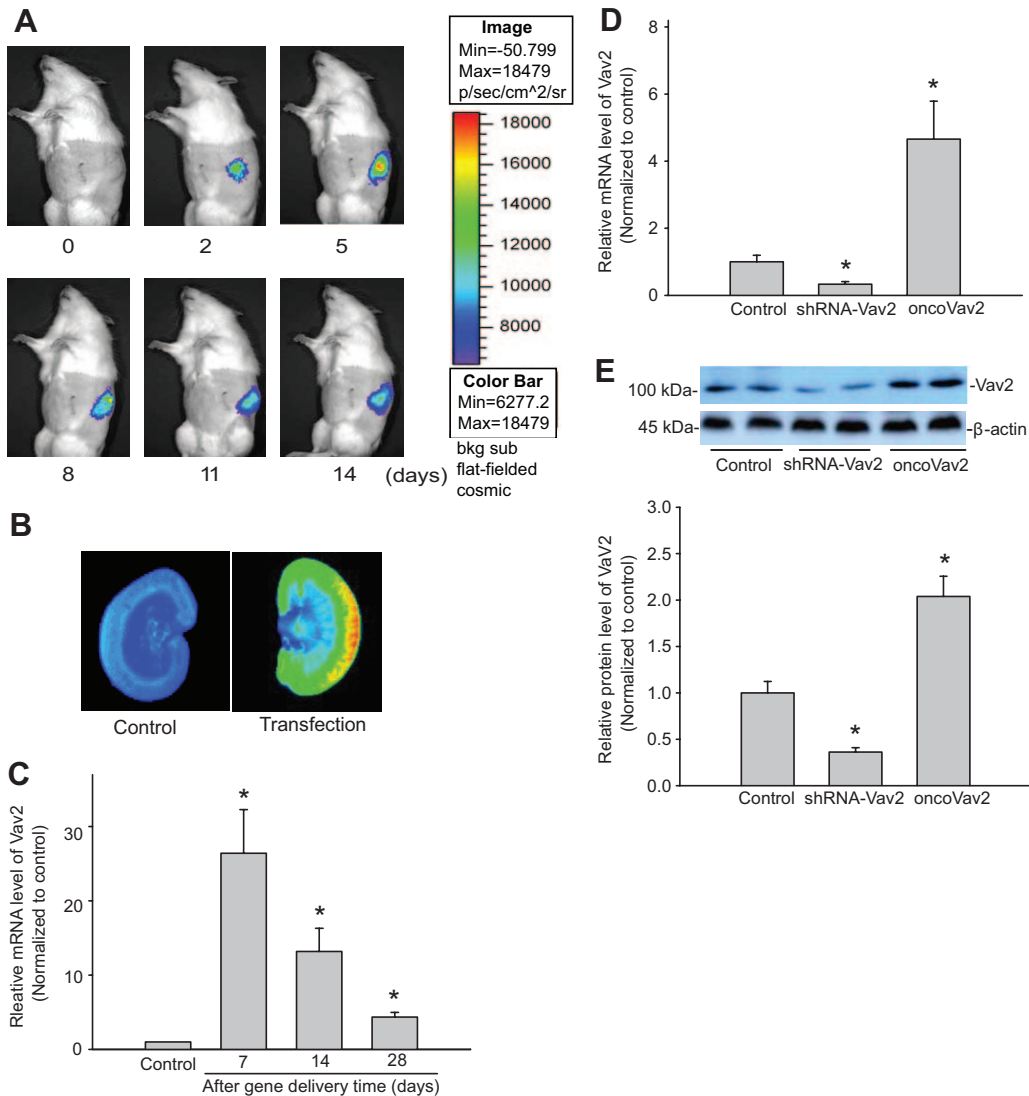


Figure 2. In vivo and in vitro determination of gene transfection efficiency in the kidney. A, Daily imaging confirmation of gene transfection in the kidney by an in vivo molecular imaging system. B, Localization of transfected gene expression in the hemidissected kidney at day 5 after gene delivery. C, Real-time RT-PCR detection of Vav2 mRNA after oncoVav2 gene delivery at different time points. D, Quantitative RT-PCR analysis of Vav2 mRNA levels in glomeruli from control, shRNA-Vav2-, and oncoVav2-transfected rats after 4-week gene transfection. E, Representative Western blot gel document (top) and summarized data (bottom) showing relative Vav2 protein levels in different groups after 4-week gene transfection (n=8). *P<0.05 vs control.

tion, the gene expression could be detected. In the hemidissected kidney, it was shown that all of the cortical regions exhibited efficient gene transfection and consequent expression, as shown in green fluorescence compared with the control area (dark blue). It should be noted that the strong signal (red color) in our semidissected kidney image does not mean that the transfection was confined in the superficial cortex. In such detection, all of the green areas should be considered as efficiently transfected. However, in the periphery area, the expression of the transfected gene was stronger, which may be because of its rich in blood flow and glomerular cells, where more plasmids could be trapped for transfection during injection of the plasmid-microbubble mixture via renal arteries (Figure 2B). These results were consistent with previous studies showing that ultrasound-microbubble gene introduction is an efficient technique for delivery of the gene into the glomerular cells, vascular endothelial cells, and

fibroblasts.¹⁸ By RT-PCR analysis, it was found that transfected gene expression could last for a relatively long period and peaked on approximately days 5 to 7 (Figure 2C). At 4 weeks, when rats were euthanized, Vav2 mRNA and protein levels were found decreased by 63% and 60% in glomeruli isolated from shRNA-Vav2-transfected rat kidneys compared with those kidneys from control or mock vector-transfected kidneys. However, Vav2 mRNA and protein increased by ≈4.5- and 2.1-fold in oncoVav2-transfected rat kidneys, respectively, when compared with control kidneys (Figure 2D and 2E).

Increased Plasma tHcys Levels in Rats With the FF Diet

By high-performance liquid chromatography analysis, a 4-week FF diet significantly increased plasma tHcys levels in uninephrectomized Sprague-Dawley rats. Neither shRNA-

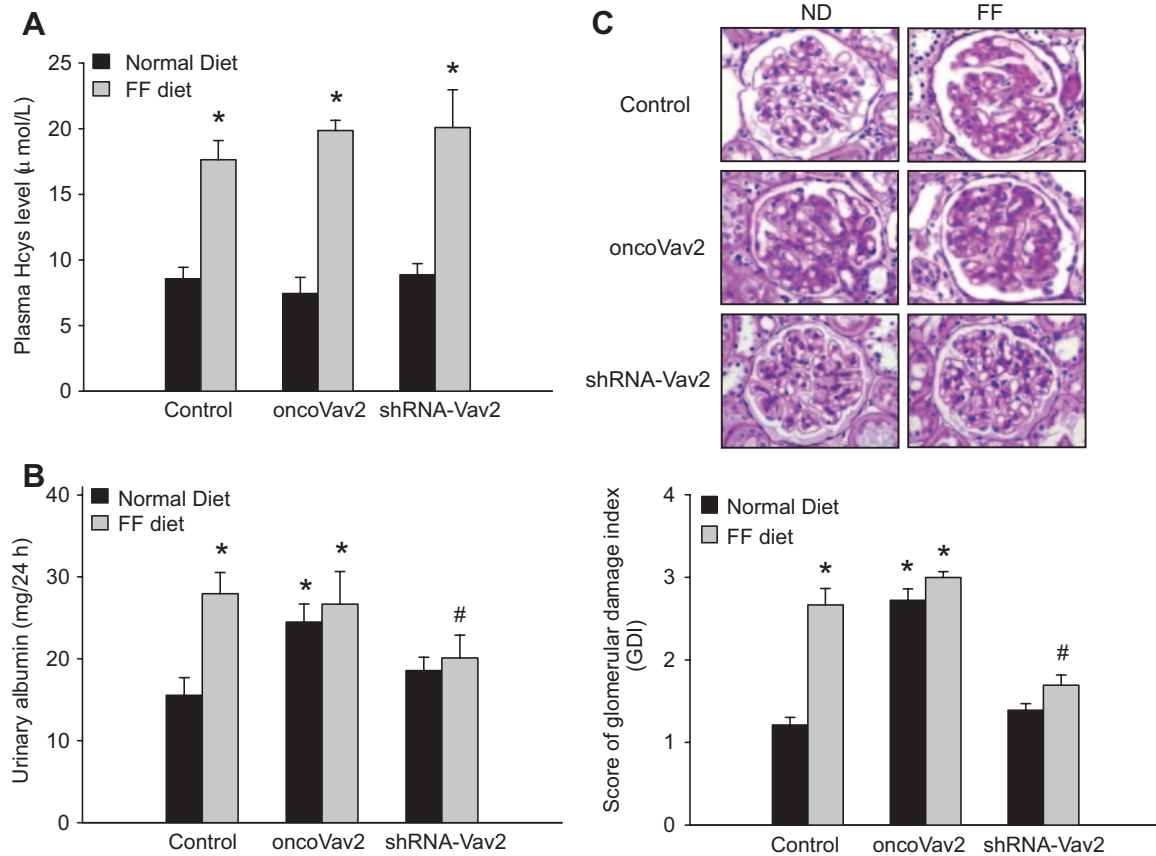


Figure 3. Effects of Vav2 on glomerular injury in hHcys rats. A, Average plasma total Hcys levels in 6 different groups of rats on a normal diet with or without shRNA-Vav2 or oncoVav2 transfection and on an FF diet with or without shRNA-Vav2 or oncoVav2 transfection. B, Urinary albumin excretion in 6 different groups of rats as indicated. C, Photomicrographs (original magnification, ×250) showing typical glomerular structure and summarized glomerular damage index (GDI) by semiquantitation of scores in 6 different groups of rats as indicated (n=8). *P<0.05 vs control; #P<0.05 vs values obtained from vehicle-treated hHcys rats.

Vav2 nor oncoVav2 transfection had an effect on the increase in tHcys levels in these rats. It is clear that Vav2 gene manipulations do not alter plasma Hcys levels (Figure 3A).

Role of Vav2 in Glomerular Damage Induced by hHcys

As shown above, in parallel to elevations of plasma tHcys, urinary albumin excretion was significantly increased in rats with an FF diet (Figure 3B, control). Morphological analysis showed a typical pathological change in glomerular sclerotic damage, showing expanded glomerular mesangium with hypercellularity, capillary collapse, and fibrous deposition in glomeruli in these rats under the FF diet (Figure 3C, control). The average glomerular damage index was substantially higher in these hyperhomocysteinemic rats (Figure 3C, bottom). In shRNA-Vav2-transfected rats, however, the FF diet produced much less glomerular damage, as shown by attenuated albuminuria and glomerular damage index (Figure 3B and 3C, bars and representative glomeruli with labels of shRNA-Vav2). In another series of experiments, we further determined whether transfection of oncoVav2 to increase Vav2 could mimic or enhance Hcys-induced glomerular injury. Indeed, overexpression of Vav2 led to increased urinary albumin excretion and glomerular mesangial expansion, which was similar to what occurred in the kidney from

rats under the FF diet. Under such condition with overexpressed Vav2 gene in the kidney, the FF diet did not further enhance pathological damages compared with those observed in rats with an FF diet but with mock vector transfection (Figure 3, bars and glomeruli with labels of oncoVav2).

Involvement of Vav2 in Enhanced Rac Activity and Consequent Activation of NADPH Oxidase Induced by hHcys

As depicted in Figure 4A, hHcys significantly increased Rac activity (control of the FF diet) compared with control (control of normal diet), which was shown as increased GTP-bound Rac on the gel document. Transfection of shRNA-Vav2 attenuated this enhanced Rac activity by the FF diet in glomeruli (shRNA-Vav2 versus control under an FF diet). In contrast, transfection of oncoVav2 enhanced Rac activity even under normal diet (oncoVav2 on both normal and FF diet). These results were summarized in a bar graph of Figure 4A by quantitation of detected specific gel band density.

We also determined the effect of Vav2 manipulations on NADPH oxidase activity during hHcys induced by the FF diet. As shown in Figure 4B, electronic spin resonance analyses indicated that O₂⁻ production was significantly increased in glomeruli isolated from rats on the FF diet.

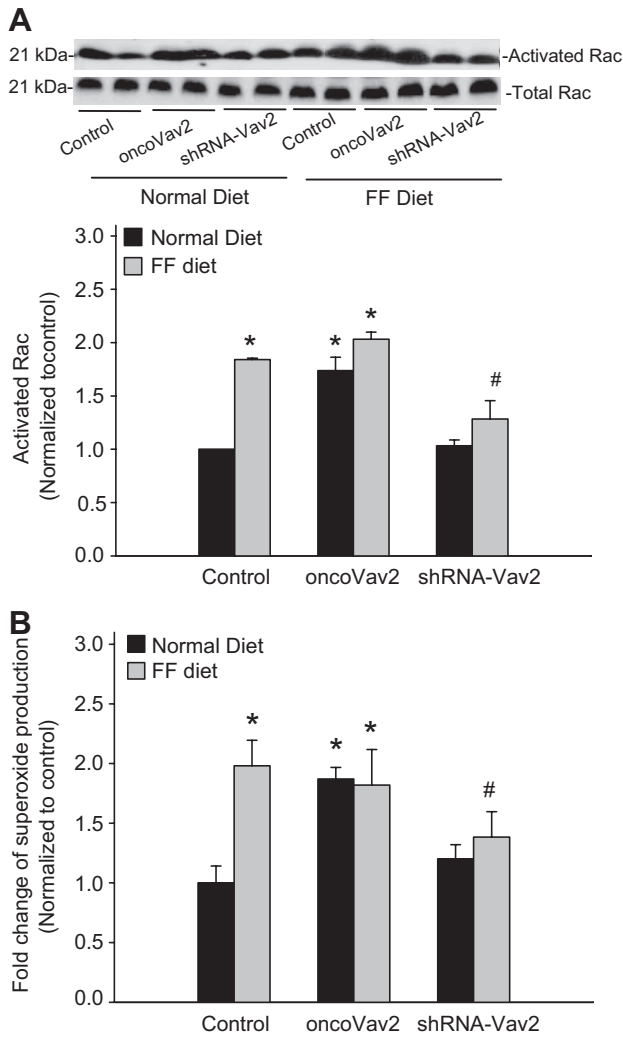


Figure 4. Effects of Vav2 gene manipulations on Rac and NADPH oxidase activities. A, Immunoblot analysis of activated Rac by pull-down assay and total Rac expression levels by general Western blot analysis. Bottom, Summarized data showing changes in Rac activity in glomeruli from 6 different group rats. B, Summarized data depicting $O_2^{\cdot-}$ production in glomeruli from 6 different group rats by electronic spin resonance analysis (n=8). * $P < 0.05$ vs control; # $P < 0.05$ vs the values obtained from vehicle-treated hHcys rats.

shRNA-Vav2 markedly attenuated this hHcys-induced increase in $O_2^{\cdot-}$ production, whereas oncoVav2 enhanced $O_2^{\cdot-}$ production when rats were on either a normal or FF diet, which was corresponding with the changes in Rac activity as presented above.

Changes in Extracellular Matrix Metabolism Induced by the FF Diet With and Without Vav2 Gene Manipulations

To further explore the mechanism mediating the role of Vav2 signaling in hHcys-induced glomerular damage, we determined whether abnormal extracellular matrix metabolism during hHcys is associated with Vav2 dysfunction. As illustrated in Figure 5A and 5B, TIMP-1, a major endogenous MMP regulator, in glomeruli from rats with the FF diet was increased by 88.0% and 47.5%, respectively. shRNA-Vav2

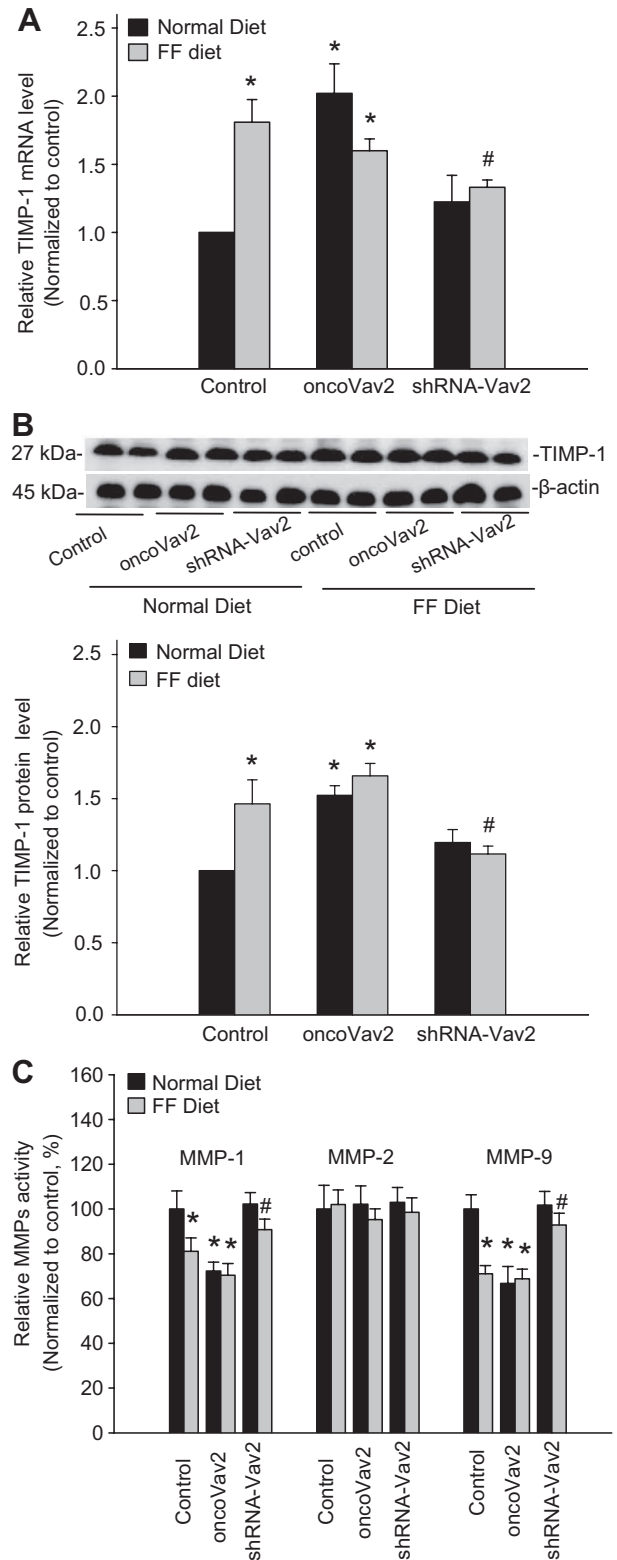


Figure 5. Effects of Vav2 gene manipulations on TIMP-1 expression and MMP activities. A, Changes in TIMP-1 mRNA expression levels in glomeruli detected by real-time RT-PCR (n=8). B, Western blot analysis of TIMP-1 (top) and summarized data (bottom) showing changes in TIMP-1 protein levels in glomeruli isolated from 6 different groups of rats as indicated. C, Summarized data showing changes in MMP-1, MMP-2, and MMP-9 activities in glomeruli isolated from these rats. * $P < 0.05$ vs control; # $P < 0.05$ vs values obtained from vehicle-treated hHcys rats.

significantly blocked the Hcys-induced increase in the TIMP-1 level in glomeruli from these hHcys rats. Similarly, overexpression of Vav2 led to an increase in TIMP-1 expression in glomeruli from rats on both normal and FF diets.

Among 3 important MMPs in glomeruli, MMP-1 and MMP-9 activities in glomeruli from hHcys rats were markedly reduced, which could be partially restored by shRNA-Vav2. Similarly, decreased MMP-1 and MMP-9 activities were observed in rats with oncoVav2 transfection. However, MMP-2 activity was not altered by either silencing the Vav2 gene or overexpression of this gene (Figure 5C).

Discussion

In the present study, we found that, among 3 members of the guanine nucleotide exchange factor-Vav subfamily, Vav2 and Vav3 are expressed in glomeruli of the rat kidney. It is suggested that both Vavs may participate in the detrimental action of hHcys on glomeruli. A focus on Vav2, rather than on Vav3, in our functional studies was primarily attributed to its relevance to Rac1-mediated NADPH oxidase activity, because Vav2 has been reported as a major Vav isoform to regulate Rac-NADPH oxidase activity.¹¹ So far, little is known regarding the linkage of Vav3 to Rac1-NADPH oxidase activity in mammalian cells. In addition, our previous studies also demonstrated that Vav2 plays a contributing role in the Hcys-induced increase in Rac1 activity *in vitro*.

To test the role of Vav2 in mediating hHcys-induced glomerular injury or sclerosis, an animal hHcys model induced by the FF diet was used, and local gene silencing or overexpression of the Vav2 gene in the kidney was conducted. A 4-week FF diet produced hHcys and resulted in a remarkable glomerular damage or sclerosis. To silence or overexpress the Vav2 gene in this animal model, an ultrasound microbubble-mediated plasmid delivery was used to introduce Vav2 shRNA or its dominant-positive variant, oncoVav2, into the kidney. Our results demonstrated that this method was highly efficient in delivering plasmids into renal cells *in vivo*, which led to gene transfection and expression in most renal cells, as also demonstrated in other previous studies.^{19–22} Vav2 mRNA or protein levels were significantly reduced by gene silencing and largely increased by the introduction of oncoVav2, as detected by real-time RT-PCR and Western blot analysis. Moreover, the present study used an *in vivo* molecular imaging system to monitor daily the efficiency of Vav2 gene transfection and expression in the kidney in living animals. It was shown that the transgene or shRNA expression vector (with luciferase gene as an indicator) could be detected even 24 hours after gene transfection and lasted ≤ 4 weeks. This *in vivo* transgene monitoring importantly guided our functional studies to define the role of the Vav2 gene in mediating glomerular damage associated with hHcys.

One of the most important findings of this study is that hHcys-induced glomerular injury in shRNA-Vav2-transfected rats was markedly ameliorated, as shown by reduced albuminuria and blunted disturbance of ECM metabolism. This action was found to be associated with attenuation of hHcys-induced activation of Rac and NADPH oxidase in the glomeruli. In addition, in experiments with transfection of a

dominant-positive Vav2 variant, overexpression of Vav2 induced glomerular injury to an extent similar to that induced by hHcys. However, under such Vav2 overexpression condition, the FF diet did not further enhance glomerular injury. These results provide strong evidence that hHcys-induced glomerular injury may share the same mechanisms with Vav2-mediated glomerular injury in rats.

There was a concern over the specificity of such Vav2-mediated signaling mechanism to hHcys-induced glomerular injury and the influence of uninephrectomy on Vav2-mediated signaling. To address this issue, we compared the roles of Vav2 in this hHcys-induced glomerular injury with that in another animal model, namely, deoxycorticosterone acetate-salt hypertensive rats. This model is often produced under an uninephrectomy condition, and glomerular injury and fibrosis are commonly observed.^{23,24} These additional experiments showed that, similar to hHcys rats, uninephrectomized deoxycorticosterone acetate-salt rats suffered from increased urinary albumin and glomerular sclerosis with enhanced $O_2^{\cdot -}$ production. However, neither total Rac-Vav2 expression nor Rac activity was changed, which was different from a significant increase in Rac activity observed in hHcys rats (Figure S1). These results indicate that Vav2 is one of the mechanisms responsible for Hcys-induced NADPH oxidase activation but not for deoxycorticosterone acetate-salt-induced enhancement of NADPH oxidase activity. It appears that the involvement of Vav2 is not ubiquitous during oxidative stress-mediated renal injury in different models. Although these results may not be extended to other models of renal injury, the specificity of such Vav2-mediated mechanism may help develop specific interventions to prevent or reverse hyperhomocysteinemic renal injury. Given failures in many massive antioxidant therapeutic trials for renal injury under different pathological conditions,²⁵ an early mechanistic intervention of NADPH oxidase activation may be beneficial in that it will block the production of $O_2^{\cdot -}$ or other reactive oxygen species rather than scavenging them.

It should be noted that a local knockdown of the Vav gene attenuates Hcys-induced glomerulosclerosis possibly by *in situ* suppression of oxidative stress. This view was also supported by many other studies indicating that, more localized in cells or organs, this Vav-mediated Rac activation is fibrotic or sclerotic.^{11,26} However, a very recent study reported that, in Vav2 knockout mice, a mild collagen accumulation could happen in several organs, including kidneys.²⁷ Although controversial, it is not surprising to us that these mice with a globally deficient Vav2 may have generous injurious pathology given that many other Vav2-regulated signaling pathways may be malfunctioning, such as a chronic stimulation of the renin/angiotensin II and sympathetic nervous systems, as they proposed. Therefore, we should be cautious in explaining the pathogenic role of systemic action of Vav2 or global knockout.

To further determine the role of Vav2 in hHcys-induced glomerulosclerosis, we analyzed the effects of both silencing and overexpressing the Vav2 gene on extracellular matrix metabolism by examining the action on expression of TIMPs and activity of MMPs in the glomeruli.²⁸ Although the spatial expression of MMPs and TIMPs in the kidney is complex and

has not been completely characterized, MMP-1, -2, and -9 and their inhibitor TIMP-1 are the most abundant in rat glomeruli.²⁹ We demonstrated that hHcys induced upregulation of TIMP-1, which could be blocked by silencing the Vav2 gene and consequent inhibition of NADPH oxidase activity. Furthermore, MMP-1 and -9 activities were found decreased by hHcys, which were reversed by shRNA-Vav2. All of these results together support our view that Vav2 serves as a sclerogenous mechanism that may initiate the sclerotic cascade in glomeruli during hHcys that relates to activation of Rac and NADPH oxidase, local oxidative stress, abnormal extracellular matrix metabolism, and consequent sclerosis.

Perspectives

The present study addressed the role of Vav2 in the development of hHcys-induced glomerular injury in an experimental hHcys animal model produced by feeding rats an FF diet. The findings for the first time demonstrate that Vav2 in the kidney is importantly implicated in the development of glomerulosclerosis associated with hHcys, which represents one of the critical initiating mechanisms in the cascade of pathogenic factors resulting in glomerular injury and sclerosis. As a new pathogenic factor contributing to glomerular injury in hHcys, Vav2 may be an ideal target for therapeutic intervention in end-stage renal disease related to hHcys, which could be extended to the development of effective therapeutic strategy of degenerative diseases associated with hHcys.

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Disclosures

None.

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Online Supplements

Contribution of Guanine Nucleotide Exchange Factor-Vav2 to Hyperhomocysteinemic

Glomerulosclerosis in Rats

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Running title: Vav2-mediated renal injury in hyperhomocysteinemia

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Expanded Methods:

Gene transfer into the kidney by ultrasound-microbubble technique. The procedures for the ultrasound-microbubble gene transfer technique include: (1) mixing luciferase and designated plasmid (50 µg) in saline and microbubble (Optison, GE HealthCare) at a ratio of 3:1 vol/vol in 0.5 ml and injecting the mixed solution by 30G needle into the left renal artery with temporary clipping of the renal artery and vein (<5 min); (2) applying the ultrasound transducer (Ultax UX-301; Celcom Medico Inc., Japan) directly onto one side of the left kidney with a continuous-wave output of 1 MHz ultrasound at 10% power output, for a total of 60 s at 30-s intervals; (3) Finally, the renal artery and vein were unclipped after needle was taken off, and renal blood flow recovered. At the same time, one or two cotton tips were used to prevent bleeding at the puncture point of the renal artery ¹.

Immunohistochemical analysis for Vav isoforms in the rat kidney: The immunohistochemical (IHC) analysis was performed as described previously ^{2, 3}. Anti-Vav1, anti-Vav2 (Invitrogen, 1:100) and anti-Vav3 (Epitomics, 1:100) antibodies were used in this study.

Western blot analysis: Western blotting was performed as described previously ⁴. Anti-Vav2 (Invitrogen, 1:500), anti-Rac (Upstate, 1:1000), anti-TIMP-1 (R&D systems, 1:1000) and anti-β-actin (Sigma, 1:2000) antibodies were used in this study.

RNA extraction and real time RT-PCR: Total RNA was isolated from renal glomeruli using TRIzol reagent (GIBCO, Life Technologies). The mRNA levels for target genes were analyzed

by real-time RT-PCR using a Bio-Rad iCycler system. The mRNA levels were calculated in accordance with the delta-delta Ct method using levels of 18S ribosomal RNA as endogenous controls.^{4,5} The primers for Vav2 (Accession number XM_216030): 5'-CCATAGTCAACCACACCAAGCA-3' and 5'-GAGCATCGACATCGGGAAGGC-3'. The primers for TIMP-1 (Accession number NM_003254): 5'-CCAGAAATCATCGAGACCAC-3' and 5'-CGGAAA CCTGTGGCATT-3'. The primers for 18S: 5'-GCGCTAGACTCCGAGAACAT-3' and 5'-TG GCCACTTAC TACCTGACCC TT-3'.⁶

Morphological examination: The fixed kidneys were paraffin-embedded, and sections were prepared and stained with periodic acid–Schiff stain. Glomerular injury index was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion as described⁴. In general, we counted 80-100 glomeruli in total in each kidney slice under microscope, when each glomerulus was graded level 0-4 damages. 0 represents no lesion, 1+ represents sclerosis of <25% of the glomerulus, while 2+, 3+, and 4+ represent sclerosis of 25% to 50%, >50% to 75%, and >75% of the glomerulus. A whole kidney average sclerosis index was obtained by averaging scores from counted glomeruli. This observation was examined by 3 investigators and averaged under blind conditions without prior knowledge as to which section belonged to which rat.

Hyperhomocysteinemia Rats and DOCA-salt-hypertensive Rats: Experiments were performed using Sprague-Dawley (SD) rats (200 g, 6 weeks old) (Harlan, Inc). All experimental rats were uninephrectomized. After a 1-week recovery period from uninephrectomy, animals were divided into three groups to receive three different diets for 4 weeks: normal diet, folate

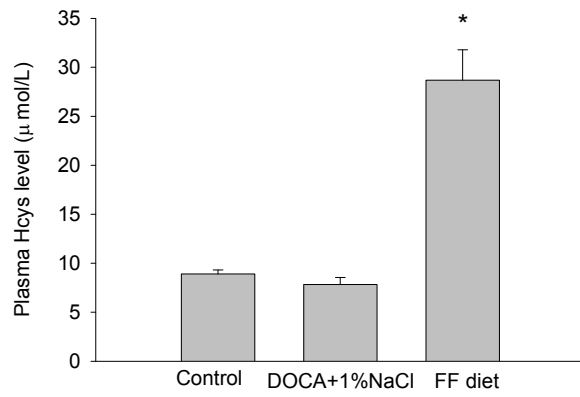
free (FF) diet and normal diet with twice/week subcutaneous injections of DOCA (30 mg/kg body wt; Sigma) suspended in sesame oil and 1% NaCl drinking water⁷. Over the experimental days, blood and a 24-hour urine sample were collected. Plasma total homocysteine (tHcys) was measured by fluorescence HPLC analysis, and urinary albumin excretion was measured using a rat albumin ELISA quantitation kit (Bethyl Laboratories)⁴.

References

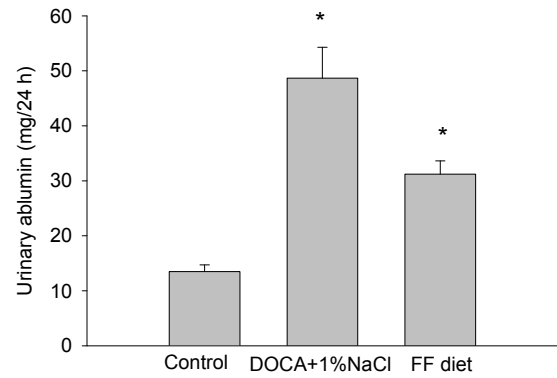
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Supplemental Results:

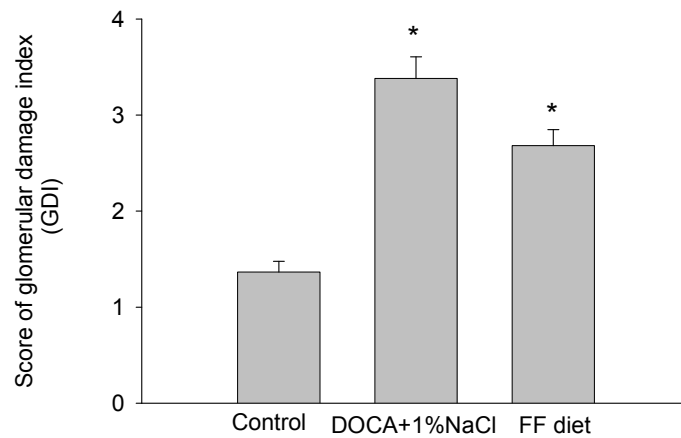
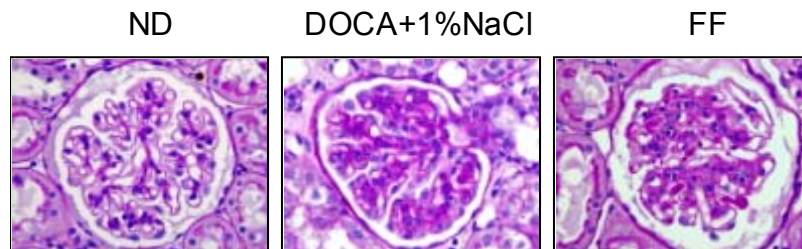
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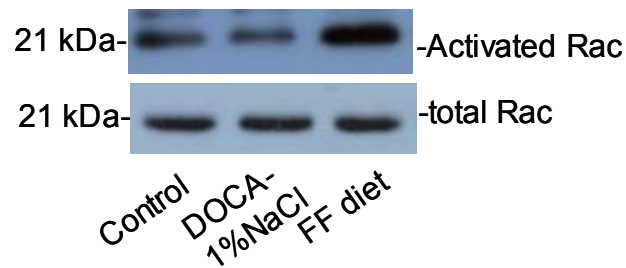
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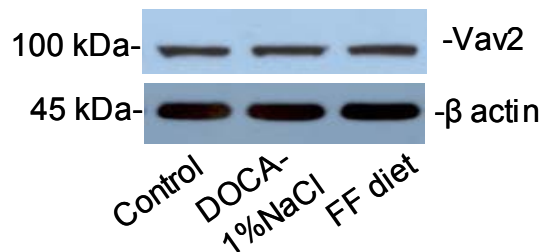


Figure. S1. Comparison of glomerular injury and related signaling in hyperhomocysteinemia induced by folate free (FF) diet and DOCA-salt treated (1% NaCl) rats. A: Average plasma total Hcys level in different groups including uninephrectomized rats on a normal diet, uninephrectomized DOCA-salt treated (1% NaCl) rats and uninephrectomized rats on a folate-free (FF) diet. B: Urinary albumin excretion in these different group rats as indicated. C: Photomicrographs (original magnification x250) showing typical glomerular structure and summarized glomerular damage index (GDI) by semiquantitation of scores in these different group rats as indicated. D: Immunoblot analysis of activated Rac by pull down assay and total Rac expression by general Western blot analysis in different group rats as indicated. E: Immunoblot analysis of Vav2 expression through Western blot analysis in these different group rats as indicated (n=5). * $P < 0.05$ compared with control.