Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;20:909-918

Accepted: August 16, 2007

Mechanism of Homocysteine-Induced Rac1/ NADPH Oxidase Activation in Mesangial Cells: Role of Guanine Nucleotide Exchange Factor Vav2

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Key Words
Small G -protein • Redox signaling • Sphingolipids • Hyperhomocysteinemia • Kinase

Abstract
We have demonstrated that homocysteine (Hcys) stimulates *de novo* ceramide synthesis and thereby induces NADPH oxidase activation by increase of Rac GTPase activity in rat mesangial cells (RMCs). However, which isofrom of Rac GTPases is involved in Hcys-induced NADPH oxidase activity and what mechanism mediates Hcys-induced Rac GTPase activation remain unknown. The present study first addressed the role of Rac1 and then determined the contribution of a subfamily of Guanine Nucleotide Exchange Factors (GEFs), Vav, to the action of Hcys on Rac and NADPH oxidase activities in RMCs. By small interfering RNA (siRNA), it was found that Rac1-siRNA attenuated Hcys-induced superoxide ($O_2^{\cdot-}$) production. To explore the mechanism activating Rac by Hcys, GEF-Vav was examined. Vav2 was found to be a predominant isoform among Vav family in RMCs. In Vav2-siRNA transfected RMCs, Hcys-induced Rac activity was blocked, which was accompanied by significant reduction of Hcys-induced $O_2^{\cdot-}$ production. This Vav2-siRNA also blocked Rac activation induced by C16-Ceramide (C16-Cer), an intermediate lipid product stimulated by Hcys. Furthermore, we found that Hcys induced Vav2 phosphorylation in a time-dependent manner, which could be induced by C16-Cer and blocked by inhibition of *de novo* ceramide synthesis. These results suggest that Vav2 importantly contributes to Hcys-induced increase in Rac1 activity and consequent activation of NADPH oxidase in RMCs via ceramide-associated tyrosine phosphorylation.

Introduction

Hyperhomocysteinemia (hHcys) is known as a critical pathogenic factor in the progression of end-stage renal disease (ESRD) and in the development of cardiovascular complications related to ESRD [1-4]. We and others have demonstrated that oxidative stress mediated by NADPH oxidase is importantly involved in the progressive glomerular injury associated with hHcys [5-8]. NADPH oxidase is a multiprotein complex in which cytosolic subunits ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and Rac

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GTPase) assemble with membrane-associated subunits (NOX and p22^{phox}) to generate superoxide ($O_2^{\cdot-}$). Nox proteins are a family of gp91^{phox}-like proteins which include several known members, namely, NOX1, NOX2 (gp91^{phox}), NOX3, NOX4, and NOX5, DUOX1 and DUOX2 [9-12]. Activation of this redox signaling enzyme complex is dependent on the assembly of all subunits to the membrane that involves several regulatory mechanisms, such as p47^{phox} phosphorylation and translocation, Rac GTPase activation and protein–protein interactions between subunits [9, 13, 14]. Previous studies in our laboratory have demonstrated that Hcys stimulated *de novo* ceramide synthesis and thereby induced NADPH oxidase activation by enhancing Rac GTPase activity in RMCs [6, 7]. However, the mechanism by which Hcys increases Rac GTPase activity and consequent activation of NADPH oxidase in these glomerular cells remains unknown.

Rac GTPases are members of Rho-family GTPases which serve as an important molecular switch in cells regulating many biological activities such as cell growth, apoptosis, differentiation, adhesion, migration and vesicle trafficking [15, 16]. This diverse range of activities is paralleled by the interaction of Rac GTPases with different signaling molecules or effectors such as serum response factor (SRF), nuclear factor kB (NF-kB) transcription factors, c-*jun* N-terminal kinase (JNK), p38 mitogen-activated protein kinase and NADPH oxidase complex [15-18]. It has been documented that there are three highly homologous Rac GTPases in mammals, including Rac1 ubiquitously distributed, Rac2 expressed in neutrophils, and Rac3 predominantly found in the central nervous system [10]. These Rac GTPases control cellular processes by cycling between two conformational states: a GTP-bound "active" state and a GDP-bound "inactive" state. The transition between these two conformations is tightly regulated by three classes of proteins, which are GTPase activating proteins (GAPs) stimulating the intrinsic GTPase activity and thereby pushing the switch towards the inactive state, guanine nucleotide dissociation inhibitors (GDIs) that keep the GTPases in the GDP-bound inactive conformation, and guanine nucleotide exchange factors (GEFs) facilitating the exchange of GDP for GTP and thus activating Rac GTPase [19]. Among these regulatory proteins, GEFs are directly responsible for the activation of Rac GTPase and therefore play a central role in the regulation of Rac GTPase activity. So far, a well-studied subfamily of GEFs regulating the activity of Rac GTPases is the Vav family [10, 20, 21]. Very recent studies have indicated the critical role of Vav family in the regulation of NADPH oxidase activity. For example, Miletic *et al* reported that Vav family as critical mediators of LPS-induced MyD88-dependent activation of Rac GTPase and NADPH oxidase [22]. Other studies also demonstrated that the essential role of Vav for the regulation of NADPH oxidase activity by activation of Rac GTPase in neutrophils [23, 24]. However, whether this Vav family is involved in Hcys-induced NADPH oxidase activity associated with activation of Rac GTPase is still unknown. The present study was designed to first elucidate the role of ubiquitously distributed Rac1 in Hcys-induced NADPH oxidase activity in RMCs by gene silencing and then to determine the contribution of the Vav family to Hcys-induced increase in Rac GTPase activity and NADPH oxidase activity in RMCs. Our results suggest that Vav2 is a predominant isoform of GEF-Vav subfamily in RMCs and that Vav2 participates in Hcys-induced increases in Rac1 activity and consequent activation of NADPH oxidase. The *de novo* ceramide synthesis is importantly involved in Hcys-induced Vav2 activity, which is associated with its tyrosine phosphorylation.

Materials and Methods

Culture of RMCs

RMCs were obtained from the American Type Culture Collection (ATCC) and incubated and propagated in Dulbecco's modified Eagle's medium (DMEM) containing 18 mM sodium bicarbonate, 25 mM glucose, 0.6 μM G418, and 15% fetal Bovine serum (FBS) with 4 mM L-glutamine at 37°C in 5% CO_2 atmosphere as we described previously. In the present study, the concentration and the incubation time of L-Hcys treatment were chosen based on previous studies [6].

RNA extraction and real time RT-PCR

Total RNA was isolated from renal tissue or RMCs using TRIzol reagent (GIBCO, Life Technologies, Carlsbad, CA) according to the protocol described by the manufacturer. The mRNA levels for Vav isoforms were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) according to the protocol described by the manufacturer. The mRNA level was normalized to the 18S mRNA. The specific primers for Rac-1 based on the core sequence of rat Rac1 cDNA (Accession number NM_134366) were 5'-GCG TTC CCT GGA GAG TAC CT-3' (Rac-1-Forward) and 5'-AAT CTT CCT GTC CAG CTG TGT (Rac-1-Reverse). The primers for Vav1 based on the core sequence of rat Vav1 cDNA (Accession number NM_012759) were 5'-ATT TCT GCG GCT CAA CCC T-3' (Forward) and 5'-GTA TTT CTT CCC TCC CAC CAG-3' (Reverse). The primers for Vav2 based on the core sequence of rat Vav2 cDNA (Accession number XM_216030) were 5'- GTT CAA ACT CGG AAG TCA GG -3' (Forward) and 5'-

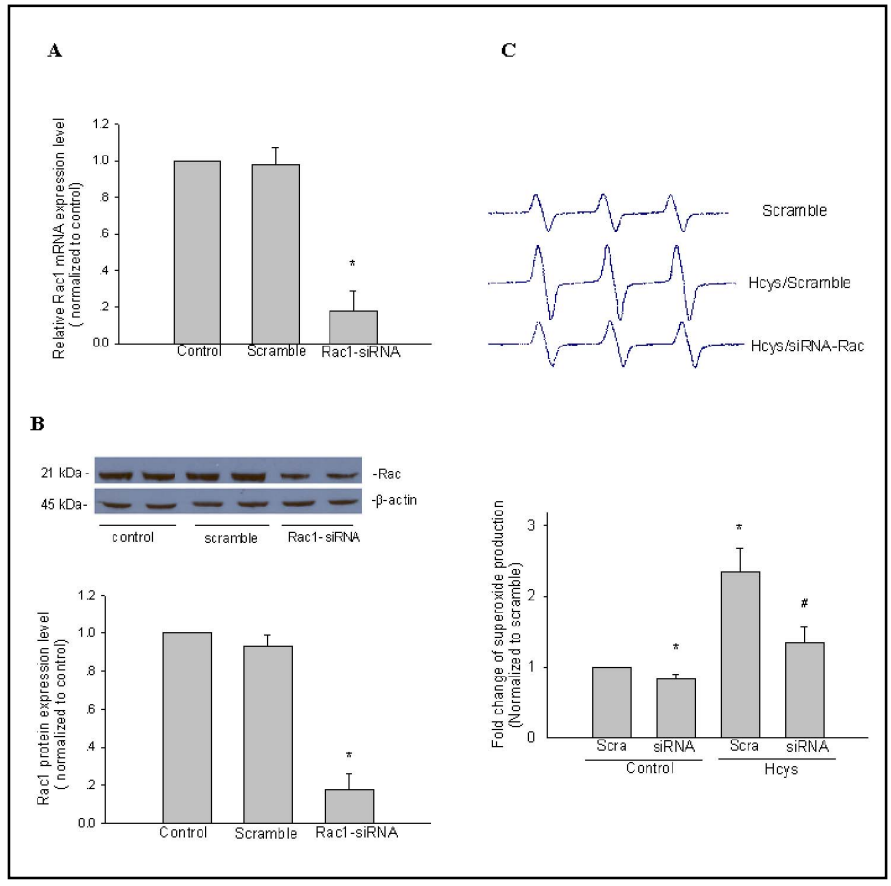


Fig. 1. Effects of Rac1-siRNA on Hcys-induced NADPH oxidase activity. A: Quantitative RT-PCR analysis of Rac1 mRNA levels in control, scrambled RNA and siRNA-Rac1 transfected RMCs. B: Representative Western blot gel documents (upper panel) and summarized data (bottom) showing relative Rac1 protein levels in control, scrambled RNA- or siRNA-Rac1-treated RMCs after normalized to β-actin. C: Representative Eelectronic Spin Resonance (ESR) spectra (upper panel) and summarized data (bottom) depicting $O_2^{\cdot-}$ production in Rac1-siRNA transfected RMCs with Hcys treatments. * P<0.05 vs. control, # P < 0.05 vs. vehicle of Hcys treatment (n=6).

CCA CGG GTA TGC AGT GTA AT -3' (Reverse). The primers for Vav3 based on the core sequence of rat Vav3 cDNA (Accession number XM_227600) were 5'-TCC CGA ACA CCA ATA GCA T-3' (Forward) and 5'- ACG GGT TTC ATC TAT CAG GTC-3' (Reverse). The primers for 18S were 5'-GCG CTA GAC TCC GAG AAC AT-3' (18S-Forward) and 5'-TGG CCA CTT ACT ACC TGA CCC TT-3' (18S-Reverse). Relative mRNA expression levels were normalized to control group.

RNA interference

Small interference RNAs (siRNAs) were purchased from QIAGEN. The DNA target sequence for Rac1-siRNA is 5'-AAG AGA TCG GTG CTG TCA AAT-3', which is from the C-terminal of region Rac1. The DNA target sequence for Vav2-siRNA is 5'- AAG GAG AGG TTC CTT GTT TAT-3'. The scrambled small RNA (AAT TCT CGA ACT GTC ACG T) has been confirmed as non-silencing double stranded RNA and was used as control in the present study. In these experiments, siRNA transfection was performed according to the manufacturer's instruction in Bio-Red siLentFect™ Lipid transfection kit.

Rac GTPase activation assay

A specific pull-down experiment was performed to determine Rac GTPase activation using a Rac activation assay kit (Upstate, Lake Placid, NY) as described previously [6]. Briefly, the RMCs were lysed in Mg^{2+} lysis buffer (MLB). After precleaned by glutathione agarose, the pull-down proteins (500 µg per sample) were incubated with 10 µg of PAK-1 PBD agarose for 1 h at 4°C to bind Rac-GTP. Then, the pelleted beads were washed three times with MLB, resuspended in 25 µL of 2X Laemmli reducing sample buffer, and boiled for 5 minutes. The sample mixtures were then loaded on SDS-PAGE gels. The bound active GTP-Rac was analyzed by Western blotting using anti-Rac monoclonal antibody.

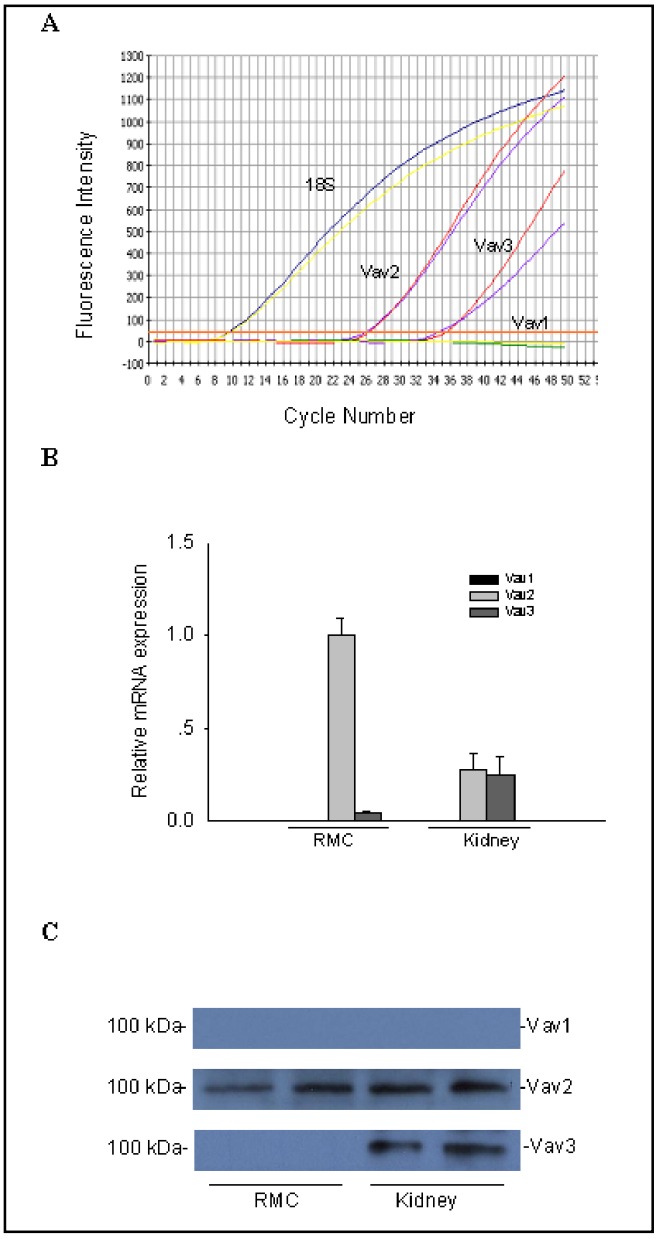
Western blot analysis

Western blotting was performed as described previously [7]. Briefly, 50 µg proteins were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked. The membrane was probed with primary polyclonal antibodies: anti-Vav1, anti-Vav2 (Invitrogen, 1:500), anti-Vav3 (Epitomics, 1:500) and pVav2 (Tyr^{172}) (Santa Cruz, 1:500) overnight at 4°C followed by incubation with horseradish peroxidase-labeled anti-rabbit IgG (1:5000). The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat film. All films with immunoreactive blots were scanned by a densitometer and the intensity of corresponding protein bands was quantitated using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT, USA). Relative protein expression levels were normalized to control group.

$O_2^{\cdot-}$ detection by Eelectronic Spin Resonance (ESR)

The measurement of $O_2^{\cdot-}$ by ESR was followed by our previous studies [25]. Gently collected RMCs were suspended in modified Kreb's/HEPEs buffer containing deferoximine (25 µM, metal chelator). Approximately 1 x 10^6 RMCs were mixed

Fig. 2. Expression profile of Vav isoforms in RMCs and in dissected renal tissue. A: Representative fluorescence monitored applification curves of PCR products for different isoforms of Vav. B: Relative quantitation of mRNA levels in Vav2 and Vav3 by real-time RT-PCR analysis in RMC and renal tissue. C: Representative Western blot for the detection of protein levels of the Vav family (n=6).



with 1 mM spin trap 1-hydroxy-3-methoxycarbonyl-2, 2, - 5, 5-tetrame-thyl-pyrrolidine (CMH) in the presence or absence of 100 units/ml polyethylene glycol (PEG)-conjugated superoxide dismutase (SOD). The cell mixture loaded in glass capillaries was immediately analyzed by ESR (Noxygen Science Transfer & Diagnostics GmbH, Denzlingen, Germany) for production of $O_2^{\cdot-}$ at each minute for 10 min. The ESR settings were based on our previous studies. The changes of production of $O_2^{\cdot-}$ were normalized to control group.

Immunofluorescent microscopy

Immunoflurescence staining was performed as described previous [4]. General, RMCs treated with Hcys or C16-Cer at different time were incubated for 1 hour at room temperature with rabbit anti-pVav2 (1:150, Santa Cruz) by incubation with Texas Red–conjugated anti-mouse antibody (Santa Cruz) for an additional 2 hours at room temperature. The slides were examined by fluorescent microscopy with a Nikon 40 Plan Apo oil-immersion lens. The images were captured with a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and exported into Adobe Photoshop 7.0. All exposure settings were kept constant for each group of cells.

Statistics

Data are presented as means ± SE and normalized to control. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test Student's t-test was used to evaluate the significant differences between two groups of observations. $P < 0.05$ was considered statistically significant.

Results

Effect of Rac1 gene silencing on Hcys-induced NADPH oxidase activity

Although previous studies have demonstrated the involvement of Rac GTPase in Hcys-induced activation of NADPH oxidase in RMCs, it remains unknown which isoform of Rac is mainly responsible for the action of Hcys. Given the ubiquitous expression of Rac1, we examined the role of this Rac isoform in Hcys actions using specific gene silencing. As shown in Figure 1A, real time RT-PCR analysis revealed that Rac1 mRNA level decreased by 81% in Rac1-siRNA transfected RMCs. Western blot analysis showed that Rac1 protein level was reduced by 76% (Figures 1B). In these siRNA transfected RMCs, Hcys-induced increase in $O_2^{\cdot-}$ production was blocked by 67%. This Rac1 RNA interference also led to a 12% reduction of basal $O_2^{\cdot-}$ production in RMCs (Figure 1C).

Vav2: a predominant isoform of Vav family in RMCs

To detect the expression of Vav family in cultured RMCs, real-time quantitative RT-PCR and Western blot analysis of isoforms of Vav family were performed. In these experiments, renal tissue was used to detect the expression of Vav family members. As shown in Figure

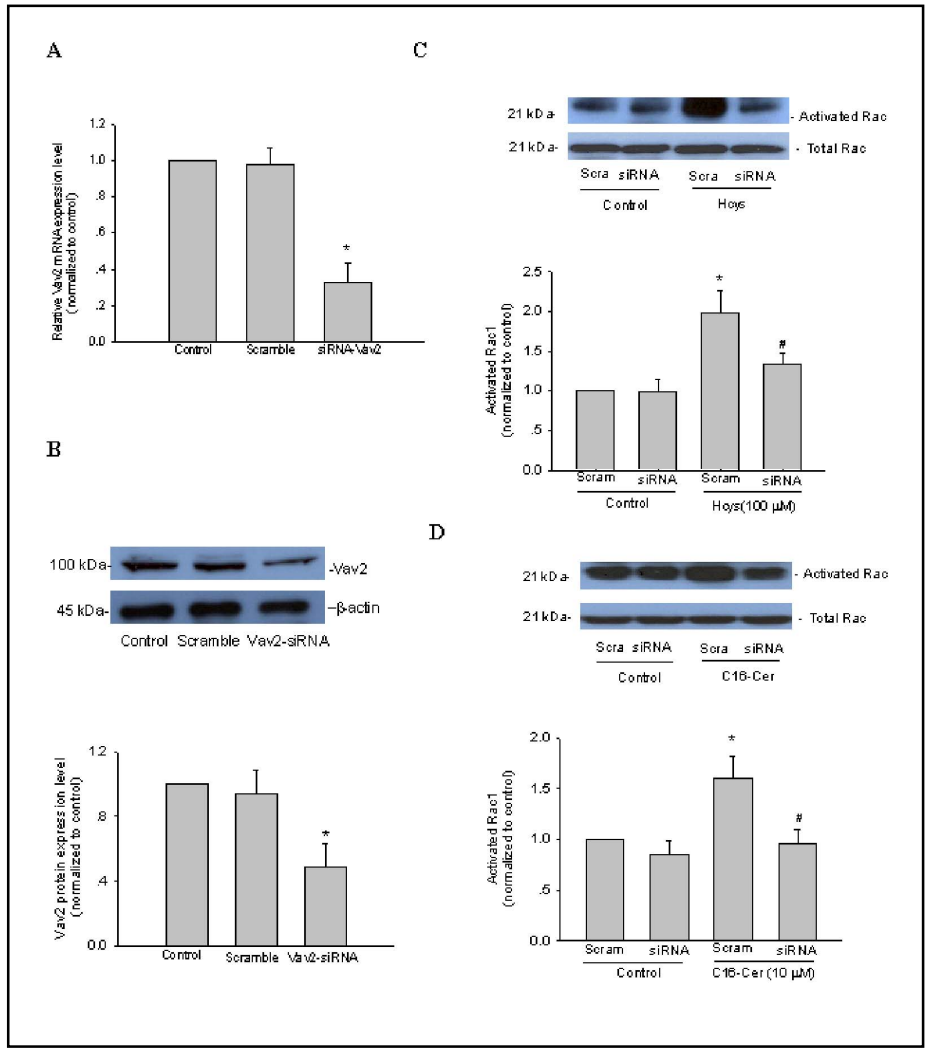


Fig. 3. Effect of Vav2 silencing on Hcys-induced Rac1 activity in RMCs. A: Quantitative RT-PCR analysis of Vav2 mRNA levels in control, scrambled RNA and siRNA-Vav2 transfected RMCs. B: Representative Western blot gel document (upper panel) and summarized data (bottom) showing relative Vav2 protein levels in control, scrambled RNA or Vav2 siRNA-treated RMCs after normalized to β-actin. C: Representative Western blot gel document (upper panel) and summarized data (bottom) showing Hcys-induced Rac1 activity in Vav2 siRNA transfected RMCs. D: Representative Western blot gel document (upper panel) and summarized data (lower panel) showing C16-Cer-induced Rac1 activity in Vav2 siRNA transfected RMCs. * $P < 0.05$ vs. control, # $P < 0.05$ vs. vehicle (n=6).

2A and B, by real time RT-PCR analysis it was found that Vav2 and Vav3 but not Vav1 were detected in both RMCs and renal tissue. The mRNA level of Vav2 was 20-fold higher than that of Vav3 in RMCs, but both Vav2 and Vav3 had similar abundance in renal tissue. Western blot analysis showed that Vav2 and Vav3 could be detected in renal tissue; however, only Vav2 could be detected in RMCs as shown in Figure 2C. Based on these results, it seems that Vav2 is a predominant isoform of Vavs in RMCs.

Contribution of Vav2 to Hcys-induced Rac1 activation

Since there is no specific inhibitors for Vav2, siRNA for Vav2 gene silencing was used to determine the role of Vav2 in the regulation of Hcys-induced Rac GTPase and NADPH oxidase activity. By real-time RT-PCR and Western blot analysis, Vav2 mRNA and protein expressions in RMCs were decreased by 63% and 52 %, respectively when these cells were transfected

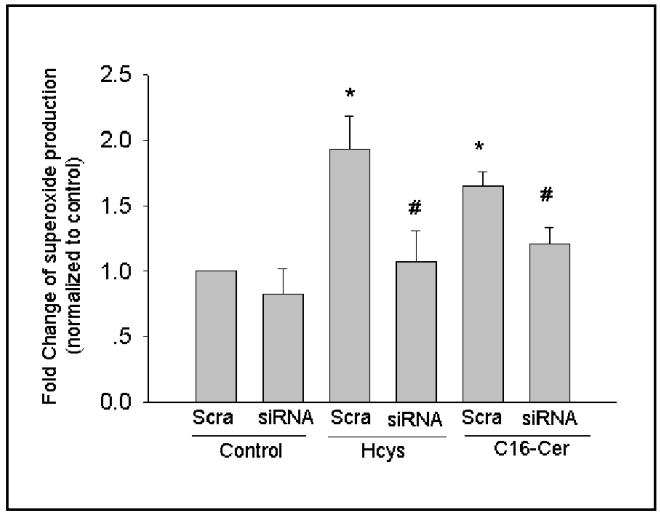


Fig. 4. Effect of Vav2 siRNA on Hcys-induced NADPH oxidase activity. Summarized data depicting O_2^- production in Vav2-siRNA transfected RMCs with Hcys treatment, which was analyzed by ESR spectrometry. * $P < 0.05$ vs. control, # $P < 0.05$ vs. vehicle (n=6).

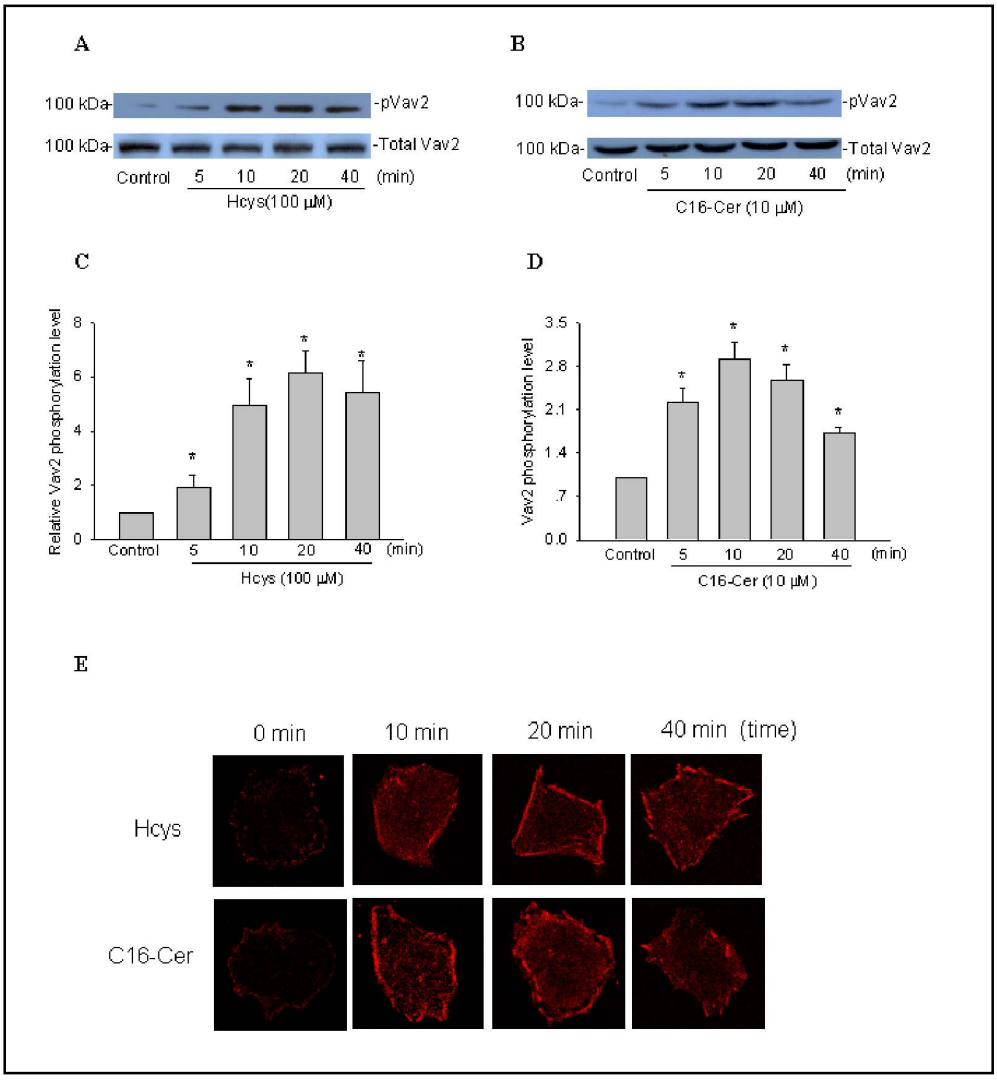


Fig. 5. Effect of Hcys or C16-Cer on Tyr[172] phosphorylation of Vav2. A: Immunoblot for pVav2 (upper) and total Vav expression levels (bottom) in Hcys-treated RMCs. B: summarized data showing changes in Vav2 phosphorylation in Hcys-treated RMCs. C: Immunoblot for pVav2 (upper) and total Vav expression levels (bottom) in C16-Cer-treated RMCs. D: Summarized data showing changes in Vav2 phosphorylation in C16-Cer-treated RMCs. E. Immunofluorescent microscopic images showing that phosphorylated Vav2 was mainly located on the membrane after Hcys and C16-Cer treatments * $P < 0.05$ vs. control (n=6).

with Vav2-siRNA (Figure 3A and B). As shown in Figure 3C, in RMCs treated with scrambled small RNA (control), Hcys produced a significant increase in GTP-bound Rac (activated Rac) protein levels, which was significantly blocked by Vav2-siRNA. Similar results were obtained from C16-Cer-treated RMCs as shown in Figure 3D. C16-Cer was used because this sphingolipid was confirmed to mediate the action of Hcys on activation of Rac-GTPase [6].

Inhibition of Hcys-enhanced NADPH oxidase activity by Vav2 siRNA

In previous studies, we reported that the consequence of Hcys-induced activation of Rac GTPase is increases in NADPH oxidase activity. Therefore, we then determined the effect of Vav2 siRNA on NADPH oxidase activity. By ESR analysis, we found that both Hcys and C16-Cer significantly increased $O_2^{\cdot-}$ in RMCs by 93% and 72% in RMCs, respectively, When these cells were transfected with Vav2-siRNA, Hcys- or C16-Cer-induced increases in $O_2^{\cdot-}$ production were markedly blocked (Figure 4).

Association of Tyrosine-phosphorylation with Hcys-induced Vav2 activity

To explore the mechanism by which Hcys and its intermediate action product, ceramide increase Vav2 activity, we first examined whether the effect of Hcys is associated with increased expression of this GEF in RMCs. It was found that neither mRNA nor protein expression of Vav2 had significant change when these cells were treated with Hcys or C16-Cer (data not shown). Next, we tested whether the phosphorylation of an analogous residue Tyr[172] in Vav2 is altered by Hcys, since the activity of Vav1 is regulated by phosphorylation of a conserved tyrosine 174 (Tyr[174]) [26]. Western blot analysis and immunofluorescence analysis were performed for this purpose. As shown in Figure 5A-D,

both Hcys and C16-Cer induced Tyr[172] phosphorylation of Vav2 in a time-dependent manner. These Vav2 phosphorylation was further confirmed by immunofluorescence of RMCs with anti-pVav2 (Tyr[172]) antibody as shown in Figure 5E. It was shown that Vav2 was evenly spread throughout the cell under control condition (0 min) as indicated by weak diffuse fluorescence (Texas red). Upon stimulation with Hcys or C16-Cer, there were aggregated pVav2 detected on the cell membrane. These strong fluorescence signals turned weak in 30 min after C16-Cer treatment. However, red fluorescence kept relative strong in Hcys-treated RMCs for a long time period.

Blockade of Hcys-induced Vav2 phosphorylation by inhibition of de novo ceramide synthesis pathway

To further examine whether Hcys-stimulated Vav2 phosphorylation is associated with enhanced production of ceramide in RMCs, we examined the effects of inhibition of *de novo* ceramide synthesis on the increase in Vav2 phosphorylation induced by Hcys. As shown in Figure 6, Western blot analysis of pVav2 demonstrated that Hcys significantly increased Vav2 phosphorylation levels. In the presence of fumonisin B_1 (FB_1), an inhibitor of *de novo* ceramide synthesis, Hcys-induced increase in Vav2 phosphorylation was substantially attenuated. The results were summarized in Figure 6B, where the density of pVav2 specific blots was higher in cells treated with Hcys alone, both not in cells treated with Hcys plus FB_1.

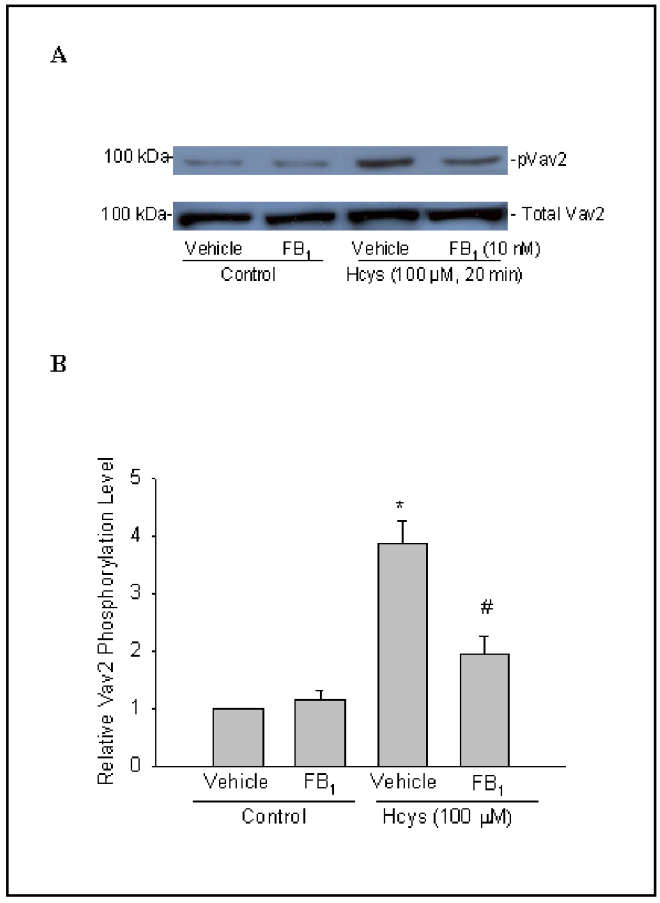


Fig. 6. Effect of fumonisin B_1 (FB_1, 10 nM) on Tyr[172] phosphorylation of Vav2 in RMCs treated with Hcys. A: Immunoblot for pVav2 (upper) and total Vav expression levels (bottom) in RMCs. B: Summarized data showing changes in Vav2 phosphorylation in RMCs. * $P < 0.05$ vs. control, # $P < 0.05$ vs. vehicle.

Discussion

In the present study, we examined the role of Vav2-mediated Rac1 signaling pathway in Hcys-induced NADPH oxidase activity in RMCs. By using siRNA to knockdown Rac1, we successfully inhibited the expression of this enzyme and attenuated Hcys-induced activation of NADPH oxidase. Furthermore, we characterized the expression of Vav, a subfamily of GEFs in RMCs and renal tissue and found that Vav2 was a predominant isoform of GEF-Vav subfamily in RMCs. Finally, we demonstrated that Vav2 played a contributing role in Hcys-induced increase in Rac1 activity and consequent activation of NADPH oxidase in RMCs and that Hcys-induced *de novo* ceramide synthesis was importantly involved in Hcys-enhanced Vav2 activity, which is associated with its tyrosine phosphorylation.

It is well known that NADPH oxidase-mediated redox signaling importantly contributes to the regulation of cell functions in many mammalian cells [10, 27]. In regard to the regulation of this enzyme, numerous studies have demonstrated that the contributing role of Rac GTPase to NADPH oxidase activity [14, 28]. Rac GTPase as a regulator of NADPH oxidase was first described in phagocytes [18, 29], where Rac2 controls $O_2^{\cdot-}$ production. Recently, Rac1-mediated NADPH oxidase activity has been demonstrated to be a major isoform in nonphagocytic cells such as vascular endothelial and smooth muscle cells and cardiac myocytes, which may mediated the action of different stimuli including angiotensin II (Ang II), PDGF, and endothelin [6, 14, 30-33]. There is general agreement that activation of Rac is associated with recruitment of $p67^{phox}$, which associates with $p47^{phox}$ and with the cytochrome. Although previous

studies in our laboratory have demonstrated that Hcys increased Rac GTPase and NADPH oxidase activity using pharmacological assays such as a small GTPase inhibitor GDPβS [6] and a Rac GTPase specific inhibitor NSC23766 [34], these studies did not attempted to address which specific isoform of Rac exerts actions in activation of NADPH oxidase. In the present study, we performed gene silencing by siRNA to inhibit Rac1 expression and then detected the role of this Rac1 in the regulation of NADPH oxidase activity induced by Hcys in RMCs. This RNA interference approach is considered as a more specific and powerful method than traditional pharmacological interventions, which may more definitively define the role of for Rac1 in Hcys-induced NADPH oxidase activation. It was found that Hcys-increased $O_2^{\cdot-}$ production was significantly blocked by Rac1 siRNA. These results suggest that Rac1 importantly contributes to the action of Hcys in these RMCs.

Next, we explored the mechanisms by which Hcys activates Rac1 in RMCs. In this regard, GEFs are directly responsible for the activation of Rac/Rho GTPases in response to diverse extracellular stimuli and regulate numerous cellular responses such as proliferation, differentiation and movement [35]. Over 90 distinct GEFs in mammals, Dbl-related GEFs represent the largest family of direct activators of Rac/Rho GTPases including several subfamilies of proteins such as Vav, Tiam1, Dbs, ITSN-L, Trio and LARG [35]. Among them, Vav and Tiam1 are GEFs that exhibit high specificity to Rac GTPase-mediated NADPH oxidase activity [22, 24, 36]. In our preliminary studies, however, immunohistochemical and Western blot analyses have shown that Tiam1 is not detectable in the kidney or RMCs, suggesting that Tiam1 may not be abundantly expressed in the kidney. Therefore, we wondered whether the Vav family of GEFs is involved in Hcy-induced Rac1 and NADPH oxidase activation. It has been reported that the Vav family consists of three known members – Vav1, Vav2 and Vav3. The first member of Vav family to be described, initially termed Vav and now known as Vav1, is a hematopoietic cell-specific signal transducer protein [37]. Vav2 and Vav3 are expressed ubiquitously [20, 38]. All Vav proteins contain several characteristic structural motifs that enable their function as signal transducer proteins. From the N terminus to the C terminus, they include a calponin homology domain, an acidic-rich domain, which functions as an actin-binding domain in other proteins; a Dbl homology (DH) domain, which exhibits GEF activity towards the Rho family GTPases; a PH domain, which interacts with polyphosphoinositides; an Src homology 2 domain (SH2), and two Src homology 3 (SH3) domains that mediate protein–protein interactions. In the present study, we first determined the expression profile of the Vav family members in RMCs and renal tissues. By real time RT-PCR and Western blot analyses, Vav2 and Vav3 but not Vav1 were detected in the kidney, which is consistent with previous studies showing that Vav1 is predominantly expressed in hematopoietic cells, Vav2 and Vav3 are more widely expressed. In cultured RMCs, mRNA level of Vav2 was 20-fold higher than that of Vav3. Moreover, we failed to detect Vav3 in RMCs despite evidence its expression in whole kidney tissue. Together, it is indicated that Vav2 is a predominant isoform expressed in RMCs. Therefore, this GEF may represent a RMC-specific candidate to mediate the activation of Rac GTPase induced by Hcys in these cells. To test this hypothesis, we used small RNA interference strategy to determine the importance of Vav2 in mediating Hcys-induced activation of Rac and consequent NADPH oxidase. It was found that Hcys-induced increases in Rac1 activity were significantly attenuated by Vav2-siRNA. Similarly, NADPH oxidase activity increased by Hcys was completely abolished. These results suggest that Vav2 may determine the action of Hcys that activates Rac1 and thereby enhances NADPH oxidase activity. In fact, a very recent study from Miletic AV *et al* have identified the Vav family of GEFs as critical mediators of LPS-induced MyD88-dependent activation of Rac2, NADPH oxidase, and ROS production using mice deficient in Vav1, Vav2, and Vav3. They also found that Vav proteins including Vav2 are required for p38 MAPK activation and for normal regulation of proinflammatory cytokine production, indicating the important role of Vav2-medicated NADPH oxidase activity in the regulation of cell functions in other cells [22].

Previous studies in our laboratory have demonstrated that Hcys-induced Rac activation is associated with enhanced *de novo* ceramide synthesis [6] and that inhibition of *de novo* ceramide synthesis by FB_1 attenuated Hcys-induced increase in ceramide production, Rac GTPase and NADPH oxidase activity in RMCs. It is concluded that ceramide-mediated signaling is of importance in Hcys-induced enhancement of Rac GTPase activity in RMCs. Therefore, we hypothesized that Vav2 may contribute to the effects of Hcys-stimulated ceramide production on Rac1 activity. In the present study, exogenous C16-Cer was used to further demonstrate the action of ceramide in activation of Rac1 through Vav2. In control RMCs, this ceramide was found to stimulate Rac1 activity, while in Vav2-siRNA transfected RMCs

ceramide-induced stimulatory action on Rac GTPase disappeared. By measurement of $O_2^{\cdot-}$ production using ESR, Vav2-siRNA also blocked C16-Cer-induced increase in NADPH oxidase activity. From these results, we believe that Hcys may stimulate Vav2 and lead to activation of Rac GTPase through ceramide production in RMCs.

To further explore the mechanism by which Hcys or ceramide enhances the activity of Vav2 to activate Rac GTPase, we first examined whether increased Vav2 activity in Hcys or ceramide treated RMCs is associated with upregulation of this GEF. However, it was demonstrated that either Hcys or C16 ceramide did not alter Vav2 expression at both mRNA and protein levels. This suggests that Hcys or ceramide does not exert their action through increase in Vav2 expression. Then, we wonder whether other regulatory mechanism is involved in Hcys-induced enhancement of Vav2 activity. In this regard, there is evidence that at the resting state, the GEF activity of Vav1 is inhibited by its N-terminal arm, which occludes the DH domain essential for interactions with small G proteins. When Tyr^{174} in the N-terminal arm of Vav1 is phosphorylated by stimulation, this arm is released from the DH domain, resulting in the exposure of the site and producing GEF activity and consequent activation of Rac [20]. To determine whether this phosphorylation is involved in the action of Hcys and ceramide on Rac GTPase, we addressed the effects of Hcys on the phosphorylation of an analogous residue Tyr^{172} in Vav2. Both immunofluorescent microscopy and Western blot analysis showed that either Hcys or C16-Cer stimulated Tyr^{172} phosphorylation of Vav2 in RMCs. It is clear that that Hcys or ceramide enhances Vav2 activity through increase in its phosphorylation at Tyr^{172}.

Despite these findings that both Hcys and ceramide produced a similar action increasing Tyr^{172} phosphorylation of Vav2 in RMCs, it remains unclear at this point whether Hcys-induced Vav2 phosphorylation is associated with enhanced ceramide synthesis. To answer this question, we examined the effect of *de novo* ceramide synthesis inhibitors on Hcys-induced Vav2 phosphorylation in these cells. Our results demonstrated that the inhibitor of *de novo* ceramide synthesis, FB_1, blocked the action of Hcys on Vav2 phosphorylation. These results suggest that the *de novo* synthesized ceramide is involved in Hcys-induced increases in Vav2 activity.

It should be noted that the present study did not attempt to address how Hcys or ceramide stimulates tyrosine phosphorylation of Vav2. However, recent studies have reported that ceramide-induced enhancement of Vav2 activity may be related to activation of tyrosine kinase Src [39, 40]. In other studies, Src was reported to induce phosphorylation of Vav and other GEFs such as Tiam1 [41-43]. Therefore, it is very possible that Hcys or production of ceramide stimulated by Hcys may increase activity of Vav2 by Src-mediated phosphorylation. Further studies are needed to address this hypothesis in the future.

In summary, the present study represents the first report that characterizes the Vav family in the kidney and RMCs. Our results demonstrate that Vav2 is a predominant isoform in these kidney cells. Furthermore, Vav2 was found to importantly participate in Hcys-induced increases in Rac GTPase activity and consequent activation of NADPH oxidase. This role of Vav2 in Hcys-induced activation of Rac GTPase is associated with its phosphorylation via ceramide signaling pathway. It is concluded that Vav2 in RM Cs importantly contributes to Hcys-induced activation of Rac GTPase and consequent NADPH oxidase, thereby producing local oxidative stress and resulting in glomerular injury and end-stage renal disease associated with hHcys.

Acknowledgements

This study was supported by grants DK54927, HL075316, and HL57244 from National Institutes of Health.

References

1 Gupta A, Robinson K: Hyperhomocysteinaemia and end stage renal disease. J Nephrol 1997;10:77-84.

2 Dennis VW, Nurko S, Robinson K: Hyperhomocysteinemia: Detection, risk assessment, and treatment. Curr Opin Nephrol Hypertens 1997;6:483-488.

3 van Guldener C, Stehouwer CD: Homocysteine metabolism in renal disease. Clin Chem Lab Med 2003;41:1412-1417.

4 Yi F, Dos Santos EA, Xia M, Chen QZ, Li PL, Li N: Podocyte injury and glomerulosclerosis in hyperhomocysteinemic rats. Am J Nephrol 2007;27:262-268.
5 Yang ZZ, Zou AP: Homocysteine enhances timp-1 expression and cell proliferation associated with nadh oxidase in rat mesangial cells. Kidney Int 2003;63:1012-1020.
6 Yi F, Zhang AY, Janscha JL, Li PL, Zou AP: Homocysteine activates nadh/nadph oxidase through ceramide-stimulated rac gtpase activity in rat mesangial cells. Kidney Int 2004;66:1977-1987.
7 Yi F, Zhang AY, Li N, Muh RW, Fillet M, Renert AF, Li PL: Inhibition of ceramide-redox signaling pathway blocks glomerular injury in hyperhomocysteinemic rats. Kidney Int 2006;70:88-96.
8 Shastry S, Ingram AJ, Scholey JW, James LR: Homocysteine induces mesangial cell apoptosis via activation of p38-mitogen-activated protein kinase. Kidney Int 2007;71:304-311.
9 Takeya R, Sumimoto H: Regulation of novel superoxide-producing nad(p)h oxidases. Antioxid Redox Signal 2006;8:1523-1532.
10 Bedard K, Krause KH: The nox family of ros-generating nadph oxidases: Physiology and pathophysiology. Physiol Rev 2007;87:245-313.
11 Cai H: Nad(p)h oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. Circ Res 2005;96:818-822.
12 Gill PS, Wilcox CS: Nadph oxidases in the kidney. Antioxid Redox Signal 2006;8:1597-1607.
13 Bokoch GM, Diebold BA: Current molecular models for nadph oxidase regulation by rac gtpase. Blood 2002;100:2692-2696.
14 Hordijk PL: Regulation of nadph oxidases: The role of rac proteins. Circ Res 2006;98:453-462.
15 Evers EE, Zondag GC, Malliri A, Price LS, ten Klooster JP, van der Kammen RA, Collard JG: Rho family proteins in cell adhesion and cell migration. Eur J Cancer 2000;36:1269-1274.
16 Etienne-Manneville S, Hall A: Rho gtpases in cell biology. Nature 2002;420:629-635.
17 Bokoch GM, Zhao T: Regulation of the phagocyte nadph oxidase by rac gtpase. Antioxid Redox Signal 2006;8:1533-1548.
18 Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW: Activation of the nadph oxidase involves the small gtp-binding protein p21rac1. Nature 1991;353:668-670.
19 Ridley AJ: Rho family proteins: Coordinating cell responses. Trends Cell Biol 2001;11:471-477.
20 Tybulewicz VL: Vav-family proteins in t-cell signalling. Curr Opin Immunol 2005;17:267-274.
21 Hornstein I, Alcover A, Katzav S: Vav proteins, masters of the world of cytoskeleton organization. Cell Signal 2004;16:1-11.
22 Miletic AV, Graham DB, Montgrain V, Fujikawa K, Kloeppel T, Brim K, Weaver B, Schreiber R, Xavier R, Swat W: Vav proteins control myd88-dependent oxidative burst. Blood 2006.
23 Utomo A, Cullere X, Glogauer M, Swat W, Mayadas TN: Vav proteins in neutrophils are required for fcgammar-mediated signaling to rac gtpases and nicotinamide adenine dinucleotide phosphate oxidase component p40(phox). J Immunol 2006;177:6388-6397.
24 Ming W, Li S, Billadeau DD, Quilliam LA, Dinauer MC: The rac effector p67phox regulates phagocyte nadph oxidase by stimulating vav1 guanine nucleotide exchange activity. Mol Cell Biol 2007;27:312-323.
25 Zhang G, Zhang F, Muh R, Yi F, Chalupsky K, Cai H, Li PL: Autocrine/paracrine pattern of superoxide production through nad(p)h oxidase in coronary arterial myocytes. Am J Physiol Heart Circ Physiol 2007;292:H483-495.
26 Aghazadeh B, Lowry WE, Huang XY, Rosen MK: Structural basis for relief of autoinhibition of the dbl homology domain of proto-oncogene vav by tyrosine phosphorylation. Cell 2000;102:625-633.
27 Sorescu D, Szocs K, Griendling KK: Nad(p)h oxidases and their relevance to atherosclerosis. Trends Cardiovasc Med 2001;11:124-131.
28 Werner E: Gtpases and reactive oxygen species: Switches for killing and signaling. J Cell Sci 2004;117:143-153.
29 Knaus UG, Heyworth PG, Evans T, Curnutte JT, Bokoch GM: Regulation of phagocyte oxygen radical production by the gtp-binding protein rac 2. Science 1991;254:1512-1515.
30 Teshima S, Rokutan K, Nikawa T, Kishi K: Guinea pig gastric mucosal cells produce abundant superoxide anion through an nadph oxidase-like system. Gastroenterology 1998;115:1186-1196.
31 Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD: Cell transformation by the superoxide-generating oxidase mox1. Nature 1999;401:79-82.
32 Krotz F, Keller M, Derflinger S, Schmid H, Gloe T, Bassermann F, Duyster J, Cohen CD, Schuhmann C, Klauss V, Pohl U, Stempfle HU, Sohn HY: Mycophenolate acid inhibits endothelial nad(p)h oxidase activity and superoxide formation by a rac1-dependent mechanism. Hypertension 2007;49:201-208.
33 Cheng G, Diebold BA, Hughes Y, Lambeth JD: Nox1-dependent reactive oxygen generation is regulated by rac1. J Biol Chem 2006;281:17718-17726.
34 Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y: Rational design and characterization of a rac gtpase-specific small molecule inhibitor. Proc Natl Acad Sci U S A 2004;101:7618-7623.
35 Rossman KL, Der CJ, Sondek J: Gef means go: Turning on rho gtpases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167-180.
36 Price MO, Atkinson SJ, Knaus UG, Dinauer MC: Rac activation induces nadph oxidase activity in transgenic cosphox cells, and the level of superoxide production is exchange factor-dependent. J Biol Chem 2002;277:19220-19228.
37 Katzav S, Martin-Zanca D, Barbacid M: Vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. Embo J 1989;8:2283-2290.
38 Bustelo XR: Regulatory and signaling properties of the vav family. Mol Cell Biol 2000;20:1461-1477.
39 Ohanian J, Ohanian V: Sphingolipids in mammalian cell signalling. Cell Mol Life Sci 2001;58:2053-2068.
40 Ibitayo AI, Tsunoda Y, Nozu F, Owyang C, Bitar KN: Src kinase and pi 3-kinase as a transduction pathway in ceramide-induced contraction of colonic smooth muscle. Am J Physiol 1998;275:G705-711.
41 Fukuyama T, Ogita H, Kawakatsu T, Inagaki M, Takai Y: Activation of rac by cadherin through the c-src-rap1-phosphatidylinositol 3-kinase-vav2 pathway. Oncogene 2006;25:8-19.
42 Murata T, Ohnishi H, Okazawa H, Murata Y, Kusakari S, Hayashi Y, Miyashita M, Itoh H, Oldenborg PA, Furuya N, Matozaki T: Cd47 promotes neuronal development through src- and frg/vav2-mediated activation of rac and cdc42. J Neurosci 2006;26:12397-12407.
43 Servitja JM, Marinissen MJ, Sodhi A, Bustelo XR, Gutkind JS: Rac1 function is required for src-induced transformation. Evidence of a role for tiam1 and vav2 in rac activation by src. J Biol Chem 2003;278:34339-34346.