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# Homocysteine activates NADH/NADPH oxidase through ceramide-stimulated Rac GTPase activity in rat mesangial cells

FAN YI, ANDREW Y. ZHANG, JENNIFER L. JANSCHA, PIN-LAN LI, and AI-PING ZOU

*Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin*

## Homocysteine activates NADH/NADPH oxidase through ceramide-stimulated Rac GTPase activity in rat mesangial cells.

**Background.** We recently demonstrated that homocysteine (Hcys) increases superoxide ( $O_2^-$ ) production via NADH/NADPH oxidase in renal mesangial cells. This  $O_2^-$  production contributes to increased expression of tissue inhibitor of metalloproteinase (TIMP-1) and consequent deposition of collagen in response to Hcys. However, the mechanism by which Hcys activates NADH/NADPH oxidase remains unknown. Given that ceramide is an intracellular activator of this oxidase in several cell types, the present study tests the hypothesis that Hcys activates NADH/NADPH oxidase through a ceramide-mediated signaling pathway in rat mesangial (MG) cells, resulting in  $O_2^-$  production.

**Methods.** Rat MG cells were incubated with L-homocysteine (L-Hcys) to determine the mechanism by which Hcys activates NADH/NADPH oxidase. Thin layer chromatography (TLC), Western blot analysis, Rac GTPase activity pull down assay, and NADH/NADPH oxidase activity measurements were performed.

**Results.** TLC analysis demonstrated that L-Hcys increased de novo production of ceramide in MG cells. L-Hcys and increased ceramide did not alter the amount of NADH/NADPH oxidase subunit p47<sup>phox</sup> and p67<sup>phox</sup> in both membrane and cytosolic fractions from MG cells. However, L-Hcys or ceramide markedly increased the level of GTP-bound Rac, which was accompanied by enhanced activity of NADH/NADPH oxidase. These Hcys or ceramide-induced actions were substantially blocked by a Rac GTPase inhibitor, GDP $\beta$ S, and a de novo ceramide synthesis inhibitor, fumonisins B<sub>1</sub> (FB<sub>1</sub>).

**Conclusion.** These results indicate that Hcys activates NADH/NADPH oxidase by stimulating de novo ceramide synthesis, and subsequently enhancing Rac GTPase activity in rat MG cells. This ceramide-Rac GTPase signaling pathway may mediate Hcys-induced oxidative stress in these glomerular cells.

Homocysteine (Hcys) is a sulfur-containing amino acid formed during the metabolism of methionine. Hyperho-

mocysteinemia has been found in many patients with different cardiovascular diseases, especially in those patients with premature atherosclerosis of coronary, carotid, and peripheral arteries. Elevated plasma Hcys levels have been implicated as an independent risk factor for arteriosclerosis and coronary heart disease [1–2]. In previous experimental studies, Hcys was shown to produce endothelial dysfunction and stimulate the proliferation of vascular smooth muscle cells and other cells. It also increased the formation of extracellular matrix (ECM), which may activate or promote the sclerotic process in vessel walls and other tissues [3–5]. Recent reports in our laboratory have indicated that chronic elevations of plasma Hcys induce glomerulosclerosis with mesangial expansion, and that increased Hcys levels play an important role in the development of glomerulosclerosis associated with hypertension in the Dahl salt-sensitive rat model. Further studies have demonstrated that Hcys increases the  $O_2^-$  production via NADH/NADPH oxidase and, therefore, stimulates the formation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in renal mesangial cells, consequently producing the deposition of collagen in response to Hcys [6]. However, the mechanism by which Hcys activates NADH/NADPH oxidase remains unknown.

NADH/NADPH oxidase is a membrane-associated enzyme that uses electrons derived from intracellular NADH or NADPH to generate  $O_2^-$ . Since both NADH and NADPH are the substrates for this enzyme, it is usually named as NADH/NADPH oxidase, or NAD(P)H oxidase. Generally, the NADH/NADPH oxidase system is composed of multiple membrane-associated and cytosolic components, including gp91<sup>phox</sup>, p22<sup>phox</sup> (membrane-associated), p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, and Rac (cytosolic). Recently, different NADH/NADPH oxidase subunits have been identified in the kidney and are thought to be an important source of  $O_2^-$  in the kidney [1, 3, 7]. Studies in our laboratory and by others have demonstrated that this enzyme plays an important role in mediating  $O_2^-$  production in the kidney, especially in the glomeruli and thick ascending loop of Henle (TALH) [6, 8]. NADH/NADPH oxidase-mediated  $O_2^-$  production

**Key words:** mesangial cells, homocysteine, kidney, ceramide, Rac GTPase, NADH/NADPH oxidase, rats.

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has been implicated in the regulation of renal function under physiologic and pathologic conditions. Our findings that Hcys activates NADH/NADPH oxidase in MG cells strongly suggest that oxidative stress mediated by this enzyme may be importantly involved in the development of end-stage renal diseases associated with hyperhomocysteinemia. Therefore, it is imperative to clarify how Hcys activates this enzyme.

Because ceramide, a sphingolipid, has been reported as one of critical signaling molecules to mediate the activation of NADH/NADPH oxidase in different cells, and this enzyme contains a regulatory component, Rac protein, which processes a high affinity to lipid binding [9], the present study examined whether ceramide signaling contributes to Hcys-induced activation of NADH/NADPH oxidase in rat MG cells. Then, we went on to explore the mechanism by which ceramide production mediates the effects of Hcys on the activity of NADH/NADPH oxidase. Given the possible involvement of subunit p47<sup>phox</sup> translocation and Rac GTPase in the regulation of this enzyme, we examined the action of L-Hcys or ceramide on these two activating processes of NADH/NADPH oxidase in rat MG cells. Our results provide evidence indicating that Hcys stimulates the de novo synthesis of ceramide and consequently activates Rac GTPase, thereby resulting in O<sub>2</sub><sup>-</sup> production via enhanced NADH/NADPH oxidase activity.

## METHODS

### Culture of rat MG cells

Rat MG cells were obtained from the American Type Culture Collection (ATCC) and incubated and propagated in Dulbecco's modified Eagle's medium (DMEM) containing 18 mmol/L sodium bicarbonate, 25 mmol/L glucose, 0.6 μmol/L G418, and 15% fetal bovine serum (FBS) with 4 mmol/L L-glutamine at 37°C in 5% CO<sub>2</sub> atmosphere as we described previously [10, 11]. The cells were used when they reached 70% to 90% confluence. Between the D- and L-forms of Hcys, L-Hcys produces pathogenic effects; therefore, we treated rat MG cells with L-Hcys at concentrations of 20 to 80 μmol/L. In the present study, the concentration and the incubation time of L-Hcys treatment were chosen based on previous studies [6] and our preliminary experiments. It was found that L-Hcys (20–80 μmol/L) induced a time- and concentration-dependent increase O<sub>2</sub><sup>-</sup> production in rat MG cells, which was significantly attenuated by the NAD(P)H oxidase inhibitor diphenylene iodonium chloride (DPI) (data not shown). Therefore, in the present study, we treated rat MG cells with L-Hcys (80 μmol/L) for 16 hours to obtain the maximal effect of L-Hcys treatment. Because L-Hcys is not commercially available, it was prepared from L-Hcys thiolactone as described previously [12]. Briefly, L-Hcys thiolactone was dissolved in

water, hydrolyzed with KOH for 12 minutes at 45°C to remove the thiolactone group, then neutralized with HCl and cooled to 0°C with constant nitrogen purging. Freshly prepared L-Hcys was used in all experiments.

### Ceramide assay

Endogenous ceramide production in rat MG cells was determined by diacylglycerol (DAG) kinase assay as we reported previously [13, 14]. In brief, confluent rat MG cells were quickly frozen in liquid N<sub>2</sub>, and homogenized in 4 volumes of 10 mmol/L phosphate-buffered saline (PBS). An aliquot of homogenate was used to measure protein concentration. The supernatant was transferred to a 15 mL glass tube, and sufficient 1 mol/L NaCl was added to bring the aqueous volume to 0.8 mL. Samples were then extracted with 3 mL of chloroform/methanol (1:2, v/v), with a final chloroform-methanol-water (1:2:0.8, v/v). Samples were then sonicated three to four times to facilitate lipid extraction. After incubation at room temperature for three hours, samples were centrifuged at 3000g for 5 minutes, and the supernatant was transferred to another tube. Then, 1 mL chloroform and 1 mL 1 mol/L NaCl were added to make a chloroform-methanol-NaCl ratio of 2:1:1.8. To break the phases, samples were vortexed and centrifuged at 3000 rpm for 5 minutes. The lower layer (organic layer) was dried under N<sub>2</sub> and used for the analysis of ceramide within 72 hours.

An aliquot of dried lipid was solubilized by bath sonication into a detergent solution [7.5% n-octyl-β-D-glucopyranoside, 5 mmol/L cardiolipin in 1 mmol/L diethylenetriaminepentaacetic acid (DTPA)] and mixed with bacterial DAG kinase (Calbiochem, CA, USA) and 4 μCi [β-<sup>32</sup>P]-ATP to a final volume of 100 μL. After incubation at 25°C for 3 hours, the reaction was stopped by extraction of lipids with 600 μL chloroform:methanol (1:1, v/v), 20 μL 1% perchloric acid, and 150 μL 1 mol/L NaCl. Then, the lower organic phase was recovered, washed twice with 1% perchloric acid, and dried with N<sub>2</sub>. The <sup>32</sup>P-labeled ceramide was separated from other lipids by thin layer chromatography (TLC) with a solvent consisting of chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1, v/v/v/v/v). After autoradiographic visualization by exposure to x-ray film, the ceramide 1-<sup>32</sup>P band was recovered by scraping and counted in a Beckman model liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). The amount of ceramide in lipid extracts was calculated from a standard curve constructed with known amounts of C<sub>6</sub>-ceramide (Sigma, St. Louis, MO, USA), and expressed as nmol/mg protein.

### Sphingomyelinase assay

Sphingomyelinase (SMase) is one of the enzyme systems responsible for ceramide production, which

metabolizes sphingomyelin into ceramide and choline. The activity of sphingomyelinase was determined using radiospectrometry [15, 16]. Briefly, [N-methyl  $^{14}\text{C}$ ]-sphingomyelin was incubated with MG cell homogenates, then the metabolites of sphingomyelin,  $^{14}\text{C}$ -choline phosphate and ceramide, were quantitated. For magnesium-dependent neutral SMase (N-SMase), an aliquot of the homogenates (20  $\mu\text{g}$  protein) was incubated with a neutral reaction buffer containing 100 mmol/L Tris-HCl (pH 7.5), 5 mmol/L  $\text{MgCl}_2$ , and 0.05% Triton X-100, and mixed with 0.1 mmol/L [N-methyl  $^{14}\text{C}$ ]-sphingomyelin (0.02  $\mu\text{Ci}$ ) in a final volume of 100  $\mu\text{L}$ . After incubation at 37°C for 15 minutes, the reaction was stopped by adding 1.5 mL of chloroform/methanol (2:1, v/v), followed by addition of 0.2 mL of  $\text{H}_2\text{O}$ . The samples were then vortexed and centrifuged at 3000 rpm for 5 minutes to separate the two phases. A portion of the upper aqueous phase was transferred to scintillation vials and counted for the formation of  $^{14}\text{C}$ -choline phosphate in a Beckman liquid scintillation counter. Acidic SMase (A-SMase) activity was determined using an acidic reaction buffer containing 100 mmol/L sodium acetate (pH 5.0), 0.05% Triton X-100, and 0.1 mmol/L [N-methyl  $^{14}\text{C}$ ]-sphingomyelin (0.02  $\mu\text{Ci}$ ) in a final volume of 100  $\mu\text{L}$ . The  $^{14}\text{C}$ -choline phosphate formation rate was calculated to represent the enzyme activity.

To confirm the accumulation of ceramide in the reaction mixtures, thin layer chromatography (TLC) was performed in some experiments. Briefly, the lower organic phase after lipid extraction was collected and separated sequentially on a silica gel TLC LK6D plate (Whatman International, Ltd., Maidstone, England) with two solvent systems (chloroform/methanol/25% ammonium hydroxide/water 50:50:2:1 and 90:10:0.5:0.5, v/v/v/v). Sphingomyelin and ceramide were visualized by iodine staining, and comparison made with synthetic sphingomyelin and ceramide standards.

### Western blot analysis

The homogenates, cytosol, or membrane of rat MG cells were prepared as we reported previously [17, 18]. Western blot analysis was performed to determine the relative quantities of NADH/NADPH oxidase subunit p47<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup> in these cell fractions. Briefly, cell homogenates, cytosol or membrane (20  $\mu\text{g}$ ) were loaded and separated by a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins of these samples were then electrophoretically transferred at 100 V for 1 hour onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20, and probed at room temperature with a monoclonal antibody against p47<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup> (1:500 dilution for 2 hours, BD Transduction Laboratories,

Lexington, KY, USA). After washing, the membrane was incubated for 1 hour with a horseradish peroxidase-conjugated rabbit antimouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a dilution of 1:5000 for monoclonal primary antibodies, then washed and incubated for 1 minute with SuperSignal West Pico detection reagents (Pierce, Rockford, IL, USA). To make gel documentation, the membrane was wrapped in Saran Wrap, and then exposed to Fuji medical x-ray film (Fuji, Tokyo, Japan).

To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein,  $\beta$ -actin, after the membrane was stripped by incubating at 50°C for 30 minutes in a buffer containing Tris-HCl (67.5 mmol/L, pH 6.8),  $\beta$ -mercaptoethanol (100 mmol/L), and SDS (2%). After washing, the membrane was incubated in 5% nonfat dry milk in Tris-buffered saline-Tween 20, and subsequently with a monoclonal antibody against  $\beta$ -actin (1:4000 dilution for 2 hours; Sigma) and then a secondary antibody. The membrane was washed, and the immunoreactive band of  $\beta$ -actin visualized by reaction with detection solution as described above. 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).

### Rac GTPase activation assays

Rac GTPase is a small G protein that is reported to regulate NADH/NDAPH oxidase activity in different cells. To determine its involvement in the action of Hcys or ceramide, a specific pull-down experiment was performed, and Rac GTPase activation was measured using a Rac activation assay kit (Upstate, Lake Placid, NY, USA). Briefly, after stimulation with L-Hcys and C<sub>2</sub>-ceramide, rat MG cells were lysed in Mg<sup>2+</sup> lysis buffer (MLB). The cell lysate was precleaned by adding 100  $\mu\text{L}$  of glutathione agarose per 1 mL, and then incubating with continuous agitation for 10 minutes at 4°C. Then, the agarose beads were collected by a 5-second spin in a microcentrifuge at 14,000g. The supernatant was collected and stored in aliquots on ice, and protein concentrations were determined by BCA assay kit (Bio-Rad, Hercules, CA, USA). Each cell extract with 600  $\mu\text{g}$  protein was aliquoted into three microfuge tubes and used for Rac GTPase activity assay. GTP $\gamma$ S was used as a positive control for in vitro stimulation of Rac GTPase, while GDP was used to determine basal GTPase activity without stimulations. The control experiments were performed by using 2  $\mu\text{L}$  of 0.5 mol/L EDTA (substituted for pull-down proteins), 2  $\mu\text{L}$  of 100 $\times$  GTP $\gamma$ S and GDP. Tubes were incubated at 30°C for 15 minutes with agitation, and the reactions were terminated by placing the tubes on ice

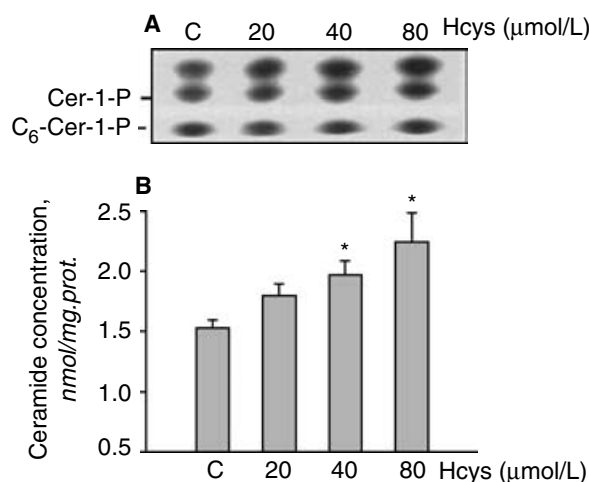
and adding 65  $\mu\text{L}$  of 1 mol/L  $\text{MgCl}_2$ . The pull-down proteins (200  $\mu\text{g}$  per sample), together with positive controls, were incubated with 8  $\mu\text{g}$  of PAK-1 PBD [Glutathione-S-Transferase fusion protein, corresponding to p21-binding domain (PBD, residues of 67–150) of human protein activating kinase-1 (PAK-1)] agarose for one hour at 4°C to bind Rac-GTP in the samples. Then, the supernatant was removed and discarded. The pelleted beads were washed three times with 0.5 mL of MLB, resuspended in 25  $\mu\text{L}$  of 2 $\times$  Laemmli reducing sample buffer, and boiled for 5 minutes. The sample mixtures were then loaded on 12% SDS-PAGE gels. The bound active GTP-Rac molecules in the beads and total Rac in the cell lysates were analyzed by Western blot using an anti-Rac monoclonal antibody.

### Measurements of basal $\text{O}_2^-$ levels and NADH/NADPH oxidase activity in rat MG cells

Fluorescence spectrometry for  $\text{O}_2^-$  production in rat MG cells was performed by using a modification of methods described previously [19, 20]. Briefly, the fluorogenic oxidation of dihydroethidium (DHE) to ethidium (Eth) was used as a measure of  $\text{O}_2^-$ . The homogenates (10  $\mu\text{g}$ ) freshly prepared from rat MG cells were incubated with DHE (10  $\mu\text{mol/L}$ ) and salmon testes DNA (0.5 mg/mL) with or without NADH (2 mmol/L) in a microtiter plate at 37°C for 30 minutes, and then Eth-DNA fluorescence was measured at an excitation of 475 nm and an emission of 610 nm by using a CytoFluor Series 4000-fluorescence microplate reader (Applied Biosystems, Foster City, CA, USA). The Eth fluorescence in the cells incubated without NADH was quantitated as basal  $\text{O}_2^-$  levels in these cells. NADH/NADPH oxidase activity to produce  $\text{O}_2^-$  in MG cells was examined by addition of NADH (0.1 mmol/L) as a substrate in the reaction mixture. Salmon DNA was added to bind to Eth and consequently stabilize Eth fluorescence, thereby increasing the sensitivity of  $\text{O}_2^-$  measurement (>40-fold). The enzyme activity of NADH/NADPH oxidase and  $\text{O}_2^-$  levels were presented as percent increases in Eth fluorescence versus control. These DHE fluorescent spectrometric assays of basal  $\text{O}_2^-$  levels and NADH oxidase activity were described in our previous studies [8, 21].

### Statistical analysis

Data are presented as mean  $\pm$  SEM. Significance of difference in mean values within and between multiple groups was examined with an analysis of variance (ANOVA) for repeated measures followed by a Duncan's post hoc test. Student *t* test was used to evaluate the significance of differences between two groups of experiments (SigmaStat; SPSS, Inc., Chicago, IL, USA). A value of  $P < 0.05$  was considered statistically significant.



**Fig. 1.** Effect of L-homocysteine (L-Hcys) on ceramide (Cer) concentrations in rat mesangial (MG) cells. Representative autoradiograph of phospholipids fractionated by thin-layer chromatography (TLC) after DG kinase assay (A). The bands shown represent ceramide-1-phosphate (Cer-1-P), which are from endogenous ceramide, and C6:0 ceramide-1-phosphate ( $\text{C}_6$ -Cer-1-P) from internal standard, respectively. Summarized quantitative changes in ceramide-1-phosphate (B). The Cer-1-P and  $\text{C}_6$ -Cer-1-P bands were scraped, and the radioactivity was counted using a liquid scintillation counter. Values are mean  $\pm$  SE from five separate experiments. \* $P < 0.05$  vs. control.

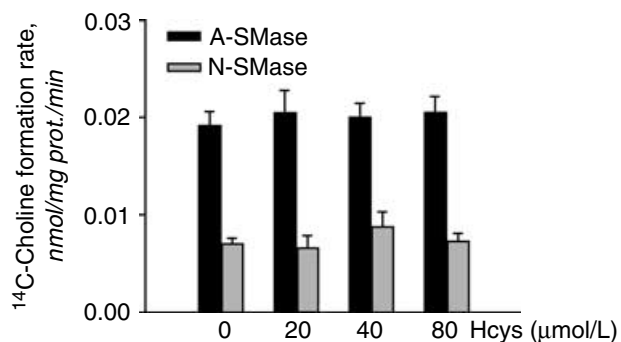
## RESULTS

### Effects of L-Hcys on ceramide production in rat MG cells

To provide direct evidence that L-Hcys stimulates production of ceramide, a DAG kinase assay was performed in rat MG cells treated with vehicle or L-Hcys (20, 40, or 80  $\mu\text{mol/L}$ ) for 16 hours. A representative TLC autoradiograph is presented in Figure 1A. In agreement with our previous reports [13, 22], two major radiolabeled phospholipid bands were detected in the extracts of rat MG cells, including phosphorylated endogenous ceramides and dihydroceramides (Cer-1-P), which comigrated with type III ceramide-1-phosphate and a phosphorylated C6:0 ceramide internal standard ( $\text{C}_6$ -Cer-1-P), respectively. By determining the radioactivity of scrapped Cer-1-P bands, we found that basal ceramide levels were detectable in control rat MG cells. L-Hcys treatment of these cells produced a significant increase in ceramide levels. As summarized in Figure 1B, the basal ceramide concentration in rat MG cells was  $1.53 \pm 0.059$  nmol/mg protein, and L-Hcys concentration dependently increased ceramide levels in these cells, with a 47% increase at a concentration of 80  $\mu\text{mol/L}$ .

### Effects of L-Hcys on SMase activity

To determine whether Hcys stimulates ceramide production through activation of SMases in rat MG cells, we measured the activity of both A-SMase and N-SMase in rat MG cells before and after treatment with L-Hcys. As shown in Figure 2, the activity of A-SMase averaged



**Fig. 2. Effect of L-Hcys on the activity of sphingomyelinases (SMases) activity in rat MG cells.** A-SMase and N-SMase represent acidic and neutral SMases, respectively. Values are mean  $\pm$  SE from five separate experiments.

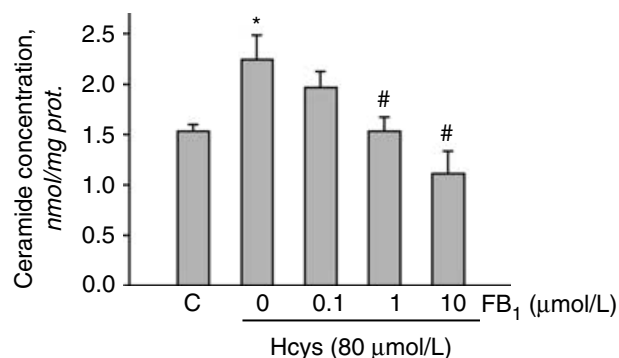
$0.02 \pm 0.0018$  nmol/min per mg protein, which was significantly greater than  $0.007$  nmol/min per mg protein of N-SMase activity. However, neither N-SMase nor A-SMase activity was altered in rat MG cells treated with L-Hcys. These results suggest that increased ceramide production by L-Hcys is not through SMase-mediated catabolism of sphingomyeline, but rather through other mechanisms.

#### Enhanced de novo ceramide synthesis in L-Hcys-treated MG cells

Recent studies have reported that ceramide can be also synthesized rapidly in response to different stimuli. We wondered whether Hcys-stimulated ceramide production is associated with activation of this de novo ceramide synthesis. To answer this question, a selective inhibitor of de novo ceramide synthesis, FB<sub>1</sub>, was used to examine whether Hcys-increased ceramide production could be blocked. FB<sub>1</sub> blocks ceramide synthase activity, and thereby, reduces ceramide production [23, 24]. It was found that FB<sub>1</sub> (0.1–10  $\mu$ mol/L) significantly blocked L-Hcys-induced increase in ceramide levels in rat MG cells in a concentration-dependent manner. The effect of L-Hcys increasing ceramide production was completely blocked at 1  $\mu$ mol/L FB<sub>1</sub>. At a high concentration of FB<sub>1</sub> (10  $\mu$ mol/L), ceramide decreased to a level even lower than basal level of ceramide in these MG cells (Fig. 3).

#### No effect of L-Hcys or ceramide on NADH/NADPH oxidase expression level and subunits translocation in rat MG cells

First, we examined the effect of L-Hcys or ceramide on the expression level of NADH/NADPH oxidase subunits in cultured rat MG cells. After treatment of rat MG cells with L-Hcys (80  $\mu$ mol/L) for 16 hours or a cell permeable C<sub>2</sub>-ceramide (15  $\mu$ mol/L) for 45 minutes, the homogenates of these cells were prepared. The subunits p47<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup> of NADH/NADPH oxidase were detected by Western blot analysis. As shown

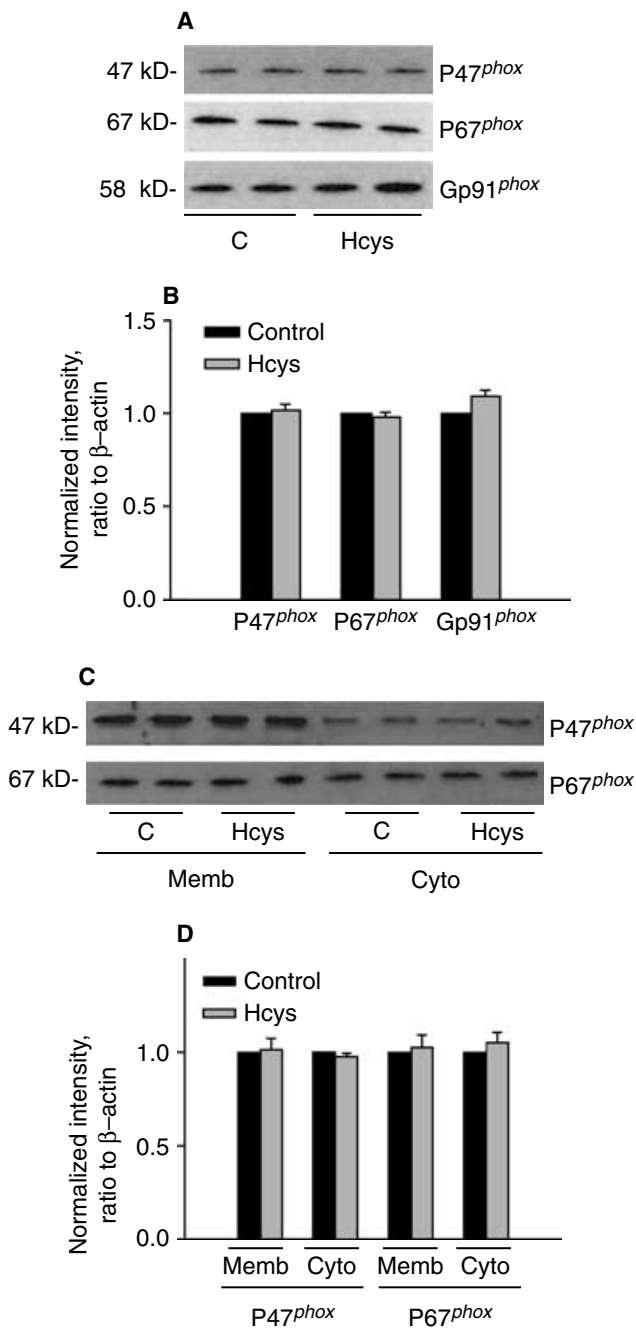


**Fig. 3. Effect of de novo ceramide synthesis inhibitor fumonisin B<sub>1</sub> (FB<sub>1</sub>) on ceramide levels in L-Hcys-treated rat MG cells.** Values are mean  $\pm$  SE from four separate experiments. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. vehicle of FB<sub>1</sub> treatment.

in Figure 4A, the amount of these subunits in rat MG cells had no significant change before and after incubation of the cells with L-Hcys. The results are summarized in Figure 4B. In our previous studies in endothelial cells, the translocation of p47<sup>phox</sup> was found to primarily mediate activation of NADH/NADPH oxidase in response to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). This p47<sup>phox</sup> translocation has also been demonstrated to be an activating mechanism of NADH/NADPH oxidase in other cells [18, 25, 26]. To determine the mechanism by which Hcys or ceramide activates NADH/NADPH oxidase, we then examined the effects of L-Hcys or C<sub>2</sub>-ceramide on the translocation of oxidase subunits from cytosol to the cell membrane. As shown in Figure 4C, the amount of p47<sup>phox</sup> in cell membrane and cytosol were similar before and after incubation of the cells with L-Hcys. Previous studies showed that the translocation of subunit p67<sup>phox</sup> from the cytosol to the membrane is essential for NADH/NADPH oxidase activity. p67<sup>phox</sup> contains two SH3 domains and binds, via its C-terminal SH3, to the praline-rich region of p47<sup>phox</sup>. This binding allows p67<sup>phox</sup> to directly associate with p47<sup>phox</sup> for the activity of NADH/NADPH oxidase. Consistent with the effect of L-Hcys on p47<sup>phox</sup>, the amount of p67<sup>phox</sup> in cell membrane and cytosol fractions was not altered by L-Hcys treatment. These results were summarized in Figure 4D. Similarly, C<sub>2</sub>-ceramide had no effect on p47<sup>phox</sup> and p67<sup>phox</sup> levels in either cytosol or cell membrane (data not shown).

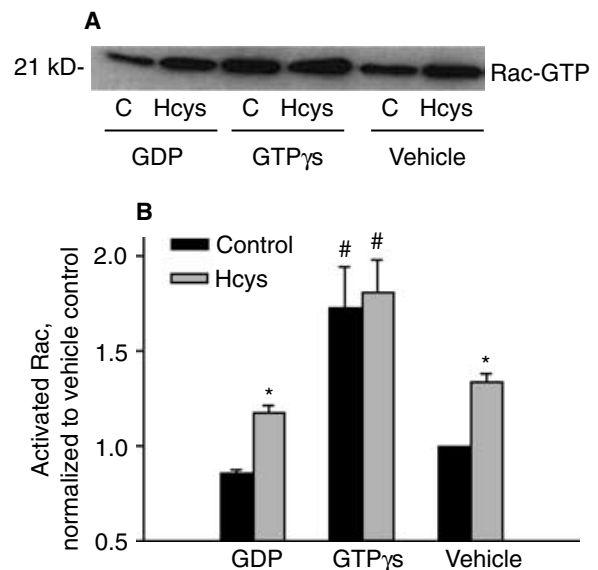
#### Activation of Rac GTPase by L-Hcys and ceramide in rat MG cells

As stated above, Rac is a regulatory element that plays an important factor in the activation of NADH/NADPH oxidase. Given that translocation of p47<sup>phox</sup> subunit seems not to be involved in the action of L-Hcys- or ceramide-induced activation of NADH/NADPH oxidase, we examined whether Hcys stimulates Rac GTPase activity through ceramide, resulting in activation of this



**Fig. 4. Effects of L-Hcys on NADH/NADPH oxidase expression level and the translocation of subunits in rat MG cells.** Immunoblot for p47<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup> in the homogenates of rat MG cells with and without treatment of L-Hcys (A). Summarized data showing changes in p47<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup> in rat MG cells ( $N = 5$ ) (B). Immunoblot for p47<sup>phox</sup>, p67<sup>phox</sup> in both cell membrane and cytosol of rat MG cells with and without treatment of L-Hcys (C). Summarized data showing changes in p47<sup>phox</sup>, p67<sup>phox</sup> in both cell membrane and cytosol of rat MG cells ( $N = 5$ ) (D).

enzyme in rat MG cells. As documented in Figure 5A, L-Hcys produced a significant increase in GTP-bound Rac protein levels in control or GDP-treated MG cells. When the cells were pretreated with a stable G-protein activa-

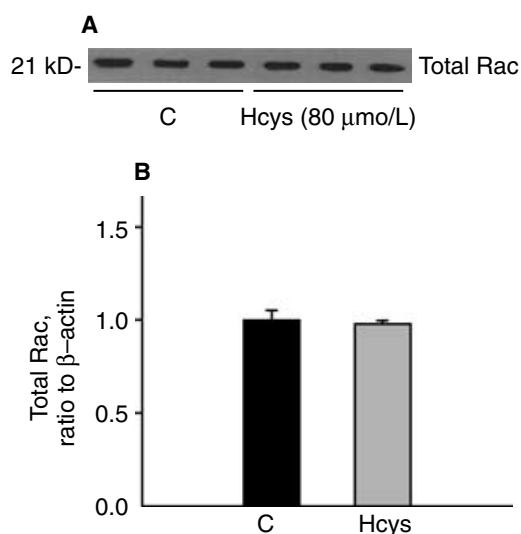


**Fig. 5. Effect of L-Hcys on Rac GTPase activity in rat MG cells.** Immunoblot for Rac GTPase activity in rat MG cells (A). GTP $\gamma$ S was used as a positive control for in vitro stimulation of Rac GTPase, while GDP was used to determine basal GTPase activity without stimulations. Summarized data showing changes in Rac GTPase activity in rat MG cells ( $N = 4$ ) (B). \* $P < 0.05$  vs. control, # $P < 0.05$  vs. vehicle of GDP treatment.

tor, GTP $\gamma$ S, GTP-bound Rac protein levels increased significantly. In the presence of GTP $\gamma$ S, L-Hcys did not have further effect on GTP-bound Rac levels. The results from these experiments are summarized in Figure 5B. L-Hcys increased GTP-bound or activated Rac levels by 37.6% and 56.4% in vehicle and GDP-treated MG cells, respectively. In the cells treated with GTP $\gamma$ S, a 1.8-fold increase in activated Rac level was observed, regardless of the absence or presence of L-Hcys treatment. However, total Rac levels, including GTP-bound and GDP-bound Rac, were not significantly altered by L-Hcys. Densitometric analysis demonstrated no difference in total Rac levels from vehicle-treated (C) and L-Hcys-treated MG cells (Fig. 6).

In rat MG cells treated with C<sub>2</sub>-ceramide (15  $\mu$ mol/L for 45 minutes), GTP-bound Rac levels were also significantly increased. As documented in Figure 7A, like L-Hcys, C<sub>2</sub>-ceramide increased the level of GTP-bound Rac in MG cells under different conditions. Summarized data in Figure 7B show that C<sub>2</sub>-ceramide significantly increased activated Rac levels. If the cells were pretreated with GTP $\gamma$ S, however, the activated Rac amount maximally increased what was not decreased or enhanced by C<sub>2</sub>-ceramide treatment. Similar to L-Hcys, C<sub>2</sub>-ceramide did not alter the level of total Rac in MG cells under any conditions (data not shown).

To further examine whether Hcys-stimulated activation of Rac is associated with enhanced production of ceramide in rat MG cells, we examined the effects of



**Fig. 6. Effect of L-Hcys on the expression levels of total Rac in rat MG cells.** Immunoblot for total Rac expression levels in rat MG cells (A). Summarized data showing changes in Rac expression in rat MG cells ( $N = 4$ ) (B).

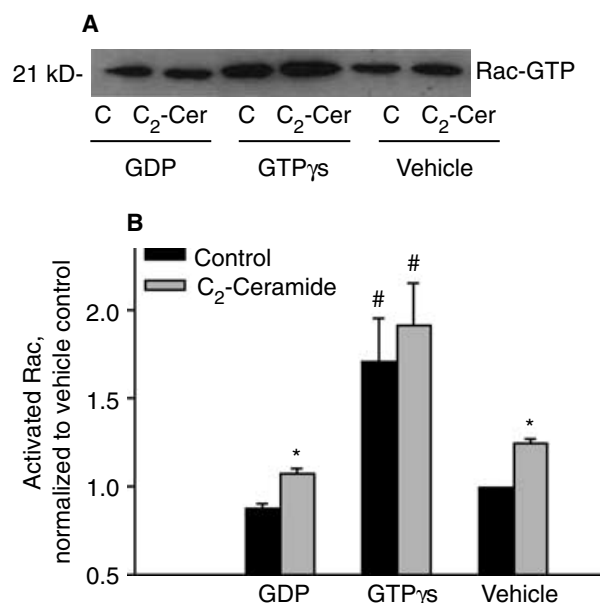
inhibition of de novo ceramide synthesis on the increase in Rac activity induced by L-Hcys. As shown in Figure 8A, L-Hcys significantly increased GTP-bound Rac levels, but it had no effect on total Rac level in the presence or absence of FB<sub>1</sub>, an inhibitor of de novo ceramide synthesis. However, in the presence of FB<sub>1</sub>, L-Hcys-induced increase in this activated Rac was substantially reduced. The results are summarized in Figure 8B, and suggest that FB<sub>1</sub> significantly blocked the L-Hcys-induced increase in activated Rac levels in these cells.

#### Blockade of L-Hcys-enhanced NADH/NADPH oxidase activity by inhibition of Rac activity and de novo ceramide synthesis in rat MG cells

Using fluorescence spectrometric assay, the activity of NADH/NADPH oxidase was determined in rat MG cells treated with L-Hcys in the absence or presence of Rac inhibitor, GDPβS, or de novo ceramide synthesis inhibitor, FB<sub>1</sub>. It was found that L-Hcys significantly increased the basal O<sub>2</sub><sup>-</sup> levels and NADH-specific activity in the homogenates of rat MG cells. As shown in Figure 9, summarized data demonstrate a significant increase in NADH/NADPH oxidase activity by L-Hcys. In the presence of GDPβS or FB<sub>1</sub>, L-Hcys-induced increase in NADH/NADPH oxidase activity was blocked completely. Both GDPβS and FB<sub>1</sub> even decreased NADH specific Eth fluorescence to a level lower than control.

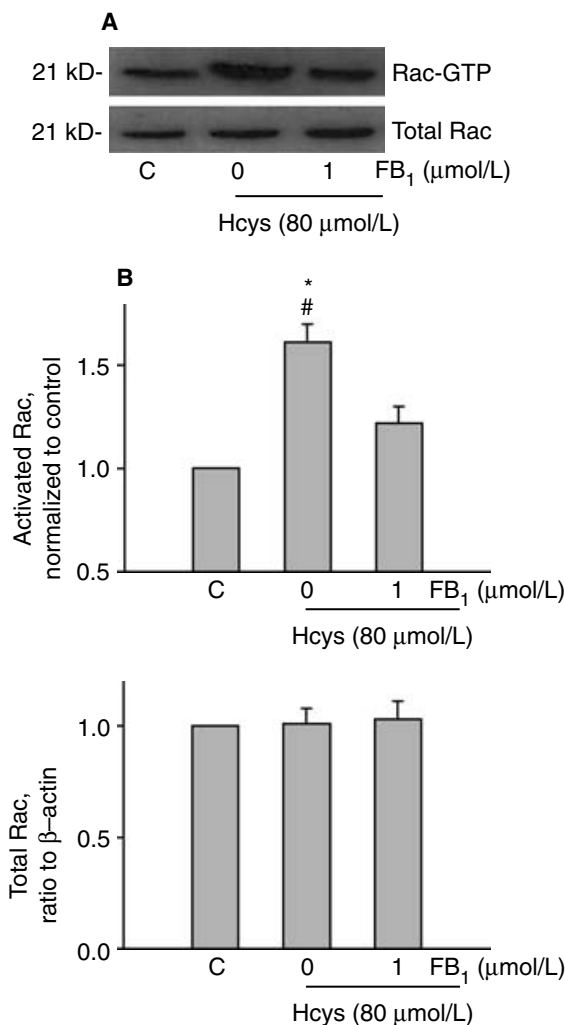
#### DISCUSSION

Recent studies in our laboratory have indicated that L-Hcys significantly increases NADH/NADPH oxidase activity, and thereby stimulates O<sub>2</sub><sup>-</sup> production in rat



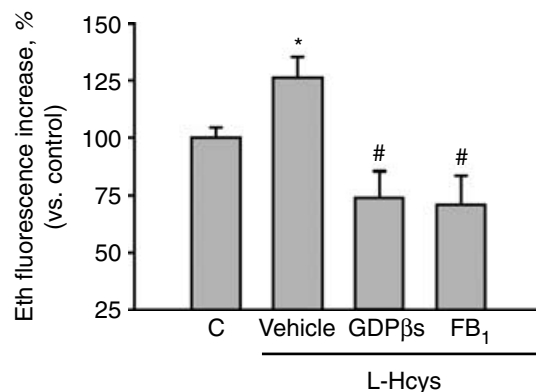
**Fig. 7. Effect of ceramide (Cer) on Rac GTPase activity in rat MG cells.** Immunoblot for Rac GTPase activity in rat MG cells (A). GTPγS was used as a positive control for in vitro stimulation of Rac GTPase, while GDP was used to determine basal GTPase activity without stimulations. Summarized data showing changes in Rac GTPase activity in rat MG cells ( $N = 4$ ) (B). \* $P < 0.05$  vs. control, # $P < 0.05$  vs. vehicle of GDP treatment.

MG cells, resulting in the abnormal metabolism of extracellular matrix and consequent glomerular sclerosis. In the present study, we performed a series of experiments to explore the mechanism by which Hcys enhances NADH/NADPH oxidase activity in these MG cells. First, we determined whether Hcys stimulates the production of ceramide, a novel lipid second messenger. This lipid-signaling molecule has been reported to mediate the actions of various stimuli, such as cytokines, chemotherapy drugs, and radiation, in a variety of mammalian tissues or cells. In addition, this signaling lipid has been implicated in the detrimental actions of many different injury factors, such as cell death factors [27–30]. Recent studies in our group have demonstrated that ceramide increased in ischemic reperfused myocardium and in endothelial cells exposed to death factors, such as TNF-α, interleukin 2 (IL-2), and endostatin [10, 14, 18]. In these studies, the ceramide signaling pathway has been confirmed to be involved in the activation of NADH/NADPH oxidase and consequent O<sub>2</sub><sup>-</sup> production, thereby leading to decreased NO bioavailability and endothelial dysfunction [31]. Therefore, we hypothesized that the ceramide signaling pathway may also contribute to the action of L-Hcys on NADH/NADPH oxidase activity in glomerular mesangial cells, and thereby mediate the action of Hcys in glomerular injury. To test this hypothesis, we incubated rat MG cells with L-Hcys for 16 hours, and then quantified cellular ceramide levels using DAG kinase/



**Fig. 8. Effect of fumonisin B<sub>1</sub> (FB<sub>1</sub>) on Rac GTPase activity in rat MG cells with L-Hcys treatment.** Immunoblot for Rac GTPase activity (upper) and total Rac expression levels (bottom) in rat MG cells (A). Summarized data showing changes in Rac GTPase activity and total Rac expression levels in rat MG cells (B). \* $P < 0.05$  vs. control, # $P < 0.05$  vs. vehicle of FB<sub>1</sub> treatment.

TLC assay. It was found that L-Hcys significantly increased ceramide concentrations in these rat MG cells. This result provides direct evidence that indicated that the ceramide signaling pathway may be involved in the action of Hcys-induced pathologic change in MG cells. In previous studies, ceramide was found to be importantly involved in renal glomerular and tubular pathology [32–34]. Ceramide can trigger apoptosis through a variety of intracellular targets: ceramide-activated protein kinase (CAPK), MAP kinases, caspases, and transcription factors, such as NF- $\kappa$ B. Activation of MAP kinases and caspases occurs during ischemia/reperfusion injury to kidneys [35]. The potential relevance of ceramide to renal injury has also been suggested by the facts that TNF- $\alpha$ , a stimulator of ceramide, induces apoptosis in renal tubule cells, and the



**Fig. 9. Effect of L-Hcys on nicotinamide adenine dinucleotide (NADH) oxidase activity and O<sub>2</sub><sup>-</sup> production in the presence or absence of inhibitors GDP $\beta$ S or FB<sub>1</sub> in rat MG cells.** Summarized data showing the effect on L-Hcys and inhibitors on NADH-dependent activity in these mesangial cells. Data were presented as percent increases in ethidium fluorescence compared to control ( $N = 4$ ). \* $P < 0.05$  vs. control, # $P < 0.05$  vs. vehicle of L-Hcys treatment.

increase in TNF- $\alpha$  can be observed in several models of acute renal failure [36]. The present study further confirms this possible pathologic relevance of ceramide in the end-stage renal diseases.

To explore the mechanism by which L-Hcys increases intracellular ceramide levels in rat MG cells, we examined the involvement of different enzymatic pathways in L-Hcys-induced ceramide production. Previous studies have demonstrated that two major important biochemical pathways are responsible for ceramide production, one of which is via sphingomyelinases (SMases). The SMases are the most extensively studied enzymes in ceramide metabolism. With the primary focus upon two major SMases, N-Smase and A-Smase, we first determined whether SMases are involved in the action of Hcys. By radiospectrometric assay, we found that L-Hcys-induced ceramide production was not associated with the activation of two major Smases, including N-SMase and A-SMase. Considering that cellular ceramide concentrations are dynamically maintained, SMases should be increased at the same time point that ceramide increases, if these enzymes involved. Therefore, failure to detect increased SMase activity suggests that L-Hcys does not alter the turnover of sphingomyelin in these MG cells through the SMase pathway.

Another important pathway to synthesize ceramide is its de novo synthesis. Previous studies reported that this de novo ceramide synthesis needs a relatively long time period to use raw materials to synthesize the product, which indicated that this pathway might not serve as a signaling mechanism. However, recent studies have reported that ceramide may be synthesized rapidly through its de novo pathway and, therefore, this de novo synthesized ceramide may also serve as a signaling molecule to mediate the actions of different stimuli [23, 37, 38].



It has been reported that de novo ceramide synthesis is stimulated by many apoptotic and cytotoxic agents, such as daunorubicin, phorbol ester, and D 609 [24, 39, 40], as well as some hormones, autocrines, and paracrines, such as thyroid hormones, angiotensin II, and others [20, 24]. Therefore, the ceramide synthesis through this pathway as a signaling mechanism may mediate the actions of these agents or factors. The first step in de novo ceramide synthesis is the condensation of palmitoyl-CoA with L-serine in a reaction catalyzed by serine palmitransferase. This is followed by the formation of ceramide resulting from the N-acylation of dihydrosphingosine or sphingosine by ceramide synthase, which is inhibited by the fungal toxin FB<sub>1</sub>. The inhibition apparently arises from the remarkable structural similarities between FB<sub>1</sub> and sphingoid bases, such as sphinganine and sphingosine, which are substrates for ceramide synthase. In the present study, we found that FB<sub>1</sub> completely abolished L-Hcys-induced ceramide production, suggesting L-Hcys-induced ceramide production through de novo ceramide synthesis in rat MG cells. Taken together, our results indicate that L-Hcys increases the de novo ceramide synthesis in the MG cells, and this lipid-signaling pathway may mediate the action of L-Hcys-induced activation of NADH/NADPH oxidase, resulting in O<sub>2</sub><sup>-</sup> production in these MG cells.

To further test this hypothesis, we explored the mechanism by which L-Hcys or ceramide activates NADH/NADPH oxidase. In the first series of experiments, we determined whether L-Hcys or ceramide increases the activity of this enzyme by stimulating translocation of its p47<sup>phox</sup> subunit from cytosol to the plasma membrane in rat MG cells. We concentrated on membrane translocation of these NADH/NADPH oxidase subunits because its translocation is considered to be an important mechanism in mediating NADH/NADPH activation in a variety of cells, including macrophages, vascular smooth muscle, and endothelial cells [18, 26, 41]. Due to the fact that p67<sup>phox</sup> together with p47<sup>phox</sup> translocates from cytosol to membrane for the function of NADH/NADPH oxidase, we also detected the abundance of p67<sup>phox</sup> in membrane and cytosol. By Western blot analysis, we found that the subunit p47<sup>phox</sup> and p67<sup>phox</sup> were detectable in the cell membrane in rat MG cells under basic culture condition without stimulation of L-Hcys or ceramide, and the abundance of p47<sup>phox</sup> NADH/NADPH oxidase subunit in this fraction at levels was even higher than its cytosolic counterpart. Neither L-Hcys nor ceramide changed the abundance of p47<sup>phox</sup> and p67<sup>phox</sup> in the cell membrane or cytosol. This indicates translocation of p47<sup>phox</sup> and p67<sup>phox</sup> seems not to be involved in L-Hcys-induced activation of NADH/NADPH oxidase in rat MG cells.

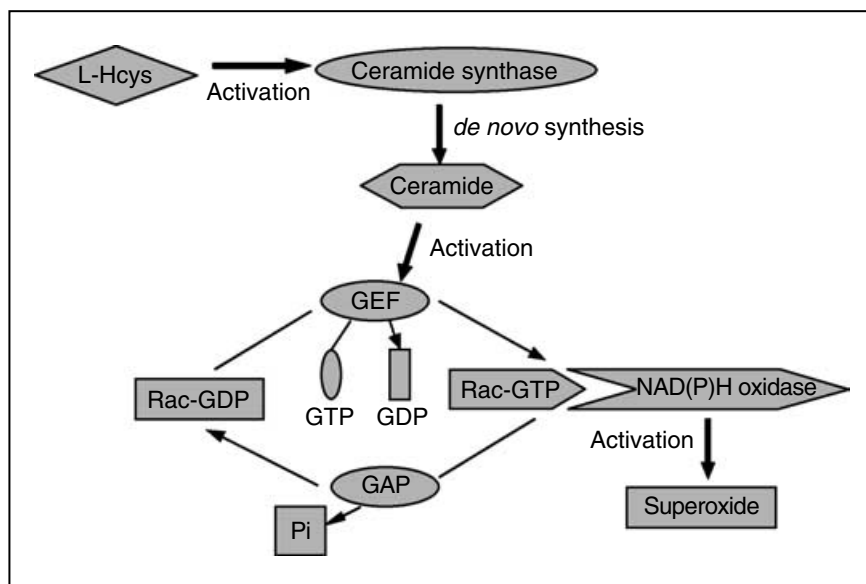
Next, we determined whether another important mechanism activating NADH/NADPH oxidase con-

tributes to the action of L-Hcys and ceramide, which is Rac GTPase-mediated activation. Recently, rapid progress has been made in unraveling the involvement of this small GTP-binding protein in regulating the NADH/NADPH oxidase activity [42–44]. Rac is a member of the Rho family of GTP-binding proteins, and it is regulated by binding GTP for activation and hydrolyzing GTP to GDP for inactivation. Thus, the Rac “activity” has been generally viewed as being synonymous with GTP binding. In previous studies, the role of Rac as a regulator of NADH/NADPH oxidase complex was described in phagocytes [45]. Recently, this Rac activation of NADH/NADPH oxidase has also been reported in nonphagocytic cells, such as vascular smooth muscle and endothelial cells. In the present study, a specific GTP-bound protein pull-down assay was used to detect activated Rac, and therefore, determined whether Rac GTPase can be activated by L-Hcys or ceramide in rat MG cells. It was found that both Hcys and ceramide significantly increased activated Rac abundance, but they had no effects on total Rac levels in rat MG cells. It is clear that Rac activity is involved in the action of L-Hcys or ceramide on NADH/NADPH oxidase activity.

Despite observations that both L-Hcys and ceramide produced a similar action increasing activated Rac in rat MG cells, it remains unclear at this point whether L-Hcys-induced Rac activation is associated with enhanced de novo ceramide synthesis. Therefore, we examined the effect of inhibition of de novo ceramide synthesis on L-Hcys-induced increase in activated Rac in these MG cells. The results demonstrated that the inhibitor of de novo ceramide synthesis, FB<sub>1</sub>, completely blocked the action of L-Hcys on Rac activity, and that in the presence of FB<sub>1</sub>, activated Rac was even lower than the basal levels. These results suggest that the de novo synthesized ceramide not only participates in the action of Hcys, but also plays a role in maintaining basal activity of Rac in rat MG cells. It is obvious that ceramide signaling is of importance in the regulation of Rac activity.

While the present study did not attempt to address how ceramide activates Rac or increases GTP binding to Rac, recent studies have reported that ceramide may stimulate Vav [46–49], a member of Ras GTP-binding protein superfamily through a protein tyrosine kinase (PTK)-independent alternative pathway, and thereby activates a guanine nucleotide exchange factor (GEF) specific to Rac. This GEF accelerates GDP exchange by GTP or Rac activation by catalyzing the release of the bound GDP. Further studies will be performed in a separate future study to address whether L-Hcys or ceramide alters Vav activity, and thereby activates GEF.

In the present study, we also determined whether L-Hcys-induced de novo ceramide synthesis, and Rac activation really enhances NADH/NADPH oxidase activity and O<sub>2</sub><sup>-</sup> production. Using fluorescence spectrometric



**Fig. 10. Proposed mechanism by which Hcys activates NADH/NADPH oxidase.** First, L-Hcys stimulated the de novo ceramide synthesis. Next, increased ceramide activated Rac GTPase via the activation of GEF (study in progress), and thereby increased NADH/NADPH oxidase activity. Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate;  $P_i$ , inorganic phosphate.

assay, the formation rate of ethidium from dihydroethidine in the homogenates of rat MG cells specific to NADH incubation was determined to represent NADH/NADPH oxidase activity. As reported previously [6], L-Hcys was found to significantly increase NADH/NADPH oxidase activity. In the presence of Rac inhibitor,  $GDP\beta S$ , L-Hcys-induced increase in NADH/NADPH activity was completely blocked, suggesting that Hcys-induced activation is associated with Rac activity. In addition, de novo ceramide synthesis inhibitor,  $FB_1$ , also blocked L-Hcys-induced increase in NADH/NADPH oxidase activity. These findings together confirmed that Hcys stimulated NADH/NADPH oxidase through activation of ceramide-Rac signaling pathway in MG cells. The de novo ceramide synthesis is importantly involved in L-Hcys-induced activation of Rac GTPase.

## CONCLUSION

The present study demonstrated that a lipid-signaling molecule, ceramide, increased through its de novo synthesis activation by L-Hcys in rat MG cells, and that ceramide activated Rac GTPase, and thereby enhanced NADH/NADPH oxidase activity. As shown in Figure 10, it has been concluded that L-Hcys activates ceramide synthase to enhance de novo ceramide synthesis. Increased ceramide stimulates Rac activity by enhancing exchange of GDP and GTP through GEF, and thereby leads to NADPH oxidase activation, producing superoxide using NADPH or NADH as substrate. This represents a new pathway mediating Hcys-induced glomerular injury.

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Reprint requests to Ai-Ping Zou, M.D., Ph.D., Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.  
E-mail: pli@mcw.edu

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