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Homocysteine enhances TIMP-1 expression and cell proliferation associated with NADH oxidase in rat mesangial cells

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Background. Recent studies in our laboratory demonstrated that chronic hyperhomocysteinemia (hHcys) induced glomerular sclerosis. The mechanism mediating hHcys-induced glomerular damage remains unknown. The present study was designed to test a hypothesis that homocysteine (Hcys) increases the $O_2^{\cdot-}$ production by nicotinamide adenine dinucleotide (NADH) oxidase and thereby stimulates the formation of tissue inhibitor of metalloproteinase (TIMP-1) in rat mesangial cells, consequently leads to glomerulosclerosis.

Methods. Rat mesangial cells were incubated with L-homocysteine (L-Hcys) to determine the effects of Hcys on cell proliferation and metabolism of extracellular matrix (ECM). Northern blot, Western blot, oligonucleotide transfection, measurements of NADH oxidase activity and $O_2^{\cdot-}$ levels, and cell proliferation assay were performed.

Results. In cultured rat mesangial cells, treatment with L-Hcys (40 to 160 μmol/L) markedly increased the mRNA levels of TIMP-1 and Gp91 and led to accumulation of collagen I, which were accompanied by enhanced cell proliferation and NADH oxidase activity in mesangial cells. These Hcys-induced biochemical and functional changes were substantially blocked by a NADH oxidase inhibitor, diphenylene iodonium chloride (DPI) or a superoxide dismutase (SOD) mimetic, hydroxyltetramethylpiperidin-oxyl (TEMPOL). Moreover, blockade of NADH oxidase subunit, phox22, by its antisense oligodeoxynucleotide also eliminated the increase in NADH oxidase activity induced by L-Hcys.

Conclusion. These results indicate that Hcys-induced alterations of ECM metabolism in mesangial cells are associated with enhanced NADH oxidase activity and that oxidative stress-stimulated up-regulation of TIMP-1 may play an important role in the deposition of collagen or ECM elements in the glomeruli during hHcys.

Homocysteine (Hcys), a sulfur-containing amino acid, is an intermediate metabolite of methionine. Human plasma total Hcys (tHcys) concentrations normally range from 5 to 15 μmol/L, and patients with plasma tHcys concentrations above 15 μmol/L are considered to have hyperhomocysteinemia (hHcys). It has been demonstrated that elevated plasma levels of Hcys are associated with the development of hypertension, stroke, and other cardiovascular diseases and, therefore, hHcys is emerging as an independent risk factor for arteriosclerosis and cardiovascular diseases [1–8]. In the experimental studies, Hcys has been shown to produce endothelial dysfunction, stimulate the proliferation of vascular smooth muscle cells and other cells, and increase the formation of extracellular matrix (ECM) [9–15], which may activate or promote sclerotic process in vessel wall and other tissues. More recently, we have reported that chronic elevations of plasma tHcys induced glomerulosclerosis with mesangial expansion and that increased Hcys levels play an important role in the development of glomerulosclerosis associated with hypertension in Dahl salt-sensitive rat model [16]. However, the mechanism by which elevations of plasma tHcys induce glomerulosclerosis has yet to be determined.

Recent studies have indicated that the formation and deposition of ECM are importantly involved in the development of glomerulosclerosis associated with diabetes and hypertension. Among the components of ECM, proteinases and antiproteinases, elastin, collagens, proteoglycans, and many growth factors are of importance in determining the formation and metabolism of ECM under physiologic or pathologic conditions [17, 18]. It has been demonstrated that matrix metalloproteinases (MMPs) can break down collagen and thereby importantly regulate the amount of this major protein in ECM. This MMPs-mediated effect degenerating collagen is normally controlled by a family of endogenously produced tissue inhibitors of metalloproteinase (TIMPs) present in a variety of tissues and body fluids. This TIMP family consists of four structurally related members, including TIMP-1, -2, -3 and -4. Among these TIMPs, TIMP-1 is a ubiquitous 28.5 kD–secreted glycoprotein and a potent inhibitor, which inactivates most of MMPs [10, 19, 20]. It has been reported that TIMP-1 plays a key role in the regulation of ECM accumulation. Excessive

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TIMP-1 expression markedly reduced the MMP activity, resulting in the accumulation of collagen, deposition of other ECM elements and consequent fibrosis and sclerosis in many organs or tissues such as arterial wall, liver or glomeruli [10, 20, 21].

In the present study, we hypothesized that Hcys stimulated the expression of TIMP-1 and thereby increased the formation of ECM in mesangial cells, leading to glomerulosclerosis. Given that oxidative stress associated with nicotinamide adenine dinucleotide (NADH) oxidase activity participates in the regulation of TIMP-1 expression and activity [22, 23], we examined whether Hcys-induced TIMP-1 increase is attributed to NADH oxidase–mediated oxidative stress in mesangial cells. These experiments provided evidence that Hcys enhances oxidative stress in mesangial cells, resulting in enhanced expression of TIMP-1 in these cells. Hcys-induced oxidative stress in mesangial cells is associated with increased expression and activity of NADH oxidase.

METHODS

Culture of rat mesangial cells

To explore the possible mechanism producing hHcys-induced glomerulosclerosis, we used rat mesangial cells to examine the effects of Hcys on the formation or expression of ECM elements. Rat mesangial cells at passage 8 were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mmol/L L-glutamine. These cells were incubated and propagated in DMEM containing 1.5 g/L sodium biocarbonate, 4.5 g/L glucose, 0.4 mg/mL G418, and 15% fetal calf serum (FCS) at 37°C in 5% carbon dioxide (CO_2) atmosphere. The cells with a confluence on regular culture dish were used for experiments. Since previous studies found that some stimuli produced different responses in cells grown on plastic bottom from those on laminin-coated culture dishes [24], another group of experiments was performed using the cells grown on laminin-coated surface to examine whether Hcys-induced alterations are different from those cells grown on regular plastic dishes.

Since D- and L-forms of Hcys have different effects on myocardium versus vessels [25] and L-Hcys primarily participates in the pathogenic effect of Hcys, we treated the cells with L-Hcys at concentrations of 40 to 160 μmol/L. Because L-Hcys was not commercially available, we prepared it from L-Hcys thiolactone as described previously [26]. Briefly, L-Hcys thiolactone was dissolved in water, hydrolyzed with potassium hydroxide (KOH) for 12 minutes at 45°C to remove thiolactone group, then neutralized with HCl and cooled to 0°C with constant nitrogen purging. Freshly prepared L-Hcys was used in all experiments. NADH oxidase inhibitor, 10 μmol/L diphenylene iodonium chloride (DPI) and superoxide dismutase (SOD) mimetic, hydroxyl tetramethylpiperidinoxyl (TEMPOL, 0.1 mmol/L) were used 6 hours prior to L-Hcys treatment. Doses of DPI and L-Hcys used in the present study were chosen based on previous studies [27] and our preliminary experiments. In preliminary experiments, we examined the effects of DPI on $O_2^{\bullet-}$ production via different pathways using dihydroethidium (DHE) fluoremetric assay. It was demonstrated that 10 to 50 μmol/L DPI significantly inhibited NADH oxidase activity with a maximal inhibition of 60%. However, DPI had no significant effects on $O_2^{\bullet-}$ production via other two major pathways, xanthine oxidase and mitochondrial enzyme systems at the same concentrations. Dose of TEMPOL chosen to remove $O_2^{\bullet-}$ was based on previous studies from our laboratory and others [28–30], showing that TEMPOL itself had no toxic effect, but specifically scavenge $O_2^{\bullet-}$. It is known that if tissue or cell catalase is normal, TEMPOL-induced H_2O_2 production does not produce detrimental effect at doses used in the present study.

RNA extraction and Northern blot analysis

Total RNA was extracted using TRIzol solution (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. Northern blot analyses of mRNA levels of TIMP-1 and a subunit of NADH oxidase, Gp91, were performed as we described previously [31, 32]. In brief, total RNA (10 to 20 μg) was fractionated on 1.0% formaldehyde-agarose gel, stained with ethidium bromide (0.5 μg /mL), washed, photographed, transferred onto nylon membrane (Pierce, Rockford, IL), and cross-linked to the membrane by ultraviolet irradiation. The nylon membranes were first prehybridized with Rapid Hyb buffer (Amersham Pharmacia, Piscataway, NJ) and then probed with ^{32}P-labeled rat TIMP-1 and Gp91 cDNA at 65°C for 2.5 hours, respectively. After washed once at room temperature (RT) and then twice at 65°C, the membranes were autoradiographed at −80°C for 24 or 36 hours. The autoradiographed films were scanned with a laser densitometer (Hewlett Packard ScanJet ADF, Boise, ID) and then digitized by a UN-SCAN-IT software package (Silk Scientific, Inc., Orem, UT). The densitometric values of those specific bands for corresponding gene expression were normalized to 28S rRNA.

The rat Gp91 cDNA probe was made from a polymerase chain reaction (PCR) II vector constructed in our laboratory, and the rat TIMP-1 probe was made from a pCMVSPORT vector purchased from the rat Unigene set of Research Genetics (Huntsville, AL). All these probes were dissected by PCR using T3 and T7 primers, purified, and stored at −80°C until hybridization. The sizes of Gp91 and TIMP-1 cDNA probes were 456 and 425 bp, respectively. The sequences of these cDNA probes were identical to those deposited in GenBank (Gp91: AF298656; TIMP-1: U06179).

Measurements of basal $O_2^{\cdot-}$ levels and NADH oxidase activity in rat mesangial cells

Fluorescence spectrometry of $O_2^{\cdot-}$ production in rat mesangial cells was performed by using a modification of methods described previously [33, 34]. More recently, we employed it to examine NADH oxidase activity in renal tissues using this modified method [28]. Briefly, the fluorogenic oxidation of dihydroethidine to ethidium was used as a measure of $O_2^{\cdot-}$. The homogenates (20 μg) freshly prepared from rat mesangial cells were incubated with 10 μmol/L DHE and salmon testes DNA (0.5 mg/mL) with or without NADH (0.2 mmol/L) in a microtiter plate at 37°C for 30 minutes, and then ethidium-DNA fluorescence was measured at an excitation of 475 nm and an emission of 610 nm by using a FL600-fluorescence microplate reader (Key Scientific, Mt. Prospect, IL). The ethidium fluorescence in the cells incubated without NADH was quantitated as basal $O_2^{\cdot-}$ levels in these cells. NADH oxidase activity to produce $O_2^{\cdot-}$ in mesangial cells was examined by addition of 0.1 mmol/L NADH as a substrate in the reaction mixture. Salmon DNA was added to bind to ethidium and consequently to stabilize ethidium fluorescence, thereby increasing the sensitivity of $O_2^{\cdot-}$ measurement (>40-fold). The enzyme activity of NADH oxidase and $O_2^{\cdot-}$ levels were presented as percent increase in ethidium fluorescence vs. control. These DHE-based assays of $O_2^{\cdot-}$ levels and NADH oxidase activity were described in our previous studies [27, 28].

Western blot analysis

Western blotting was performed as we described previously [31, 35]. Briefly, 100 μg homogenates were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Then, the membrane was washed and probed with 1:100 specific anticollagen type I antibody (Calbiochem, San Diego, CA) and subsequently with 1:1000 horseradish peroxidase–labeled goat antirabbit immunoglobulin (IgG). Finally, 10 mL enhanced chemiluminescence detection solution (Pierce) was added, and the membrane was wrapped and exposed to Kodak Omat film (Kodak, Rochester, NY). A band at 115 kD was recognized by this specific antibody against collagen type I. Protein concentration of the cell homogenates was measured with the use of a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the procedures described by the manufacturer.

NADH oxidase subunit, phox22 antisense oligodeoxynucleotide (P22-AS) transfection

A phosphorothioation-modified phox 22 antisense oligodeoxynucleotide (P22-AS) was synthesized based on a cDNA sequence of phox22 (AJ295951) and it contained 5′-GCCCACTCGATCTGCCCCAT-3′ (antisense, OPERON, Santa Clarita, CA). The modification of five nucleotides on each side of P22-AS by phosphorothioation increased the stability and prevented this oligonucleotide from being degraded by intracellular nucleotide enzymes. The fluorescein attachment at 5′-end was used as an indicator for transfection into the mesangial cells. The P22-AS was wrapped by cationic liposome (Avanti Polar Lipids, Inc., Alabaster, AL) and transfected into mesangial cells as described by the manufacturer. P22-AS (10 μg) was first mixed with 50 μL liposome and then added into 5 mL serum-free incubation medium. The transfection efficiency was evaluated by using a fluorescence microscope (Olympus, Tokyo, Japan) 3 hours after incubation of mesangial cells with liposome-P22-AS mixtures. Positively transfected cells (70% to 80% cells) indicated by a remarkable intracellular fluorescence were used to determine the effects of phox22 blockade on NADH oxidase activity in mesangial cells in response to L-Hcys in the presence or absence of diphenylene iodonium (DPI). These gene transfection procedures were described in detail in our previous study [31].

Measurement of proliferation of rat mesangial cells

Rat mesangial cell proliferation was measured by using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA). In this assay system, a proprietary green fluorescent dye, CyQUANT GR is used to determine the fluorescence intensity when it bound to cellular nucleic acids. To perform this assay, mesangial cells cultured in 96-well microplates were rapidly frozen after removal of incubation medium and stored at −70°C for 24 hours, then thawed and lysed by addition of 200 μL of CyQUANT GR dye/cell lysis buffer. After a 5-minute incubation at room temperature, the fluorescence intensity was measured using a FL600-fluorescence microplate reader with filters appropriate for ~480 nm excitation and ~520 nm emission. The time course of the fluorescence intensity changes represented the profile of cell proliferation.

Statistical analysis

Data are presented as mean ± 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.

RESULTS

Effects of L-Hcys on TIMP-1 and Gp91 mRNA levels in rat mesangial cells

First, we examined the effects of L-Hcys on TIMP-1 and Gp91 mRNA expression in cultured rat mesangial

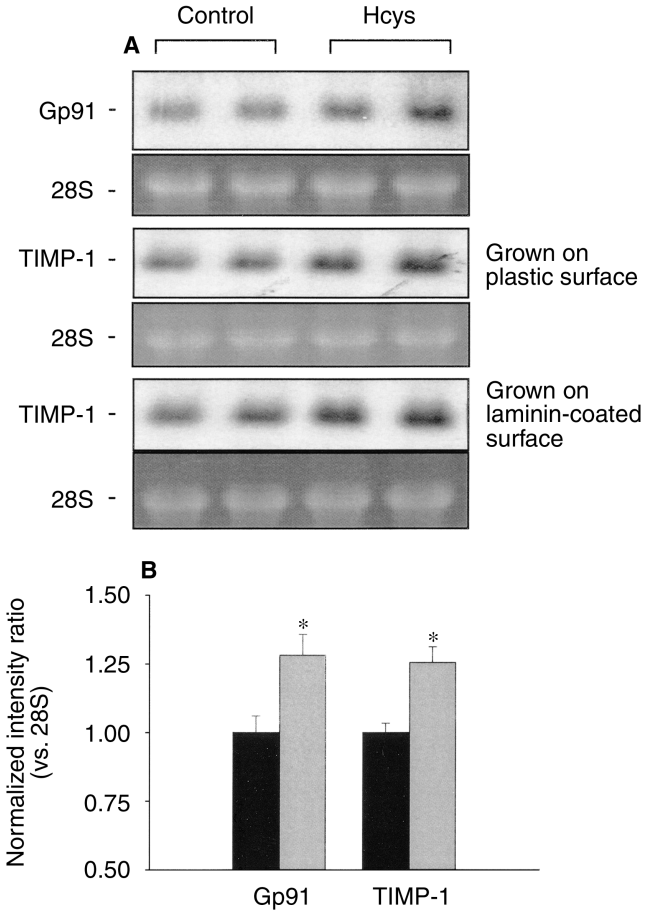

Fig. 1. Effects of L-homocysteine (L-Hcys) on Gp91 and tissue inhibitor of metalloproteinase (TIMP-1) mRNA expression in rat mesangial cells. (*A*) Typical autoradiographic documents of Northern blot analysis showing the effects of L-Hcys on the mRNA levels of Gp91 and TIMP-1 in rat mesangial cells grown on regular plastic or laminin-coated surface. (*B*) Summarized data showing the intensity ratio of Gp91 and TIMP-1 mRNA to 28S blots in rat mesangial cells grown on regular plastic surface under control condition (■) and during treatment of L-Hcys (□). $^*P < 0.05$, compared with control.

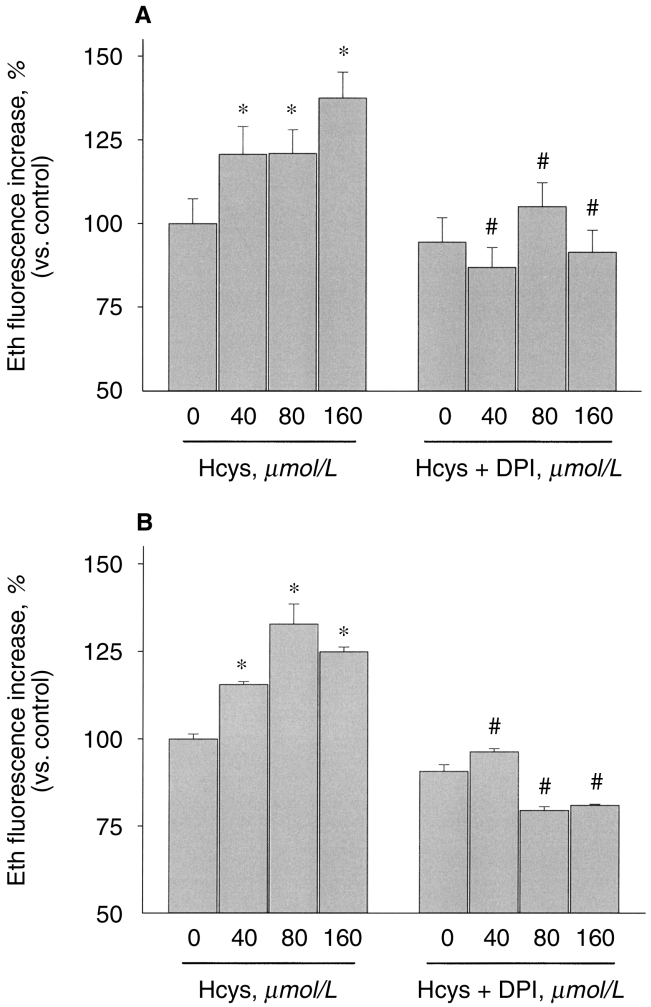

Fig. 2. Effects of L-homocysteine (L-Hcys) on nicotinamide adenine dinucleotide (NADH) oxidase activity and $O_2^{\cdot-}$ production in the presence or absence of diphenylene iodonium chloride (DPI) in rat mesangial cells. Summarized data showing the effects of L-Hcys on basal $O_2^{\cdot-}$ levels (*A*) and NADH-dependent activity (*B*) in mesangial cells in the presence or absence of DPI. Data were presented as ethidium fluorescence increase percentage compared to control. $^*P < 0.05$ compared with control; $\#P < 0.05$ compared with the values obtained during L-Hcys treatment alone.

cells. Treatment of rat mesangial cells with L-Hcys for 48 hours markedly increased TIMP-1 mRNA levels. Gp91 mRNA abundance was also found to significantly increase in parallel in these mesangial cells treated with L-Hcys (Fig. 1A). As summarized in Figure 1B, L-Hcys produced a 1.3- and 1.4-fold increase in TIMP-1 ($N = 12$) and Gp91 ($N = 8$) mRNA abundance in these mesangial cells, respectively. In previous studies, cells grown on plastic surface were found to have opposite response to some stimuli compared to those cells grown on laminin-coated surface [24]. We performed another group of experiments to examine whether mesangial cells grown on different surfaces produced different response with respect to the expression of TIMP-1 in response to L-Hcys. It was found that L-Hcys-induced increase in TIMP-1 expression was not different in mesangial cells grown on laminin-coated surface compared to that in mesangial cells on regular plastic surface (Fig. 1A).

Effects of L-Hcys on NADH oxidase activity and $O_2^{\cdot-}$ production in rat mesangial cells

Since the expression of NADH oxidase increased during treatment of mesangial cells with L-Hcys, we were wondering whether the activity of this enzyme and consequent $O_2^{\cdot-}$ production is enhanced by L-Hcys. To answer this question, we assessed activity of NADH oxidase and $O_2^{\cdot-}$ levels in the homogenate from mesangial cells treated with L-Hcys. It was found that L-Hcys significantly increased the basal $O_2^{\cdot-}$ levels as measured by ethidium fluorescence in cells without NADH in incubation mixture (Fig. 2A). Similarly, enhanced NADH oxidase activity in these cultured mesangial cells was observed, as shown by ethidium fluorescence intensity in

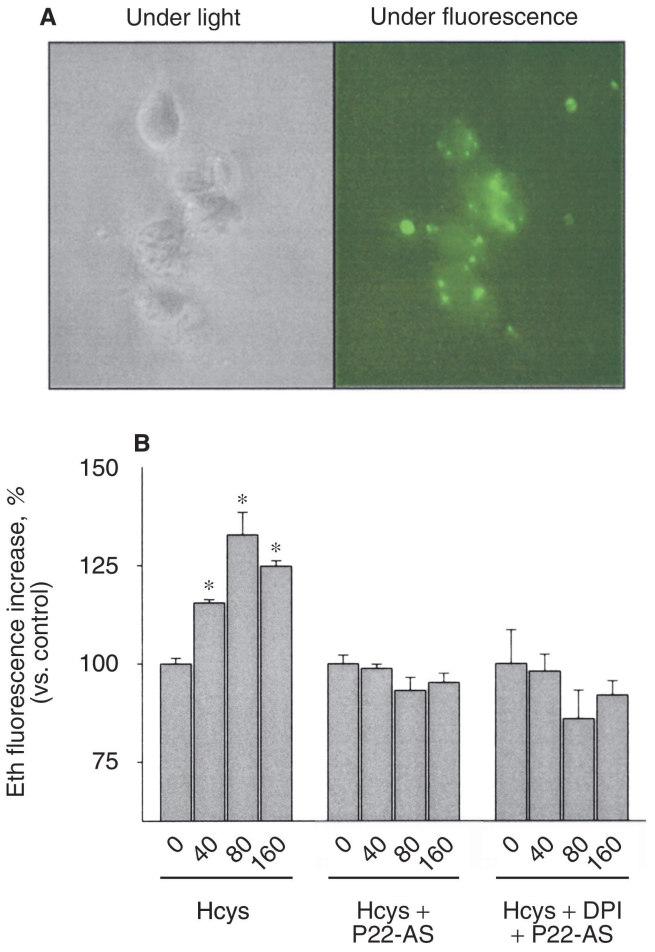

Fig. 3. Effects of phox22 blockade on L-homocysteine (L-Hcys)–induced increase in nicotinamide adenine dinucleotide (NADH) oxidase activity in the mesangial cells in the presence or absence of diphenylene iodonium chloride (DPI). (*A*) Light microscopic photomicrograph (×200, left panel under light) and fluorescence microscopic photomicrograph of the mesangial cells (×200, right panel under fluorescence) transfected with fluorescein-attached phox22 antisense oligodeoxynucleotide (P22-AS). (*B*) Summarized data showing the effects of phox22 blockade on NADH oxidase activity in the mesangial cells in response to L-Hcys μmol/L in the presence or absence of DPI. Data were presented as ethidium fluorescence increase percentage compared to control. $^*P <$ 0.05 compared with control.

cells incubated with NADH (Fig. 2B). In the presence of 10 μmol/L DPI, a NADH oxidase inhibitor, L-Hcys-induced increase in enzyme activity of NADH oxidase and $O_2^{\cdot-}$ levels was completely blocked.

Effects of phox22 blockade on L-Hcys-induced increase in NADH oxidase activity in the mesangial cells in the presence or absence of DPI

To address a concern about the specificity of DPI inhibition of NADH oxidase in mesangial cells, we utilized antisense oligodeoxynucleotide approach to examine the effect of phox22 blockade on NADH oxidase activity in the mesangial cells. Figure 3A shows a photomicrograph of the mesangial cells transfected with fluorescein-attached P22-AS oligodeoxynucleotide. A high efficient transfection (70% to 80%) was reached by using cationic lipsome from Avanti Polar Lipids, Inc. (Alabaste, AL). In these P22-AS–transfected cells, L-Hcys-induced increase in NADH oxidase activity was completely blocked. Treatment of these P22-AS–transfected mesangial cells with DPI had no further effect on NADH oxidase activity, regardless of the presence or absence of L-Hcys (Fig. 3B).

Effects of DPI and TEMPOL on L-Hcys-increased expression of TIMP-1 mRNA in rat mesangial cells

To determine whether L-Hcys-increased TIMP-1 expression is associated with $O_2^{\cdot-}$ production via NADH oxidase, additional experiments were performed to examine whether inhibition of NADH oxidase by DPI or removal of $O_2^{\cdot-}$ by a SOD mimetic, TEMPOL alters TIMP-1 response to Hcys in these mesangial cells. As shown in the typical gel documents in Figure 4A, both DPI and TEMPOL significantly attenuated L-Hcys–induced increase in TIMP-1 mRNA levels. The results of these experiments were summarized in Figure 4B. TEMPOL ($N = 6$) or DPI ($N = 6$) completely blocked L-Hcys–induced increase in TIMP-1 mRNA levels. The levels of TIMP-1 mRNA after Hcys treatment were even lower in TEMPOL or DPI-treated cells than control cells without Hcys treatment.

Effects of L-Hcys on the levels of collagen type I in the presence or absence of TEMPOL or DPI in rat mesangial cells

By Western blot analysis, we found that a major subtype of collagens in rat mesangial cells is collagen type I. Therefore, this collagen was used as a prototype to study L-Hcys–induced accumulation of collagens in mesangial cells in the absence and presence of DPI and TEMPOL. It was found that protein levels of collagen type I was doubled in mesangial cells treated with L-Hcys at 80 or 160 μmol/L for 48 hours compared to vehicle-treated mesangial cells. In the presence of DPI, L-Hcys–induced accumulation of this collagen was significantly blocked. Similarly, TEMPOL substantially attenuated the increase in collagen type I protein levels (Fig. 5A). Summarized data are presented in Figure 5B showing that TEMPOL ($N = 6$) and DPI ($N = 6$) decreased L-Hcys-induced increase in collagen type I protein by 60% and 56%, respectively.

Effects of TEMPOL and DPI on L-Hcys–induced proliferation of rat mesangial cells

The results of these experiments are presented in Figure 5. Incubation of mesangial cells with L-Hcys (40 to 160 μmol/L) for 48 hours concentration dependently enhanced the cell proliferation with a 1.5-fold maximal

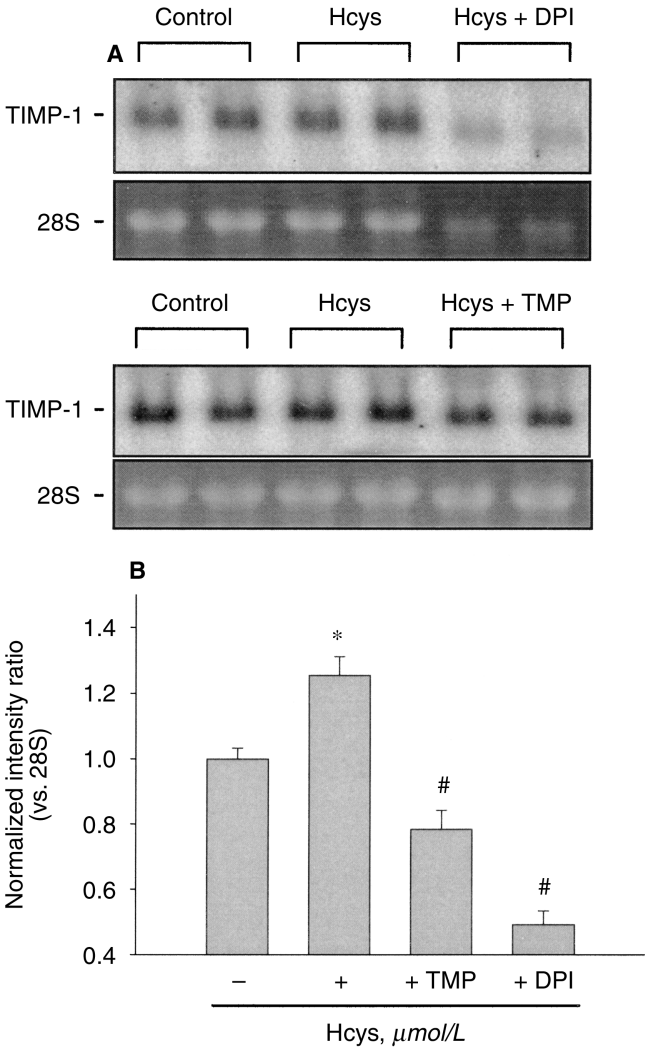

Fig. 4. Effects of diphenylene iodonium chloride (DPI) and hydroxyl tetramethylpiperidin-oxyl (TEMPOL) on L-homocysteine (L-Hcys)–induced tissue inhibitor of metalloproteinase (TIMP-1) mRNA expression in rat mesangial cells. (*A*) Typical autoradiographic documents of Northern blot analysis showing the mRNA levels of TIMP-1 in mesangial cells treated with L-Hcys in the presence TEMPOL or DPI. (*B*) Summarized data showing the intensity ratio of TIMP-1 mRNA to 28S blots in mesangial cells during treatment of L-Hcys alone and in the presence of TEMPOL or DPI. TMP indicates treatment of mesangial cells with TEMPOL, DPI indicates treatment of mesangial cells with DPI. *$P < 0.05$ compared with control; #$P < 0.05$ compared with the values obtained during L-Hcys treatment alone.

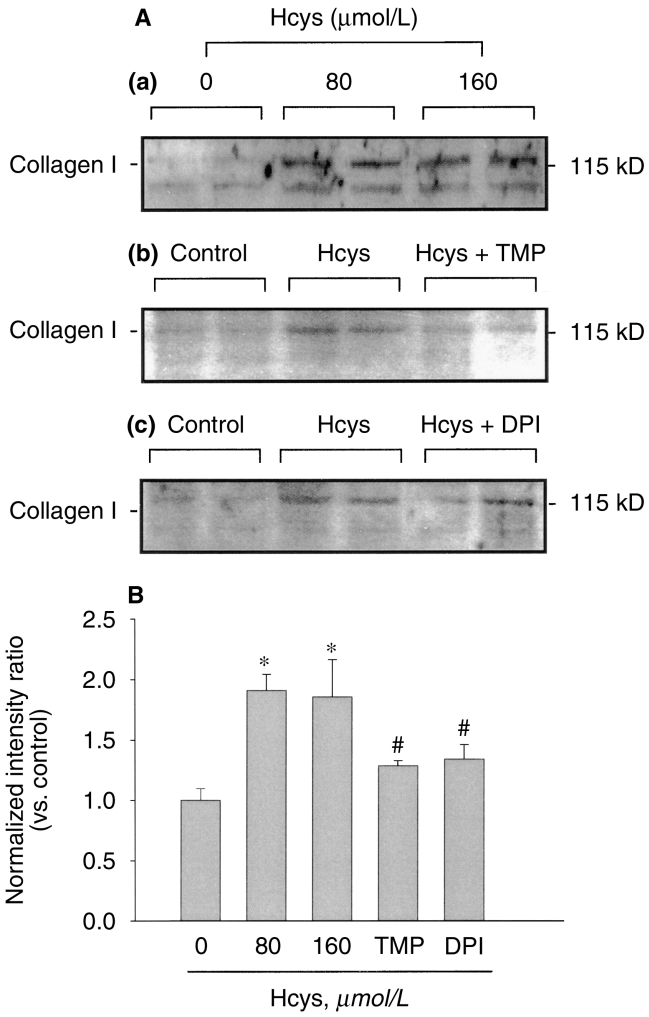

Fig. 5. Effects of L-homocysteine (L-Hcys) on levels of collagen type I in the presence or absence of hydroxyl tetramethylpiperidin-oxyl (TEMPOL) or diphenylene iodonium chloride (DPI) in rat mesangial cells. (*A*) Typical gel documents of Western blot analysis showing levels of collagen type I protein in rat mesangial cells treated with L-Hcys alone at different concentrations (a) and with Hcys (80 μmol/L) in the presence or absence of TEMPOL (b) and DPI (c). A 115 kD immunoreactive band was recognized by antibody against collagen type I. (*B*) Summarized data showing the intensity ratio of collagen type I protein to control in mesangial cells in the absence or presence of TEMPOL or DPI. *$P < 0.05$ compared with control; #$P < 0.05$ compared with the values obtained during L-Hcys treatment alone.

increase in DNA fluorescence ($N = 12$) (Fig. 6A). In the presence of 100 μmol/L TEMPOL, L-Hcys–induced mesangial cell proliferation was substantially attenuated. NADH oxidase inhibition by 10 μmol/L DPI also completely blocked L-Hcys–induced proliferation of these mesangial cells ($N = 8$) (Fig. 6B).

DISCUSSION

Despite intensive investigations, the mechanism by which hHcys causes arteriosclerosis or other pathologic changes is still poorly understood. It has been reported that hHcys-induced arteriosclerosis and thrombosis are associated with many pathologic processes such as endothelial damage, proliferation of vascular smooth muscle cells, increased lipid peroxidation, hemostatic imbalance, DNA methylation, and accumulation of collagens [9–15]. These pathologic changes may result from the production of Hcys-thiolactone, a highly reactive form of Hcys. This metabolite of Hcys activates oxidative stress, damages the lining cells, activates the growth of arterial smooth muscle cells, and stimulates the formation of fibrous tis-

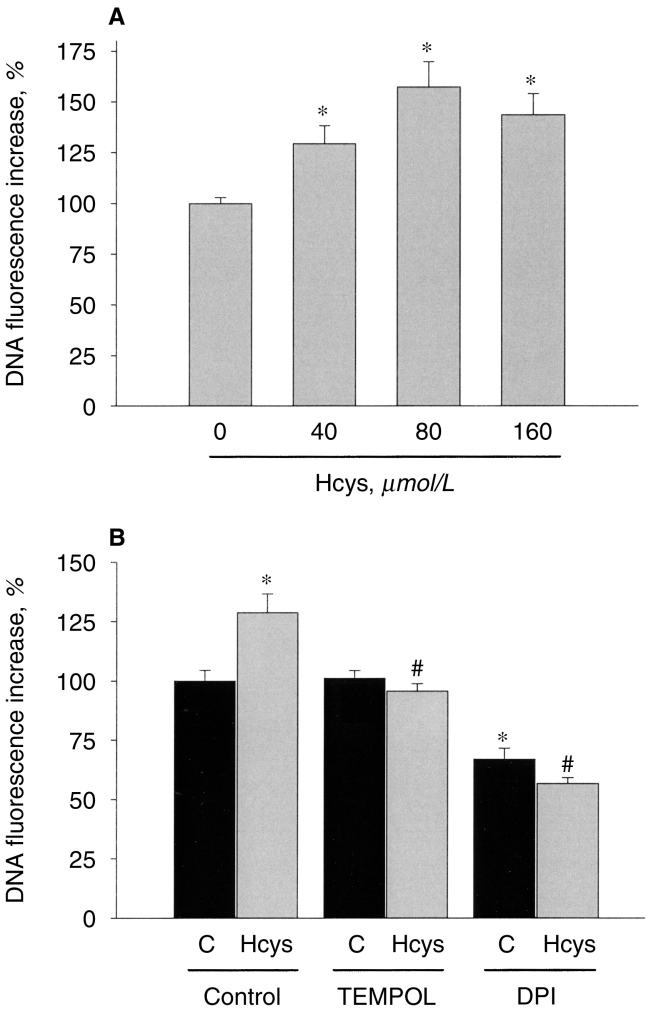

Fig. 6. Effects of L-homocysteine (L-Hcys) on rat mesangial cell proliferation in the presence or absence of hydroxyl tetramethylpiperidinoxyl (TEMPOL) and diphenylene iodonium chloride (DPI). (*A*) Summarized data showing the effects of L-Hcys (40 to 160 μmol/L) on cell proliferation of mesangial cells. (B) The effects of L-Hcys on mesangial cell proliferation in the presence of TEMPOL and DPI. $*P < 0.05$ compared with control; $\#P < 0.05$ compared with the values obtained during L-Hcys treatment alone.

sue, mucoid matrix, and degenerative elastic tissues [1]. Moreover, the auto-oxidation of Hcys may directly stimulate the production of reactive oxygen species (ROS) and exaggerate oxidative stress in the artery wall [36], leading to the development of arteriosclerosis and thrombosis. It is obvious that the pathogenic effects of Hcys are involved in a variety of cellular, biochemical, and molecular events, which are importantly related to local oxidative stress.

However, it remains unknown which pathway is responsible for Hcys-induced oxidative stress and whether this oxidative stress–mediated pathogenic mechanism contributes to Hcys-induced glomerular damage. Previous studies have shown that NADH oxidase is an important pathway for $O_2^{\cdot-}$ production in vascular tissues [37], and therefore activation of this enzyme in spontaneously hypertensive rats or during the elevation of plasma angiotensin II levels produced sustained hypertension [38, 39]. In the kidney, NADH oxidase has been demonstrated to primarily mediate the production of $O_2^{\cdot-}$ in response to angiotensin II in mesangial cells [40]. More recently, we have reported that NADH oxidase is a major enzyme responsible for $O_2^{\cdot-}$ production in the rat kidney homogenate [28] and microdissected thick ascending limb [27] and that $O_2^{\cdot-}$ production from NADH oxidase participates in the control of renal blood flow and tubular function even under physiologic conditions [27, 28]. It is possible that increased expression of NADH oxidase may represent one of the important mechanisms initiating glomerular damage during hHcys. To test this hypothesis, we performed a series of experiments using cultured rat mesangial cells and examined the NADH oxidase expression and activity and related role in the regulation of ECM metabolism. The mesangial cells were chosen since these cells have been reported to play an essential role in the development of glomerulosclerosis and mesangial expansion is considered as a featured change in glomerular pathology in end-stage renal disease. Many pathologic changes in the sclerotic glomeruli such as increased matrix formation, aggregated proteoglycan, cell proliferation or growth, and fibrosis are associated with dysfunction or damage of mesangial cells. Using these cells, we first demonstrated that incubation of rat mesangial cells with L-Hcys significantly increased the mRNA levels of both TIMP-1 and NADH oxidase subunit, Gp91. This coordinate increase in mRNA expression of TIMP-1 and NADH oxidase in Hcys-treated mesangial cells suggests that NADH oxidase may play an important role in the accumulation of ECM and mesangial expansion, since TIMP-1 is one of the most important enzymes responsible for the regulation of collagen accumulation or degradation in different tissues [10, 19, 20]. It has been reported that increased TIMP-1 expression may promote accumulation of collagen and deposition of ECM and that Hcys-induced enhancement of TIMP-1 expression in rat hepatic stellate cells and hepatocytes is involved in liver fibrosis [10]. It appears that TIMP-1 is a common mechanism participating in the sclerotic process.

By measuring $O_2^{\cdot-}$ production, we found that incubation of rat mesangial cells with L-Hcys significantly increased NADH oxidase activity, which could be completely blocked by NADH oxidase inhibitor, suggesting that this Hcys-induced $O_2^{\cdot-}$ production is derived from NADH oxidase. It is obvious that Hcys significantly enhances oxidative stress through activation of NADH oxidase in these mesangial cells. This is consistent with the findings from rat microvascular endothelial cells, suggesting that Hcys may produce oxidative stress through

induction of NADH oxidase and increase in the activity of this enzyme in mesangial cells [15, 41].

To address a concern about the specificity of DPI to NADH oxidase, we used an antisense approach to further confirm that in mesangial cells at 10 μmol/L DPI specifically blocks NADH oxidase activity. We examined the effect of phox22 blockade on NADH oxidase activity in the mesangial cells in response to L-Hcys in the presence or absence of DPI. We demonstrated that P22-AS eliminated the increase of NADH oxidase activity induced by L-Hcys. In the presence of DPI, no further decrease in NADH oxidase activity was found in the mesangial cells transfected with P22-AS. These results provide evidence that specific blockade of phox22 decreases activation of NADH oxidase by L-Hcys in the mesangial cells. Since DPI did not further inhibit NADH oxidase activity after phox22 blockade, the effect of DPI seems to share the same mechanism as phox22 blockade. Therefore, inhibition of Hcys-induced NADH oxidase activation by DPI does represent a specific effect in these mesangial cells. This antisense approach further increases the confidence to use DPI as a specific inhibitor of NADH oxidase in all other experiments.

Next, we addressed whether Hcys-induced $O_2^{\cdot-}$ production is responsible for enhanced TIMP-1 expression in mesangial cells. It was found that pretreatment of mesangial cells with NADH oxidase inhibitor, DPI completely blocked Hcys-induced increase in TIMP-1 expression. Similarly, a cell-permeable SOD mimetic, TEMPOL, also significantly attenuated Hcys-enhanced expression of TIMP-1. These results suggest that an NADH oxidase–associated oxidative stress is importantly involved in the regulation of TIMP-1 expression, thereby participating in sclerotic process. Although previous studies have indicated that Hcys may increase oxidative stress in the local tissue [15], the mechanisms increasing oxidants production or decreasing their cleavage have not been yet clarified. The results of the present studies provide the direct evidence that Hcys stimulates $O_2^{\cdot-}$ production via NADH oxidase and thereby up-regulates TIMP-1 in mesangial cells, which may result in accumulation of ECM in the glomeruli during hHcys.

Previous studies have demonstrated that TIMP-1 critically regulates the homeostasis of ECM and that its effect directly contributes to the metabolism of collagen in ECM. Therefore, the present study also examined whether Hcys-induced increase of TIMP-1 could result in accumulation of collagens. Although there are several types of collagens, such as collagen types I, III, IV, and V found in different tissues, we have found that ~95% of the total collagen synthesized in cultured mesangial cells was type I. In previous studies, induction of collagen type I in the glomerular mesangium has been documented in the experimental models of glomerulosclerosis [42]. We therefore examined the levels of collagen type I in mesangial cells treated with Hcys in the absence or presence of NADH oxidase inhibtor or SOD mimetic. As expected, L-Hcys was found to increase the levels of collagen type I in mesangial cells. Inhibition of NADH oxidase by DPI or scavenging of $O_2^{\cdot-}$ by TEMPOL substantially blocked L-Hcys–induced accumulation of collagen type I in these cells. These results further support the view that Hcys influences the homeostasis of ECM in mesangial cells through NADH oxidase–mediated $O_2^{\cdot-}$ production.

In addition to its effect on homeostasis of ECM, Hcys has been reported to stimulate the proliferation of different cells such as vascular smooth muscle cells, liver stellate cells, and fibroblast cells [6, 7]. The cell proliferation and growth also participate importantly in the sclerotic process in different tissues. However, it remains unknown whether Hcys stimulates the proliferation or growth of mesangial cells and thereby activates or accelerates glomerulosclerosis. In the present study, we examined the effects of L-Hcys on the proliferation of cultured rat mesangial cells. L-Hcys was found to significantly stimulate the proliferation of these mesangial cells. In the presence of DPI or TEMPOL, however, L-Hcys–induced proliferation of mesangial cells was completely blocked. These results suggest that Hcys-induced cell proliferation is also associated with NADH oxidase activity.

CONCLUSION

In summary, the present study provided direct experimental evidence that $O_2^{\cdot-}$ production by NADH oxidase contributes importantly to the effects of Hcys on the homeostasis of ECM and cell proliferation in mesangial cells. These NADH oxidase–induced effects on mesangial cells may represent an important mechanism producing glomerulosclerosis associated with hHcys.

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