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Activation of mesangial cell MAPK in response to homocysteine

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Background. Alteration in mesangial cell function is central to the progression of glomerular disease in numerous models of chronic renal failure (CRF). Animal models of chronic glomerular disease are characterized by mesangial cell proliferation and elaboration of extracellular matrix protein (ECM), resulting in glomerulosclerosis. Elevated plasma levels of homocysteine (Hcy) are seen in both animal models and humans with CRF, and have been proposed to contribute to the high prevalence of vascular disease in this group. Some of the pathogenetic effects of Hcy are thought to be mediated via the induction of endoplasmic reticulum stress. Thus, Hcy effects on mesangial cells could contribute to the progression of CRF. Previous work has shown Hcy- mediated induction of Erk mitogen-activated protein kinase (MAPK) in vascular smooth muscle cells (VSMCs). Erk induces increases in activator protein-1 (AP-1) transcription factor activity which may augment mesangial cell proliferation and ECM protein production. Consequently, we studied the effect of Hcy on mesangial cell Erk signaling.

Methods. Mesangial cells were exposed to Hcy after 24 hours of serum starvation and Erk activity assessed. Nuclear translocation of phospho-Erk was visualized by confocal microscopy. AP-1 nuclear protein binding was measured in response to Hcy by mobility shift assay. Hcy-induced mesangial cell calcium flux was measured in Fura-2 loaded cells. Mesangial cell DNA synthesis in response to Hcy was assessed by [^{3}H]-thymidine incorporation and proliferation by Western blotting for proliferating cell nuclear antigen (PCNA). Expression of endoplasmic reticulum stress response genes were determined by Northern and Western analysis.

Results. Hcy led to an increase in Erk activity that was maximal at 50 μmol/L and 20 minutes of treatment. Subsequent experiments used this concentration and time point. Erk activity in response to Hcy was insensitive to n-acetylcysteine and catalase, indicating oxidative stress did not play a role. However, Hcy 50 μmol/L induced a brief increase in intracellular mesangial cell calcium within 5 minutes, and the calcium ionophores A23187 and ionomycin increased Erk activity while chelation of intracellular calcium with BAPTA-AM abrogated the Erk response to Hcy. Confocal microscopy of activated Erk nuclear translocation mirrored these results as did mesangial cell nuclear protein binding to AP-1 consensus sequences. Hcy- induced increases in thymidine incorporation and PCNA expression at 24 hours were Erk dependent. The expression of endoplasmic reticulum stress response genes was significantly elevated by Hcy in an Erk-dependent manner.

Conclusion. Hcy increases Erk activity in mesangial cells via a calcium-dependent mechanism, resulting in increased AP-1 nuclear protein binding, cell DNA synthesis and proliferation and induction of endoplasmic reticulum stress. These observations suggest potential mechanisms by which Hcy may contribute to progressive glomerular injury.

Key words: mesangial cell, homocysteine, mitogen-activated protein kinase, signaling.

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The mesangial cell provides architectural support for glomerular capillary loops [1]. Proliferation of mesangial cells and elaboration of extracellular matrix protein (ECM) are important factors in the glomerulosclerosis that occurs in several models of chronic renal failure (CRF) [2–5]. Both physical and chemical factors play roles in stimulating mesangial cell proliferation and ECM production. Intraglomerular hypertension increases the magnitude of cyclic stretch to which mesangial cells are exposed, and has been demonstrated to precede the accumulation of ECM protein in CRF animal models [3–5], and interventions that decrease intraglomerular pressure attenuate sclerotic injury [6, 7]. Cytokines such as angiotensin II (Ang II) [8] and transforming growth factor-beta (TGF-β) [9] also play critical roles in the promotion of injury in these models, and transfection of either TGF-β [10] or infusion of Ang II [11] into intact animal glomeruli results in sclerosis. Sclerotic glomeruli are also characterized by resident cell loss, and evidence is accumulating that apoptosis is an important mechanism in progressive glomerular injury [12–14].

Homocysteine (Hcy) is a thiol-containing amino acid formed as an intermediate metabolite in the conversion of methionine to cysteine [15]. Elevated plasma levels of Hcy have been identified as an important and

independent risk factor for myocardial infarction [16] and atherosclerosis in humans [17, 18]. It is now well established that patients with CRF have the highest nongenetically determined plasma Hcy levels seen in clinical practice [19, 20], and this has been reported to be an independent risk factor for adverse cardiovascular outcomes in these patients [21, 22]. In Dahl salt-sensitive rats, a high methionine diet exacerbated proteinuria and glomerulosclerosis, an effect attributed to the hyperhomocysteinemia that results from methionine loading [23]. Whether elevated ambient levels of Hcy contribute to progression of glomerular disease in humans, however, is unknown.

Recent in vitro data support this possibility, however. Hcy treatment for 48 hours increases mesangial cell proliferation and collagen I in culture [24]. This is consistent with the effects of Hcy on the systemic vasculature, which has been more extensively studied. Aortic vascular smooth muscle cells (VSMC) proliferate when exposed to Hcy [25, 26] and increase their production of collagen [27, 28]. Conversely, endothelial cells show an inhibition of DNA synthesis and proliferation when exposed to Hcy [29]. Apoptosis has also been observed in endothelial cells exposed to Hcy, and induction of the unfolded protein response and endoplasmic reticulum stress appear to mediate this effect [30]. Mitogen-activated protein kinase (MAPK) signaling may also be important in mediating these effects- Hcy activated Erk in VSMC [31], but inhibited it in endothelial cells [32]. In monocytes, Hcy activated Erk in a reactive oxygen species (ROS)-dependent manner, and downstream cytokine secretion was Erk dependent [33]. We and others have established that Erk signaling induces mesangial cell proliferation in response to numerous stimuli [34–39], while data also indicate that Erk signaling may induce endoplasmic reticulum stress in response to hypoxia or intracellular calcium release [40, 41].

Accordingly, we studied whether Hcy might lead to activation of Erk MAPK in mesangial cells, and the mechanisms thereof. Furthermore, we studied whether two downstream events implicated in vascular injury, proliferation and endoplasmic reticulum stress, were also affected by Hcy in mesangial cells in an Erk-dependent manner.

METHODS

Materials and cell culture

Sprague- Dawley rat mesangial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) (Gibco BRL, Mississauga, Ontario, Canada), streptomycin (100 μg/mL), penicillin (100 U/mL), and 2 mmol/L glutamine at 37°C in 95% air/5% CO_2 as described previously [38, 39]. Experiments were carried out in cells from passages 5 to 10. DL-Hcy, catalase, n-acetylcysteine, and BAPTA-AM were from Sigma Chemical Co. (St. Louis, MO, USA). PD98059 and U0126 were from Calbiochem (San Diego, CA, USA) and Promega (Madison, WI, USA), respectively.

MAPK (Erk) activity

Protein isolation and Western blotting. Initially, the time course and concentration dependence of Erk activation in response to Hcy were studied, and subsequent experiments performed with 50 μM Hcy at 20 minutes. Cultures were serum-starved for 20 minutes prior to the addition of Hcy and/or inhibitors. After incubation with Hcy and/or inhibitors, media was removed and the cells washed once with ice-cold phosphate-buffered saline (PBS). PBS was then removed and cells harvested under nondenaturing conditions on ice by incubation for 5 minutes with 0.5 mL 1× ice-cold cell lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L egtazic acid (EGTA), 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β- glycerophosphate, 1 mmol/L sodium orthovanadate, and 1 μg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. Cells were then scraped into microcentrifuge tubes on ice and sonicated four times for 5 seconds each. After microcentrifugation at 14,000 rpm for 10 minutes at 4°C, the supernatant was transferred to a fresh microcentrifuge tube. Protein concentration was measured with the Bio-Rad Assay Kit (Bio-Rad, Hercules, CA, USA). Subsequently, 40 μg of sample was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted to a nitrocellulose membrane (Protran, Schleicher and Schuell, Keene, NH, USA). Membranes were incubated for 3 hours at room temperature with 25 mL of blocking buffer [1× Tris-buffered saline (TBS), 0.1% Tween-20 (TTBS) with 5% wt/vol nonfat dry milk], and then overnight at 4°C with Erk MAPK (thr 202/tyr 204) polyclonal antibody (1:1000) (New England Biolabs, Beverly, MA, USA) in 10 mL of antibody dilution buffer [1× TBS, 0.05% Tween-20 with 5% bovine serum albumin (BSA)] with gentle rocking overnight at 4°C. Membranes were then washed three times with TTBS and then incubated with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibody (1:2000) in 10 mL of blocking buffer for 45 minutes at room temperature. After three further TBS washes, the membrane was incubated with LumiGlo reagent (KPL Inc., Gaithersburg, MD, USA) and then exposed to x-ray film (X-OMAT, Kodak, Rochester, NY, USA).

Activity assays. After protein isolation from total cell lysate as above, 200 μg total protein was then incubated with Erk MAPK (thr 202/tyr 204) monoclonal

antibody (1:200) (New England Biolabs) with gentle rocking overnight at 4°C. Protein sepharose A beads (20 μL of 50% beads) were then added and the gentle rocking continued for 3 further hours. Lysate was then microcentrifuged for 30 seconds at 14,000 rpm to recover the beads, and the pellet washed twice with 0.5 mL of 1× lysis buffer. For the kinase assay, after immunoprecipitation, pellets were washed twice with 0.5 mL kinase buffer [25 mmol/L Tris, 5 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol (DTT), 0.1 mmol/L sodium orthovanadate, and 10 mmol/L $MgCl_2$]. Erk MAPK activity was then performed by suspending the pellet in 50 μL of 1× kinase buffer, with 200 μmol/L adenosine triphosphate (ATP) and 2 μg Elk-1 fusion protein as substrate. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 μmol/L 3× SDS sample buffer [187.5 mmol/L Tris-HCl (pH 6.8), 6% wt/vol SDS, 30% glycerol, 150 mmol/L DTT, and 0.3% wt/vol bromphenol blue], boiled for 5 minutes, vortexed, and then microcentrifuged for 2 minutes. Twenty microliters of sample was then run on an SDS-PAGE gel. After blotting to nitrocellulose, membranes were incubated for 3 hours at room temperature with 25 mL of blocking buffer (1× TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk), and then overnight at 4°C with phospho-specific anti-Elk-1 (ser 383) antibody 1:1000 in 10 mL of antibody dilution buffer (1× TBS, 0.05% Tween-20 with 5% BSA). Gels were washed 3 times with TTBS and then incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000) for 1 hour at room temperature. After three further TBS washes, the membrane was incubated with LumiGlo reagent (KPL Inc.) and then exposed to X-ray film (X-OMAT).

Assessment of intracellular oxidative stress. The H_2O_2-sensitive fluorescent probe 5-(and-6)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) (Molecular Probes, Eugene, OR, USA) was used to assess the production of intracellular H_2O_2. Intracellular esterases convert this compound to 2′,7′-dichlorofluorescein which is then oxidized by H_2O_2 to the fluorescent 2′,7′-dichlorofluorescein. Subconfluent mesangial cells were made quiescent by serum deprivation in 0.5% serum for 24 hours. Cells were then incubated with 10 μmol/L CM-H_2DCFDA for 30 min at 37°C in PBS. This was replaced by fresh media after washing, and mesangial cells were treated with 3000 U/mL catalase or 1 mmol/L n-acetylcysteine for 10 minutes prior to incubation with 50 μmol/L Hcy for 20 minutes. Mesangial cells were then fixed for 10 minutes in 3.7% formaldehyde, mounted in DAPI-containing mounting medium (Vectashield, Burlingame, CA, USA) and imaged using a fluorescent microscope (Zeiss, Thornwood, NY, USA) at an excitation wavelength of 488 nm and an emission wavelength of 510 to 550 nm.

Phospho-Erk nuclear translocation. Mesangial cells (passages 5 to 10) were grown on glass slips for these experiments exactly as above. After incubation with Hcy with or without inhibitors, cells were washed three times with PBS and fixed with 3.7% formaldehyde (300 μL/well) for 10 minutes at room temperature. Cells were washed three times with PBS and then permeabilized in 0.1% Triton for 2 minutes on ice, washed again with PBS and incubated with antiphospho-Erk (thr202/tyr204) (New England Biolabs) 1:50 in PBS for 30 minutes at room temperature. Following three further PBS washes, cells were incubated with an Alexa 488 goat antirabbit green IgG (H + L) fluorescent-conjugated antibody (Molecular Probes) 1:50 in PBS (the secondary antibody was recovered by centrifugation at 12000 rpm for 10 minutes prior to application) for 30 minutes at room temperature in the dark. After washing, a drop of antifade mount media mixture was added and mesangial cells covered with a glass slip. Slides were stored at 4°C in the dark until confocal microscopy laser scanning was performed using a Bio-Rad MRC-600 confocal microscope (Bio-Rad, Mississauga, Ontario, Canada) within 7 days.

Nuclear protein binding to activated protein-1 (AP-1) consensus sequences. After washing in cold PBS, nuclear extracts of mesangial cells were prepared by lysis in hypotonic buffer (20 mmol/L Hepes, pH 7.9, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L $Na_4P_2O_7$, 1 mmol/L DTT, 0.5 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 0.6% Nonidet P-40), homogenized and sedimented at 16,000*g* for 20 minutes at 4°C. Pelleted nuclei were resuspended in hypotonic buffer with 0.42 mol/L of $NaCl_2$ and 20% glycerol and rotated for 30 minutes at 4°C. After centrifugation for 20 minutes at 16,000*g*, the supernatant was collected and protein concentration was measured with the Bio-Rad Assay Kit.

AP-1 consensus oligonucleotides were prepared by incubating 2 μL consensus oligonucleotide (1.75 pmol/uL), 1 μL T4 polynucleotide kinase 10× buffer, 1 μL [γ-^{32}P]-ATP (3000 Ci/mL) (DuPont, Boston, MA, USA) and 5 μL nuclease-free water for 10 minutes at 37°C. The reaction was stopped by adding 1 μL of 0.5 mol/L EDTA. Unlabeled [^{32}P]-ATP was removed from the oligonucleotide mixture with D-25 Sephadex columns.

The supernatants were used as nuclear proteins for the binding assay. Three micrograms of nuclear proteins were incubated with 2 μg of poly(dI-dC).poly(dI.dC) (Pharmacia, Uppsala, Sweden) in binding buffer (20 mmol/L Hepes, pH 7.9, 1.8 mmol/L $MgCl_2$, 2 mmol/L DTT, 0.5 EDTA, and 0.5 mg/mL BSA), incubated for 30 minutes at room temperature and then reacted with radiolabeled consensus oligonucleotides at room temperature for 20 minutes (50,000 cpm to 100,000 cpm). Reaction mixtures were electrophoresed in a 6% polyacrylamide gel and autoradiographed. Competition experiments were performed with excess unlabeled consensus oligonucleotides in 100× excess.

Calcium influx. Mesangial cells grown on glass coverslips were preloaded for 30 minutes in the dark with the calcium sensitive fluorescent dye Fura-2/AM (5 μM) in DMEM with 1 mg/mL BSA. After washing, mesangial cells were mounted in a chamber with 1.5 mL Hepes-buffered NaCl solution containing 1.5 mmol/L $CaCl_2$ and 1.0 mmol/L $MgCl_2$. The chamber was mounted in a fluorescence microscope (Zeiss) and 50 μmol/L Hcy was added. Fluorescence measurements were performed at emission wavelength 500 nm and excitation wavelengths of 340 and 380 nm, representing fluorescence of calcium-bound and unbound dye, respectively. Cytosolic free calcium was then calculated from the ratio (R) of the two wavelengths according to the standard formula [42], with R_{max} and R_{min} calculated according to standard methodology [42].

[^{3}H]-thymidine Incorporation. Incorporation of [^{3}H]-thymidine was performed as we have previously published [38]. Briefly, subconfluent mesangial cells were made quiescent by serum deprivation in 0.5% serum for 24 hours. Mesangial cells were then incubated with 50 μmol/L Hcy with or without the extracellular signal-related protein kinase (ERK) (MEK-1) inhibitor U0126 or the calcium chelator BAPTA-AM (both used at 10 μmol/L) for 24 hours. [^{3}H]-thymidine was added for the last 4 hours of this period. Mesangial cells were then washed three times with ice-cold PBS, incubated in ice-cold 5% trichloroacetic acid for 25 minutes and dissolved in 500 μL 0.2 mol/LNaOH. [^{3}H]-thymidine incorporation was counted on a Beckman LS7500 Scintillation counter. Protein concentrations were determined in each well as above, and [^{3}H]-thymidine incorporation expressed as counts per minute per microgram protein.

Western Blotting for proliferating cell nuclear antigen (PCNA) and endoplasmic reticulum stress proteins. After serum starvation, mesangial cells were incubated with 50 μmol/L Hcy for 24 hours. Subsequently, protein was isolated as above, and 40 μg of sample was separated on a 12% SDS-PAGE gel, and electroblotted to a nitrocellulose membrane. Membranes were incubated for 1 hour at room temperature with 25 mL of blocking buffer (1× TBS, 0.1% Tween-20 with 5% wt/vol non-fat dry milk) and then for 1 hour at room temperature with either monoclonal anti-PCNA antibody (1:1000 in antibody dilution buffer) (Novocastra Labs., Newcastle, UK), anti-GRP 78/GRP 94 (1:3000) (StressGen, Victoria, British Columbia, Canada) as we have published [15], polyclonal antiprotein disulfide isomerase (PDI) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal anti-GADD153 (1:1000) (Santa Cruz Biotechnology). Following three TTBS washes, a 1-hour incubation with the appropriate HRP-conjugated secondary antibody 1:2000 was performed. After three further TBS washes, the membrane was incubated with LumiGlo reagent (KPL Inc.) and then exposed to x-ray film (X-OMAT; Kodak).

Northern analysis

Total RNA was isolated from Hcy-treated mesangial cells as indicated using the RNeasy total RNA kit (Qiagen Inc., Mississauga, Ontario, Canada) and resuspended in diethyl pyrocarbonate–treated water. Quantification and purity of the RNA was assessed by A260/A280 absorption, and RNA samples with ratios above 1.6 were stored at −70°C for further analysis. Total RNA (10 μg/lane) was size fractionated on 2.2 mol/L formaldehyde/1.2% agarose gels, transferred to Zeta-Probe GT nylon membranes (Bio-Rad Laboratories), and hybridized using radiolabeled cDNA probes as we have described [15, 43]. Signal intensities were quantified by densitometric scanning of the autoradiograms using the ImageMaster VDS and Analysis Software (Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada). To correct for differences in gel loading, integrated optical densities were normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA probe encoding GRP78/BiP has been described previously [15, 43].

Statistical analysis

Analyses were performed with analysis of variance (ANOVA), using Tukey's HSD for post hoc analysis to determine differences between individual groups when ANOVA was siginificant. A two-tailed $P < 0.05$ was considered significant. Analyses were performed with SPSS for Windows, version 11.0.

RESULTS

Erk protein and activity

Initially, we studied Erk activation in mesangial cells exposed to Hcy. The in vitro activity of Erk was measured in immunoprecipitates from cell lysates after incubation with an Elk-1 fusion protein. Phosphorylation of the substrate, Elk-1, was then detected by Western blot analysis with a specific antibody to phosphorylated Elk-1. Incubation of mesangial cells with Hcy for 1 hour at concentrations from 5 μmol/L to 5 mmol/L led to an increase in Erk activity that was maximal at 50 μmol/L (Fig. 1A). Accordingly, we subsequently used a concentration of 50 μmol/L, as this plasma level is commonly seen in patients with CRF. No change in the amount of Erk protein was seen under any condition. Some cytotoxicity was observed only at 5 mmol/L, with about 5% of cells floating after 1-hour incubation at this concentration (data not shown). Time course studies with Hcy 50 μmol/L revealed maximal Erk activity after 20 minutes, with decay thereafter (Fig. 1B). Consequently, subsequent experiments were performed at this time point. No activation of Erk was seen with cysteine at doses up to 1 mmol/L (data not shown).

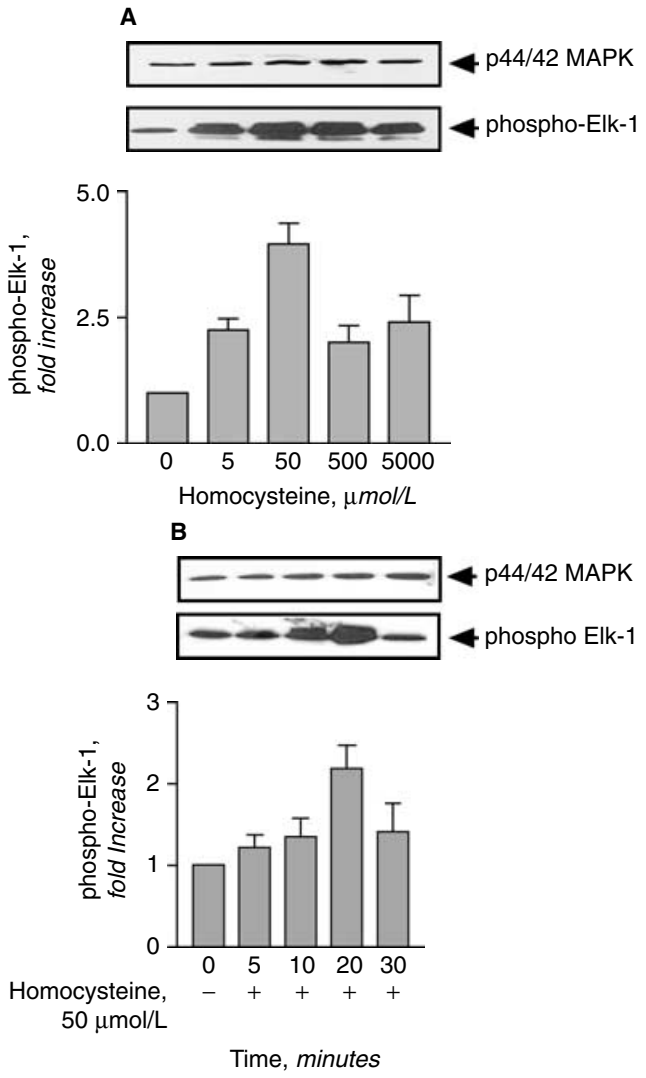

Fig. 1. Induction of Erk activity in mesangial cells by homocysteine (Hcy) is concentration- and time-dependent. The in vitro activity of Erk was measured in immunoprecipitates from mesangial cell lysates by detection of phosphorylation of an Elk-1 fusion protein by Western blotting. (*A*) Incubation of mesangial cells with Hcy for 1 hour at concentrations from 5 μmol/L to 5 mmol/L led to an increase in Erk activity that was maximal at 50 μmol/L (middle panel). No change in the amount of Erk protein was seen under any condition (top panel). Changes in activity are presented graphically with error bars (bottom panel) ($N = 4$). Erk activity at 50 μmol/L was significantly different from baseline and other concentrations ($P < 0.01$). (*B*) Incubation of mesangial cells with Hcy at 50 μmol/L for the times indicated led to an increase that was maximal at 20 minutes (middle panel). No change in the amount of Erk protein was seen under any condition (top panel). Changes in activity are presented graphically with error bars (bottom panel) ($N = 4$). Erk activity at 20 minutes only was significantly different from baseline and other time points ($P < 0.05$). MAPK is mitogen-activated protein kinase.

Mediators of Erk activity in response to Hcy

Next, we wished to explore mechanisms by which Hcy might activate Erk signaling. It is well-established that thiols, including Hcy, can generate ROS as a consequence of their rapid auto-oxidation [44], and recent data have indicated that Hcy enhances tissue-inhibitor of metalloproteinase (TIMP-1) expression in mesangial cells via the induction of oxidative stress [24]. We have also shown that Hcy impairs the generation of antioxidant enzymes in endothelial cells [43]. Consequently, we sought to determine whether oxidative stress and the generation of ROS played a role in activation of Erk by preincubating mesangial cells with n-acetylcysteine (1 mmol/L) (Sigma) or catalase (3000 U/mL) (Sigma) for 10 minutes prior to the addition of Hcy. Figure 2A shows that neither antioxidant affected the Erk activity observed in response to Hcy. In order to ensure that n-acetylcysteine and catalase were providing effective relief of oxidative stress in Hcy-treated mesangial cells, we employed the H_2O_2-sensitive fluorescent probe CM-H_2DCFDA. Figure 2B shows that 50 μmol/L Hcy for 20 minutes increased CM-H_2DCFDA fluorescence in mesangial cells when compared with baseline. Preincubation with n-acetylcysteine (1 mmol/L) or catalase (3000 U/mL) effectively prevented the Hcy-induced increase in CM-H_2DCFDA fluorescence.

Hcy has also been shown to induce calcium transients in VSMC [45], and an increase in intracellular calcium has been observed to activate Erk in several cell lines [46]. There is indirect evidence that Hcy activation of Erk in VSMC is calcium-sensitive [31]. We therefore wished to determine whether calcium played a role in the mesangial cell Erk response to Hcy. As demonstrated in Figure 2C, BAPTA-AM (10 μM) (Sigma), an intracellular calcium chelator, completely abrogated Hcy-induced Erk activity in mesangial cells when added 10 minutes prior to Hcy. Supporting this observation, exposure of mesangial cells to the calcium ionophore A23187 (1 μg/mL) (Alexis, San Diego, CA, USA) (Fig. 2C) or ionomycin (data not shown) also led to Erk activation.

Since calcium chelation was able to prevent Hcy-induced activation in mesangial cells, we sought to determine whether Hcy induced a calcium transient in mesangial cells, as has been observed by others in VSMC [45]. Figure 3 shows a representative view of the consistently-observed rapid increase in intracellular calcium, with a peak of 225 nmol/L, that was sustained above baseline (100 nmol/L) for 6 minutes ($N = 11$).

Translocation of phospho-Erk in response to Hcy

Figure 4 shows that calcium is important for translocation of phospho-Erk to the nucleus. Figure 4A shows background cytoplasmic and nuclear staining in normal mesangial cells, and Figure 4B shows the prompt nuclear translocation of phospho-Erk after 20 minutes of 50 μmol/L Hcy. However, the addition of BAPTA-AM (10 μmol/L) (Fig. 4C) virtually abrogated the phospho-Erk translocation observed in response to Hcy, recapitulating the results obtained with activity assays. Neither n-acetylcysteine (1 mmol/L) nor catalase

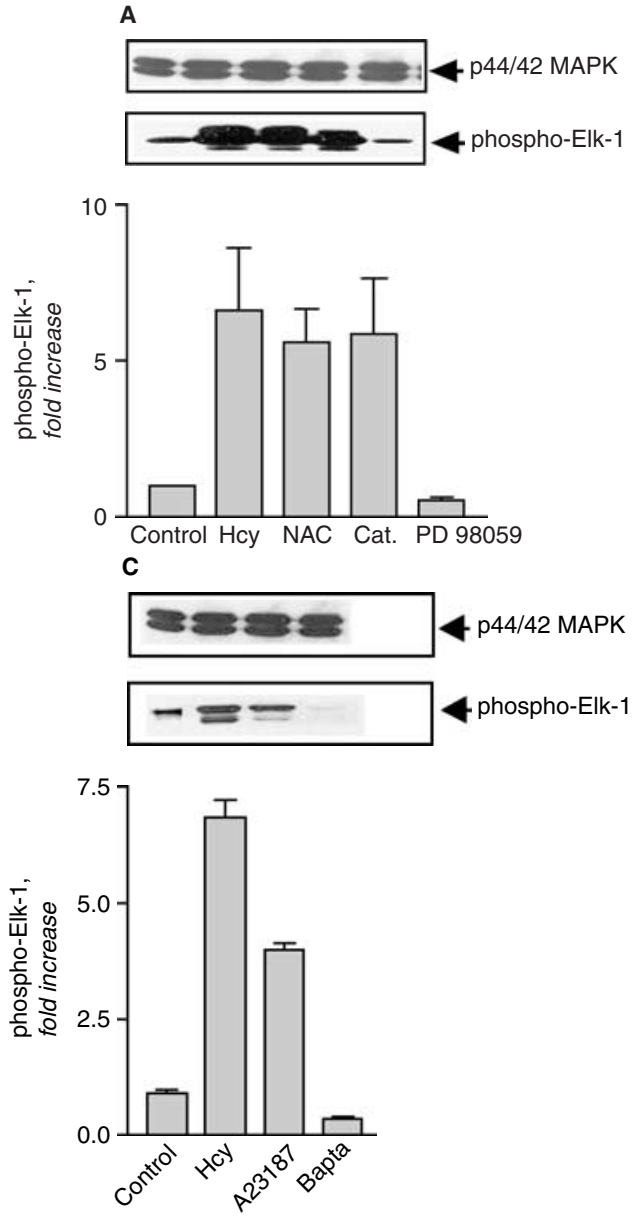

Fig. 2. Mediators of homocysteine Hcy-induced Erk activation in mesangial cells. The in vitro activity of Erk was measured in immunoprecipitates from mesangial cell lysates by detection of phosphorylation of an Elk-1 fusion protein by Western blotting. (*A*) Incubation of mesangial cells with 50 μmol/L Hcy for 20 minutes led to an increase in Erk activity that was unaffected by pre-incubation with n-acetylcysteine (1 mmol/L) nor catalase (Cat) (3000 U/mL) for 10 minutes prior to the addition of Hcy. The MEK inhibitor PD98059 effectively abrogated Erk activity, as anticipated (middle panel). No change in the amount of Erk protein was seen under any condition (top panel). Changes in activity are presented graphically with error bars (bottom panel) ($N = 4$). Erk activity in the presence of Hcy was significantly greater than baseline or Hcy with PD98059 ($P < 0.01$), but not different from Hcy with n-acetylcysteine or catalase. (*B*, located on the next page) The H_2O_2-sensitive fluorescent probe (CM-H_2DCFDA) (Molecular Probes, Eugene, OR, USA) was used to assess the production of intracellular H_2O_2 and the ability of catalase and n-acetylcysteine to relieve oxidative stress in this system. Subconfluent quiescent mesangial cells were treated with 3000 U/mL catalase or 1 mmol/L n-acetylcysteine for 10 minutes prior to incubation with 50 μmol/L Hcy for 20 minutes, then fixed in formaldehyde, mounted in DAPI-containing mounting medium, and imaged using a fluorescent microscope. Panel A shows untreated cells; panel B shows mesangial cells exposed to 50 μmol/L Hcy for 20 minutes; panel C shows mesangial cells coincubated with Hcy and catalase (3000 U/mL); and panel D shows mesangial cells coincubated with Hcy and n-acetylcysteine (1 mmol/L). (*C*) Incubation of mesangial cells with 50 μmol/L Hcy for 20 minutes led to an increase in Erk activity that was abrogated by preincubation with BAPTA-AM (10 μmol/L) an intracellular calcium chelator, when added 10 minutes prior to Hcy. The 20-minute exposure to the calcium ionophore A23187 (1 μg/mL, Alexis) also activated Erk, although not to the same extent as Hcy (all middle panel). No change in the amount of Erk protein was seen under any condition (top panel). Changes in activity are presented graphically with error bars (bottom panel) ($N = 4$). Erk activity in response to Hcy or A23187 was signficantly greater than baseline or Hcy with BAPTA-AM ($P < 0.03$).

(3000 U/mL) had any effect on phospho-Erk translocation (data not shown).

AP-1 nuclear protein binding in response to Hcy

Since increased AP-1 transactivational activity is important in both mesangial cell proliferation [47–49], and endoplasmic reticulum stress induction in response to increased intracellular calcium or hypoxia [40, 41], we next studied whether Hcy-induced changes seen in Erk activity led to downstream increases of nuclear protein binding to AP-1 consensus sequences in mesangial cells. As shown in Figure 5, Hcy 50 μmol/L for 20 minutes resulted in a marked increase in nuclear protein binding that was primarily Erk dependent, as it was largely prevented by pre-incubation with the Erk inhibitor PD98059 (10 μmol/L) (Calbiochem). The antioxidants n-acetylcysteine and catalase were without effect. However, the increase in AP-1 nuclear protein binding observed in response to Hcy could be abrogated by BAPTA-AM (Fig. 6), again supporting a role of calcium in this signaling pathway. It should be noted that nuclear protein binding to AP-1 consensus sequences may not be proportional to AP-1–mediated transactivation.

Mesangial cell DNA synthesis and proliferation in response to Hcy

To ascertain whether any functional outcome important to progressive glomerular disease might be impacted through this Hcy-activated signaling pathway, we studied mesangial cell DNA synthesis and proliferation in response to Hcy. Incubation of mesangial cells with 50 μmol/L Hcy, a plasma concentration often seen in human subjects with CRF [20, 50], for 24 hours led to an increase in [^{3}H]-thymidine incorporation by mesangial cells (Fig. 7A) and to an increase in expression of PCNA by Western blot (Fig. 7B). Preincubation with MEK-1 inhibitors U0126 (Fig. 7A) or PD98059 (Fig. 7B), both 10 μmol/L, abrogated this, demonstrating the Erk dependence of the effect. Preincubation with BAPTA-AM

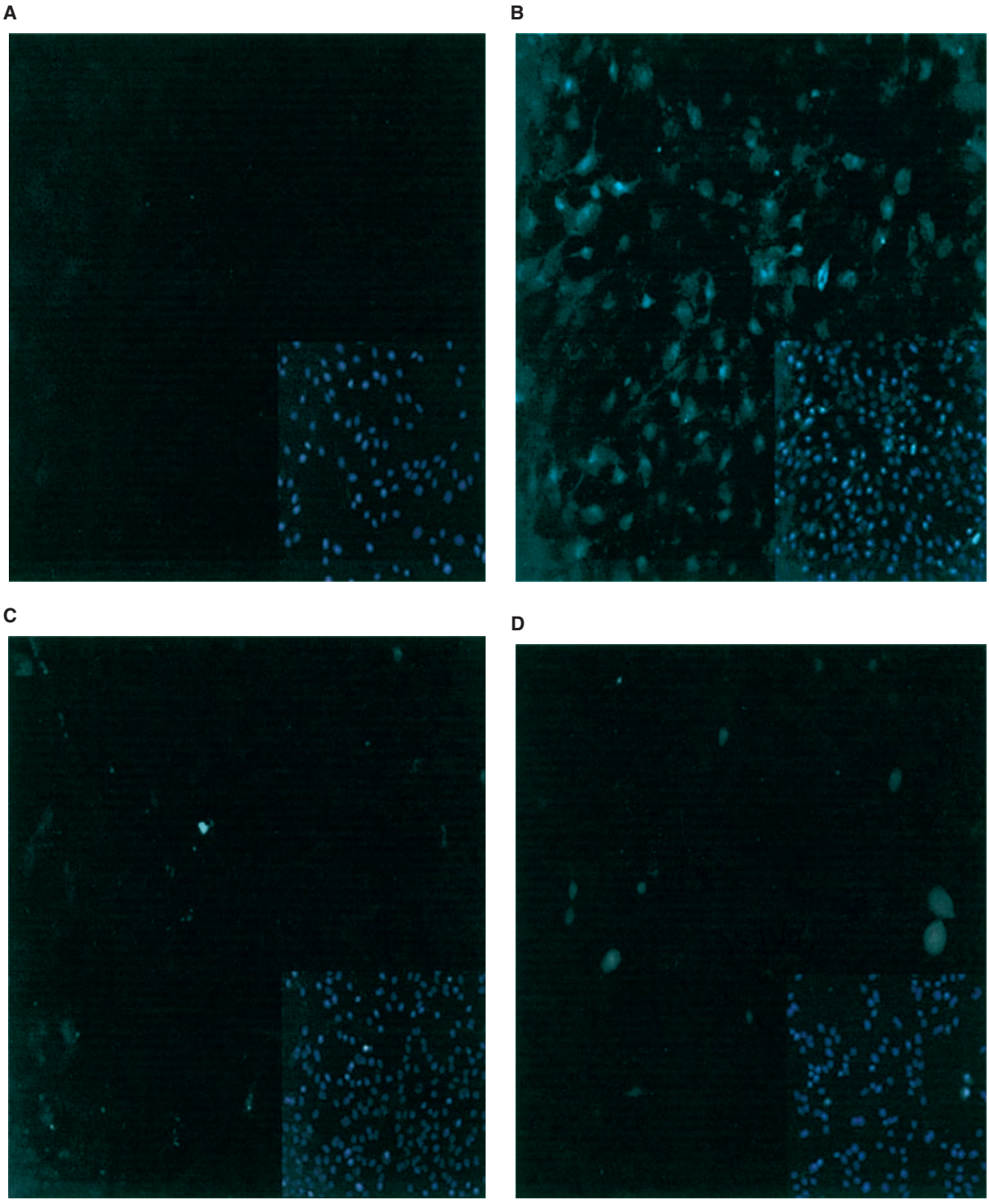

Fig. 2B.

also prevented [^{3}H]-thymidine incorporation in response to Hcy. Both MEK-1 inhibition and BAPTA-AM markedly reduced [^{3}H]-thymidine incorporation, attesting to the importance of Erk signaling and calcium in mesangial cell DNA synthesis in general.

Endoplasmic reticulum stress in response to Hcy

Induction of endoplasmic reticulum stress genes, such as the molecular chaperones Grp78 and 94, GADD 153 and PDI, are critical to the maintenance of cellular viability in response to many extracellular stresses, such as

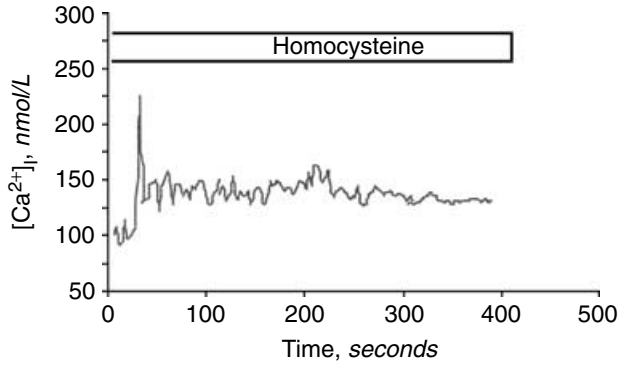

Fig. 3. Homocysteine (Hcy) induces a rise in intracellular calcium in mesangial format. Mesangial cells grown on glass slips were loaded with Fura-2/AM and intracellular calcium measured after exposure to 50 μmol/L Hcy. A rapid peak in intracellular calcium after addition of 50 μmol/L Hcy is observed ($N = 11$).

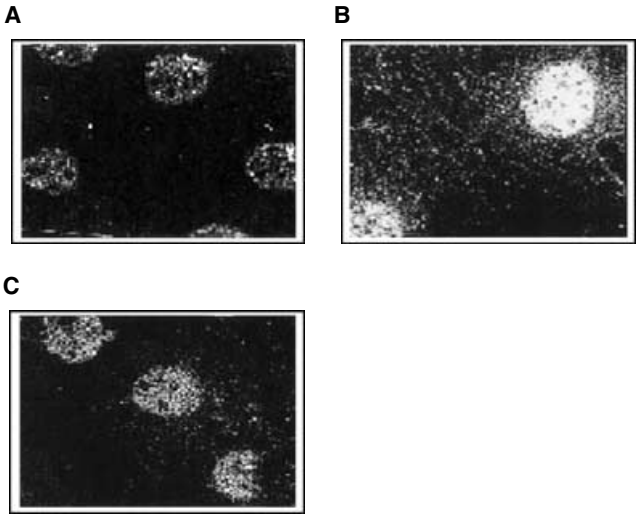

Fig. 4. Nuclear translocation of phospho-ERK is induced by homocysteine (Hcy). Mesangial cells grown on glass slips were incubated with Hcy with or without inhibitors, permeabilized, and incubated with anti phospho-Erk for 30 minutes and subsequently with a secondary goat antirabbit green fluorescent-conjugated antibody and confocal laser scanning microscopy performed. (*A*) Background cytoplasmic and nuclear staining in normal mesangial cells. The addition of 50 μmol/L Hcy for 20 minutes (*B*) led to a prompt (20 minutes) increase in staining that was almost entirely nuclear in distribution. Preincubation with BAPTA-AM (10 μmol/L for 10 minutes) (*C*) abrogated the nuclear translocation of phospho-Erk observed with Hcy. Four separate experiments were performed and representative fields shown.

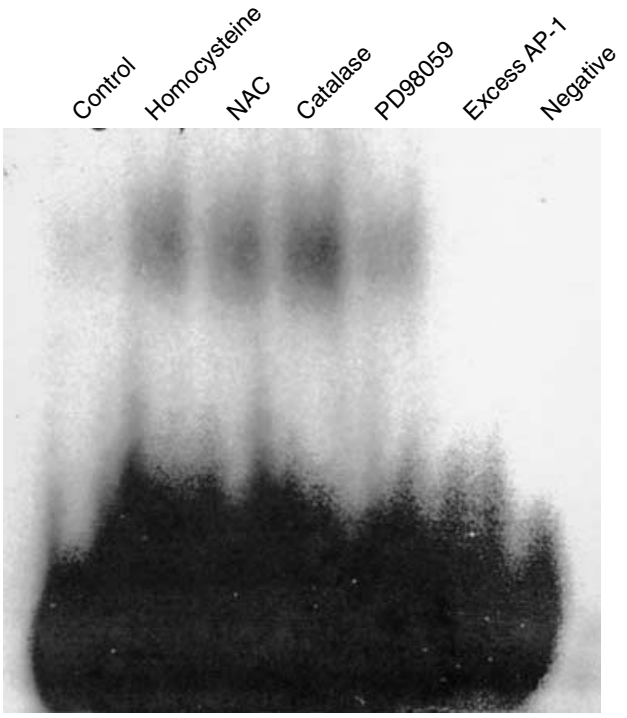

Fig. 5. Erk-dependent nuclear protein binding to activator protein-1 (AP-1) consensus sequences is induced by homocysteine (Hcy). Nuclear proteins were reacted with radiolabeled AP-1 consensus oligonucleotides for 20 minutes, electrophoresed, and autoradiographed. Lane 1 shows nuclear protein binding in normal mesangial cells. Addition of 50 μmol/L Hcy for 20 minutes led to an increase in nuclear protein binding (lane 2) that was unaffected by preincubation with n-acetylcysteine (1 mmol/L) or catalase (3000 U/mL) for 10 minutes prior to the addition of Hcy (lanes 3 and 4). The Erk inhibitor PD98059 (10 μmol/L for 10 minutes prior to Hcy) largely prevented the increase in nuclear protein binding (lane 5). Competition experiments were performed with excess unlabelled consensus oligonucleotides in 100× excess (lane 6). Four separate experiments were performed and a representative autoradiograph is shown. Densitometry revealed that nuclear protein binding to AP-1 consensus sequences in the presence of Hcy was significantly greater than baseline or Hcy with PD98059 ($P < 0.05$), but not different from Hcy with n-acetylcysteine or catalase.

treatment with calcium ionophores and oxidative stress [51, 52]. We and others have shown that induction of endoplasmic reticulum stress is seen in response to Hcy in endothelia, although studies of this effect have consistently used nonphysiologic doses of Hcy (1 mmol) [15, 43, 53, 54]. Recent data indicates that endoplasmic reticulum stress genes can be induced in response to increased intracellular calcium or chronic hypoxia through an Erk/AP-1 mechanism [51, 52]. Accordingly, we sought to determine whether endoplasmic reticulum stress genes were induced in mesangial cells exposed to physiologic elevations of Hcy, and whether this was Erk dependent. Mesangial cell treatment with 50 μmol/L Hcy led to an increase in Grp78 mRNA levels by Northern analysis, an effect completely eliminated by the specific MEK inhibitor U0126, although U0126 alone has a lesser effect to increase Grp78 (Fig. 8). This effect was specific to Hcy, as cysteine had no effect (Fig. 8). Immunoblotting indicated that all endoplasmic reticulum stress genes examined were induced by 50 μmol/L Hcy in mesangial cells (Fig. 9). These data indicate that physiologic doses of Hcy induce both proliferation and endoplasmic reticulum stress in mesangial cells, and that both of these responses are Erk dependent.

DISCUSSION

The mesangial cell is instrumental in the progression of chronic glomerular disease, and both proliferation and

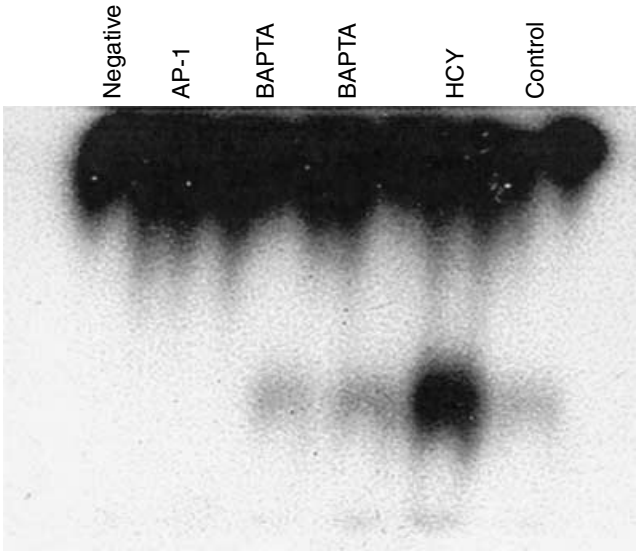

Fig. 6. Homocysteine (Hcy)-induced nuclear protein binding to activator protein-1 (AP-1) consensus sequences is calcium-dependent. Nuclear proteins were reacted with radiolabeled AP-1 consensus oligonucleotides for 20 minutes, electrophoresed, and autoradiographed. Lane 1 shows nuclear protein binding in normal mesangial cells. Addition of 50 μmol/L Hcy for 20 minutes led to an increase in nuclear protein binding (lane 2) that was prevented by preincubation with BAPTA-AM (10 μmol/L) for 10 minutes prior to the addition of Hcy (lanes 3 and 4).Competition experiments were performed with excess unlabeled consensus oligonucleotides in 100× excess (lane 5). Four separate experiments were performed and a representative autoradiograph is shown. Densitometry indicated that nuclear protein binding to AP-1 consensus sequences in the presence of Hcy was significantly greater than baseline or Hcy with BAPTA-AM ($P < 0.02$).

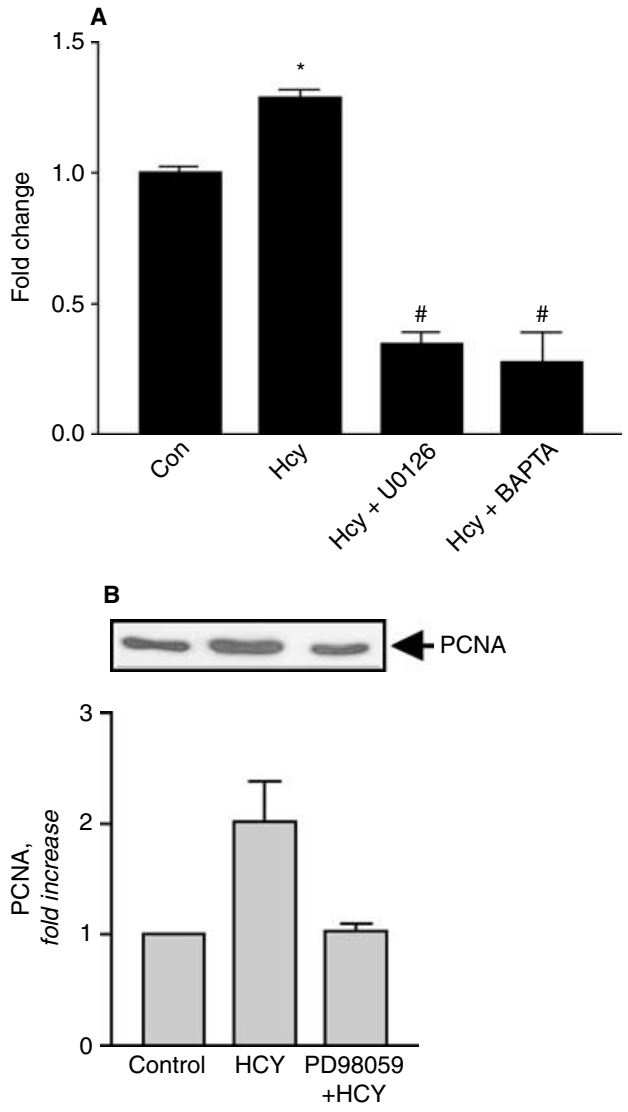

Fig. 7. Mesangial cell DNA synthesis and proliferation is induced by homocysteine (Hcy). Mesangial cells were exposed to 50 μmol/L Hcy for 24 hours. DNA synthesis was assessed by [^{3}H]-thymidine incorporation (*A*) and proliferation by Western blotting for proliferating cell nuclear antigen (PNCA) (*B*). In (A), Hcy increases mesangial cell [^{3}H]-thymidine incorporation at 24 hours by a factor of 1.3, a statistically significant result ($P < 0.05$). Preincubation with either the MEK-1 inhibitor U0126 or BAPTA-AM prevented [^{3}H]-thymidine incorporation in response to Hcy, and both inhibitors significantly prevented DNA synthesis when compared to either baseline or Hcy ($P < 0.03$). In (B), Hcy (lane 2) markedly increased PCNA expression, which was abrogated by preincubation with the Erk inhibitor PD98059 (10 μmol/L) (lane 3). Densitometric results are shown graphically below with error bars ($N = 4$). The increase in PCNA expression was significantly different in Hcy-treated cells when compared to baseline or Hcy with PD98059 ($P < 0.05$).

matrix production are observed [2, 3]. The signaling pathways leading to proliferation and synthesis of ECM have been studied, and MAPK activation has been observed in response to both physical and chemical stimuli. Prevention of intraglomerular hypertension in animal models [6, 7] and human studies [55] ameliorates progressive glomerular injury. We have demonstrated activation of MAPKs, including Erk, in response to cyclic stretch in mesangial cells [38], and activation of Erk in glomeruli in vivo has recently been demonstrated in the Dahl salt-sensitive rat [56]. The most studied chemical stimuli in models of progressive glomerular disease are the cytokines such as Ang II [8] and TGF-β [57]. Ang II signaling in VSMC results in proliferation and matrix production, and these effects are also dependent on Erk signaling [58, 59]. Inhibition of Erk attenuates responsiveness of VSMC to Ang II, further implicating this signaling pathway [60]. Most important, Ang II infusion results in activation of glomerular Erk in intact rats, providing direct evidence of the existence of this signaling pathway in kidney [61]. It appears that MAPKs are also involved in TGF-β signaling, perhaps through Erk-mediated phosphorylation of Smad1 [62].

CRF is associated with the highest levels of nongenetically determined plasma Hcy seen in routine clinical practice [19, 63]. Epidemiologic evidence links hyperhomocysteinemia to adverse cardiovascular outcomes in the end-stage renal disease (ESRD) population [22, 50, 64],

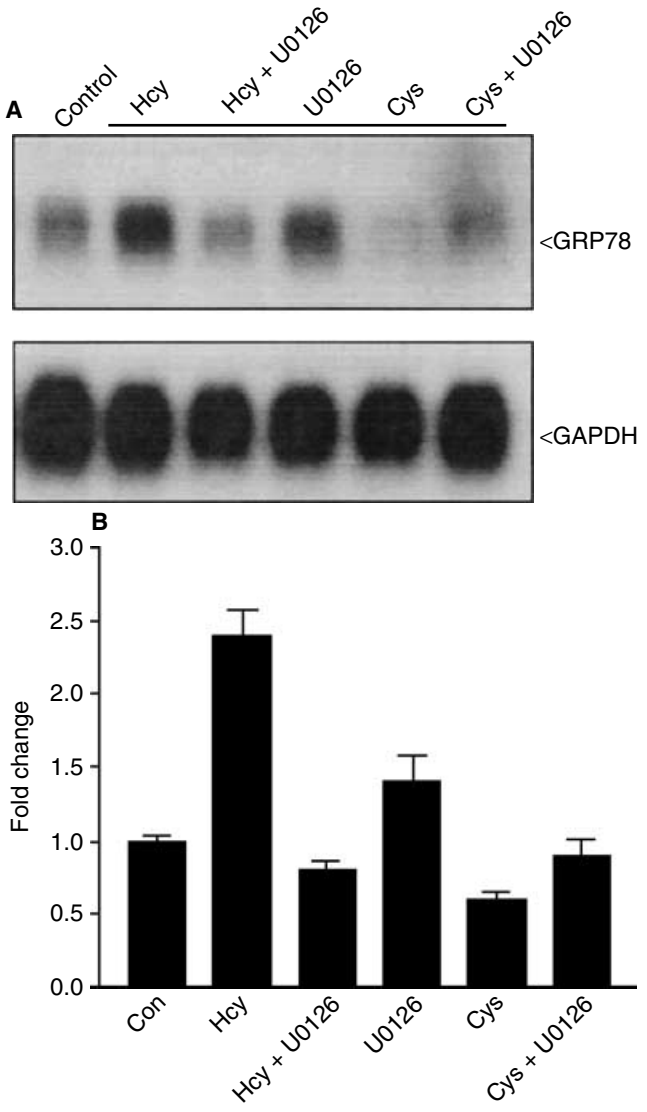

Fig. 8. Mesangial cell Grp78 induction by homocysteine (Hcy). Mesangial cells were exposed to 50 μmol/L Hcy with or without 10 μmol/L U0126 for 24 hours and RNA extracted for Northern analysis. Each lane was loaded with 5 μg RNA and hybridized using a radiolabeled cDNA probe to Grp78. (*A*) A representative autoradiograph is shown ($N = 3$). (*B*) Densitometric results are shown graphically with error bars ($N = 4$). Statistical analysis revealed that only Hcy alone significantly increased Grp78 mRNA when compared to control ($P < 0.05$). Abbreviations are: Cys, cysteine; GAPDH, glyeraldehyde-3-phosphate dehydrogenase.

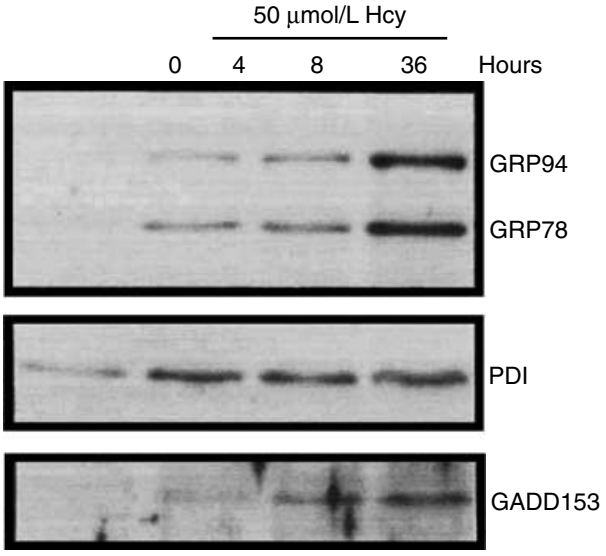

Fig. 9. Mesangial cell endoplasmic reticulum stress protein induction by homocysteine (Hcy). Mesangial cells were exposed to 50 μmol/L Hcy for the times indicated and protein lysate recovered for immunoblotting with specific antisera. Each lane was loaded with 40 μg protein. A representative autoradiograph is shown ($N = 3$).

recapitulating the findings of large trials in the general population [16, 65]. Evidently, the vascular wall is a major target for the pathologic effects of Hcy, and consequently most work has concentrated on the two major cell types of arterial walls, the VSMC and vascular endothelial cells (VEC). VSMC proliferate in the presence of Hcy, with up-regulation of cyclin A [25, 26]. This was attributed to the auto-oxidation of the free sulfhydryl groups on Hcy [26]. N-acetylcysteine was demonstrated to inhibit VSMC proliferation in response to Hcy in another study [66]. VSMC matrix production was also induced by Hcy in this and other studies [25, 66]. VEC proliferation is, by contrast, inhibited by Hcy [25, 29]. Clearly, the VSMC, phenotypically similar to the mesangial cell, displays similar responses to injurious stress, proliferation and matrix production. Recently, though, it has been demonstrated that Hcy induces type I collagen production in mesangial cells [24], indicating that Hcy may not just be an epiphenomenon of CRF affecting the systemic vasculature, but may also contribute to the progression of the underlying renal disease itself. A recent in vivo study of diet-induced hyperhomocysteinemia in Dahl salt-sensitive rats also supports this construct [23].

The mechanisms by which Hcy contributes to the development and progression of vascular disease remain obscure, but several theories have emerged. Studies have concentrated on oxidative stress induced by peroxide and free radical generation, hypomethylation, and endoplasmic reticulum stress [67]. The differential effects on VSMC have been attributed to the fact that Hcy is efficiently converted to s-adenosylhomocysteine (SAH) in VEC, an inhibitor of methyltransferase, but not in VSMC. Hypomethylated $p21^{ras}$ was observed [29] and this was subsequently associated with diminished Erk activity in VEC, lending credence to this hypothesis [32]. This effect is not seen in VSMC and, indeed, activation of Erk has been demonstrated by Hcy in VSMC [31]. It is critical to note that many studies of the effect of Hcy on vascular cells used extremely high levels of Hcy (1 mmol/L), levels not attained even in the presence of genetic defects in Hcy handling.

Differential display studies suggested a third possible mechanism for detrimental effects of Hcy, endoplasmic reticulum stress. Exposure of VEC to Hcy led to up-regulation of an array of mRNA transcripts primarily involved in the response of the endoplasmic reticulum to protein misfolding [53], an effect that has variably been attributed to oxidative [53] or reductive [15] stresses. This observation, also termed the unfolded protein response has been confirmed in vivo by our group, who observed induction of endoplasmic reticulum stress proteins and the unfolded protein response in VSMC and hepatocytes in a hyperhomocysteinemic mouse model [68]. It was noted over 10 years ago that Hcy could inhibit the biosynthesis of proteins normally secreted by some cells, and that this was due to endoplasmic reticulum retention of the proteins and their ultimate degradation [69]. Induction of Grp78 and other chaperones accompany the unfolded protein response, and protect cells from apoptotic death in response to endoplasmic reticulum stress stimuli. Although we and others have observed endoplasmic reticulum stress in response to Hcy, the mechanism by which this occurs has remained elusive [68, 69].

Thus, we explored whether Hcy-induced Erk activation occurred in mesangial cells, and the mechanism(s) thereof. We observed dose-dependent Erk activation in mesangial cells, maximally at the physiologically relevant concentration of 50 μmol/L. Hcy-induced oxidative stress did not appear to play a role in the induction of Erk, as the antioxidants catalase and n-acetylcysteine were without effect. AP-1 nuclear protein binding was also unaffected by antioxidants. These results differ from the oxidative stress mechanism whereby Hcy induced collagen I production by mesangial cells [24], suggesting that multiple signaling pathways might potentially be activated by Hcy in mesangial cells. We did note, however, that chelation of intracellular calcium with BAPTA-AM efficiently abrogated Hcy-induced Erk signaling and markedly decreased AP-1 nuclear protein binding. In support of this construct, we also observed that 50 μmol/L Hcy induced a prompt increase in intracellular calcium in mesangial cells. The data presented here, therefore, indicate that Hcy elicits mesangial cell proliferation via Erk signaling, and that calcium is indispensable for this process. Subsequently, we sought to determine whether mesangial cell proliferation would be observed in the presence of Hcy, and if this was Erk-dependent, as has been proposed in VSMC [31]. We observed that 50 μmol/L Hcy, a plasma concentration commonly observed in the setting of ESRD, increased mesangial cell expression of PCNA at 24 hours. This increase could be abrogated by PD98059, an inhibitor of Erk, suggesting that the effect of Hcy of mesangial cell proliferation was signaled through Erk. We also wished to determine if Hcy induced endoplasmic reticulum stress in mesangial cells, given recent observations of the importance of this effect by ourselves [68] and others [54]. There is ample data to suggest that Hcy up-regulates several genes associated with the accumulation of misfolded proteins in endoplasic reticulum [15, 43, 53], and we observe herein the same effect in mesangial cells, although at physiologically relevant concentrations for the first time in cell culture. Furthermore, Erk inhibition also prevented the up-regulation of Grp78 mRNA, indicating that the Hcy-induced endoplasmic reticulum stress occurred through Erk activation.

These data support the hypothesis that mesangial cells respond to Hcy in a similar fashion as VSMC [25, 26, 31], by proliferation and the induction of endoplasmic reticulum stress. Since mesangial cells have also been recently shown to increase their production of matrix in response to Hcy, in vitro evidence now exists to suggest that Hcy may play a role in the progression of glomerular disease in the same way that it plays a role in the progression of vascular disease. As our studies are the first to show these effects in cultured cells at Hcy levels routinely observed in vivo, attention should be given to the potential role for Hcy in the progression of chronic glomerular disease.

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