HSP72 attenuates renal tubular cell apoptosis and interstitial fibrosis in obstructive nephropathy

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Mao H, Li Z, Zhou Y, Li Z, Zhuang S, An X, Zhang B, Chen W, Nie J, Wang Z, Borkan SC, Wang Y, Yu X. HSP72 attenuates renal tubular cell apoptosis and interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol 295: F202–F214, 2008. First published April 16, 2008; doi:10.1152/ajprenal.00468.2007.—Although heat shock protein 72 kDa (HSP72) protects tubular epithelium from a variety of acute insults, its role in chronic renal injury and fibrosis is poorly characterized. In this study, we tested the hypothesis that HSP72 reduces apoptosis and epithelial-to-mesenchymal transition (EMT), important contributors to tubular cell injury in vitro and in vivo. In rats, orally administered geranylgeranylacetone (GGA), an agent that selectively induces HSP72, markedly reduced both apoptosis and cell proliferation in tubular epithelium and decreased both interstitial fibroblast accumulation and collagen I deposition after unilateral ureteric obstruction, a model of chronic renal tubulointerstitial fibrosis and dysfunction. In cultured renal NRK52E cells, exposure to TGF-β1 induced EMT and apoptosis, major causes of renal fibrosis and tubular atrophy, respectively. Exposure to a pancaspase inhibitor (ZVAD-FMK) prevented TGF-β1-induced apoptosis but did not reduce EMT. In contrast, selective HSP72 expression in vitro inhibited EMT caused by TGF-β1 as indicated by preserving the E-cadherin expression level and α-smooth muscle actin induction. Small interfering RNA directed against HSP72 blocked the cytoprotective effects of HSP72 overexpression on EMT in TGF-β1-exposed cells. Taken together, our data indicate that HSP72 ameliorates renal tubulointerstitial fibrosis in obstructive nephropathy by inhibiting both renal tubular epithelial cell apoptosis and EMT.

heat stress protein 72 kDa; epithelial-to-mesenchymal transition, α-smooth muscle actin, E-cadherin, transforming growth factor-β1

Tubulointerstitial fibrosis (TIF) is the common final pathway of diverse forms of chronic renal disease that contributes to both organ insufficiency and failure. Although a number of experimental studies have attempted to clarify the pathogenesis of TIF, its evaluation and management remain poorly defined. TIF is characterized by leukocytic infiltration, tubular atrophy, fibroblast proliferation, and the accumulation of extracellular matrix proteins (3). Transforming growth factor-β1 (TGF-β1) is believed to play an important role in TIF by inducing both epithelial-to-mesenchymal transition (EMT) and apoptosis of renal tubular cells, which promotes tubular atrophy in many forms of progressive renal disease (38, 44).

The extent of tubular apoptosis in animal models of ureteric obstruction correlates with the severity of tubular injury and subsequent TIF (5, 23, 28, 50). In contrast, inhibition of initial tubular cell apoptosis by either neutralizing the activity of apoptosis-inducing molecules or supplementing with prosurvival factors effectively prevents inflammation and attenuates progression to fibrosis in the unilateral ureteric obstruction (UUO) model (24, 36). These data provide evidence for an apparent interplay between early apoptosis and subsequent fibrosis, and the apoptosis could be an early event that occurs before the onset of frank fibrosis. EMT describes a phenotypic change characterized by the loss of epithelial markers including E-cadherin, the gain of mesenchymal markers such as α-SMA, and epithelial cell migration across damaged tubular basement membrane to the interstitial space, where cells become activated myofibroblasts (13). The process of EMT eventually generates extracellular matrix associated with a loss of viable tubular epithelial cells and nephrons. Although both EMT and apoptosis contribute to fibrosis and reduction in renal parenchyma, the cellular mechanisms that regulate apoptosis and EMT are yet to be fully elucidated.

Heat shock protein 72 kDa (HSP72), a molecular chaperone, protects renal epithelial cells from acute lethal injury including necrosis and apoptosis in vivo and in vitro (31, 45, 46) and also ameliorates sublethal injury by preventing cytoskeletal collapse, by improving cell-cell junction function and by enhancing cell-matrix interaction (7, 19, 21, 42). GGA is a nontoxic agent that ameliorates acute, ischemic renal injury in the rat by selectively inducing the expression of HSP72 (35). Furthermore, UUO increases endogenous HSP72 generation in the kidney of both rats and humans (5, 18). It is still not known whether an increment of HSP72 in a chronic fibrosis setting is merely a marker of nonspecific repair mechanisms due to cellular injury or a potential cytoprotective mechanism that protects renal epithelial cells from apoptosis, prevents EMT, and thus facilitates repopulation of injured tubules.

In the present study, we tested the hypothesis that GGA-induced HSP72 expression reduces renal TIF in UUO rats by inhibiting renal tubular apoptosis and EMT. We find that selective HSP72 expression reduces EMT in vitro and decreases tubular cell apoptosis, interstitial fibroblast infiltration, and collagen I deposition in the rat kidney in a model of ureteric obstruction.

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MATERIALS AND METHODS

Reagents and Antibodies

A normal rat kidney proximal tubular epithelial cell line (NRK52E) was purchased from American Type Culture Collection (Rockville, MD). Recombinant human TGF-β1 and ZVAD-FMK were purchased from R&D Systems (Minneapolis, MN). GGA was obtained from Eisai China (Shanghai, China). Scrambled and HSP72-specific small interfering RNA (siRNA) products were purchased from Shanghai GenePharma (Shanghai, China), and Lipofectamine 2000 transfection reagent was purchased from Invitrogen Life Technologies (Paisley, UK). TdT-mediated dUTP nick end labeling (TUNEL) Assay Kits (Fluorescent), an annexin V-FITC Apoptosis Detection Kit, protease inhibitors, and anti-collagen I were obtained from Calbiochem (San Diego, CA). In addition, the following antibodies were used: anti-E-cadherin antibody (BD Biosciences Pharmingen, San Jose, CA); anti-α-SMA and anti-PCNA (DAKO, Cupertino, CA); anti-HSP72, anti-HSP27, anti-HSP60, and anti-HSP90 (Stressgen Biotechnologies, Victoria, BC, Canada), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Boster, Wuhan, China). Horseradish peroxidase-conjugated anti-mouse IgG, horseradish peroxidase-conjugated anti-rabbit IgG, and Cy3-conjugated anti-mouse IgG were obtained from Jackson Immunoresearch (West Grove, PA). All remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cells and Treatments

NRK52E cells were cultured at 37°C in a 5% CO2 atmosphere in DMEM mixed 1:1 (vol:vol) with F12 medium (Invitrogen-GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were grown to 70–80% confluence and subjected to serum deprivation for 24 h before experimental manipulation. The effects of TGF-β on cell phenotype and apoptosis were induced by adding recombinant TGF-β1 (10 ng/ml) to cells for 72 h. To inhibit the apoptotic signal pathway, cells were pretreated with various concentrations (25–150 μM) of ZVAD-FMK, a pan-caspase inhibitor, for 1 h before and during exposure to TGF-β1. All experiments were performed under serum-free conditions.

To increase HSP72 content in vitro, NRK52E cells were treated with either GGA or adenoviruses containing HSP72 cDNA (Adv-HSP72), which was kindly provided by Dick Mosser (University of Guelph, Ontario, Canada). GGA is an emulsion with 5% gum arabic and 0.008% tocopherol. Cells were incubated for 2 h with increasing concentrations of GGA that varied between 0 and 200 μmol/l or with vehicle at the same concentrations. The time course of HSP72 expression was also studied after administration of 100 μmol/l GGA or the same doses of vehicle in culture medium. Furthermore, NRK52E cells were coinfected with adenoviruses containing wild-type human HSP72 and green fluorescent protein (Adv/TR5/HSP72-GFP) located on separate cistrons induced by a tetracyclin-regulated promoter (Adv/CMV/tTA) as described previously (21). Control cells were coinfected with Adv/TR5/GFP and Adv/CMV/tTA. Infection efficiency for both adenoviruses was >95% as determined by direct visualization of GFP in cells infected with adenovirus at multiplicities of infection (MOI) that varied between 40 and 60. The effect of each adenoviral dose and GGA on HSP72 expression was assessed by Western blot analysis.

siRNA for HSP72

Incorporation of siRNA targeted against HSP72 was achieved using Lipofectamine 2000 according to the manufacturer’s instructions. The cells were transfected for 4 h before replacement of the transfection medium with DMEM/F12 medium. Initial studies focused on the identification of the target siRNA that would produce the lowest levels of HSP72 expression in NRK52E cells. Preliminary results (n = 3) showed that silencing was evident at 24, 48, and 72 h after transfection. Therefore, 24 h after transfection, cells were stimulated with TGF-β1. The effect of HSP72 silencing on EMT was observed at 72 h after TGF-β1 exposure. For determination of the efficiency of HSP72 knockdown, Western blot analysis for HSP72 was performed. Several concentrations of HSP72 siRNA (10–50 nM) were used to determine the optimal knockdown concentration.

Animals

The experiments were performed with male Sprague-Dawley rats weighing 200–250 g maintained with free access to water and standard rat food. UUO was performed by using the protocol approved by Animal Care and Use Committee of the Sun Yat-sen University (Guangzhou, China). Rats were anesthetized by injection of chloral hydrate (400 mg/kg ip) and allowed to breathe spontaneously. The left ureter of each animal was located and double-ligated with 4-0 silk thread after a midline abdominal incision. Sham-operated rats had their ureters exposed but not ligated. To induce optimal renal HSP72 expression, the GGA protocol described by Suzuki et al. (35) was modified by our laboratory, and additional studies on dose- and time-dependent induction of HSP72 were performed using doses of 0, 200, and 400 to 800 mg/kg daily and time points of 12, 24, and 48 h after a signal dose of GGA (400 mg/kg). Rats received daily oral administration with optimal 400 mg/kg GGA, starting 1 day before the operation and continuing throughout UUO or sham surgery. Rats in the control group were given the same dose of vehicle. Rats (n = 5 each) were killed on day 7 after UUO, and both kidneys were harvested and were then subjected to the studies described below.

Analysis of Morphology and TIF

Kidney tissues were fixed in 10% phosphate buffer formalin, dehydrated through graded alcohol and xylene, embedded in paraffin, sectioned at 3-μm thickness, and then stained with periodic acid-Schiff or Masson’s trichrome. Histological examinations were performed in a blinded manner. A point-counting method was used to evaluate chronic tubular injury and TIF (40). Briefly, under high magnification (×400), 10 nonoverlapping fields from each section of the renal cortex were photographed. A grid containing 117 (13 × 9) sampling points was superimposed on each photograph. The number of points overlying chronic tubular injury and TIF was counted and expressed as a percentage of all sampling points (40). Chronic tubular injury was defined as tubular atrophy with interstitial fibrosis and infiltrate with/without tubular dilatation, tubular cast formation, flattening of tubular epithelial cells, and thickening of the tubular basement membrane (25).

Immunohistochemical and Immunofluorescence Stainings

All immunostainings were performed on 4-μm paraffin-embedded kidney sections. Antigen retrieval was performed by microwave treatment. Negative controls were performed with nonimmune mouse serum substituted for the specific primary antibodies.

In immunohistochemical staining, sections were exposed to 3% H2O2 for 20 min to destroy endogenous peroxidase activity, rinsed with PBS once, blocked with 10% nonimmune sheep serum in PBS for 1 h, incubated with monoclonal mouse anti-HSP72 or PCNA, respectively, at 4°C overnight, rinsed with PBS three times for 5 min each, and then incubated for 1 h with either sheep or horse horseradish peroxidase-conjugated anti-mouse IgG. The reaction was stopped by rinsing with PBS when the color was fully developed using diamobenzidine for ~5 min. The PCNA-positive tubular and interstitial cells in the cortex were calculated in 10 high-power fields at ×400 magnification within the same section of kidney from an individual animal. Results were expressed as percentage of the total nuclei in cortex.

To detect E-cadherin, α-SMA, or collagen I, cells or tissue sections were incubated with monoclonal mouse anti-E-cadherin, anti-α-SMA, and anti-collagen 1 at 4°C overnight followed by Cy3-conjugated anti-mouse IgG. To identify nuclei, cells or sections were counterstained with 4,6-diamidino-2-phenylindole for 2 min. The positive
stainings were detected using a laser-scanning confocal microscopy (Zeiss LSM 510 META, Carl Zeiss). For semiquantitative analysis of collagen I expression, all cortical fields were scored from 0 to 4 for each field, with an average for each kidney calculated. The score of collagen I accumulation was assessed as 0 for 0%, 1 for <25%, 2 for 25–50%, 3 for 50–75%, and 4 for >75% of each field occupied by a collagen I-positive area. Quantification of immunostaining was performed on blinded slides.

Detection of Apoptosis

TUNEL. Apoptosis was quantified in histological sections using a commercially available in situ Apoptosis Detection Kit according to the manufacturer’s instructions. To this end, paraffin-embedded kidney sections were treated with 20 μg/ml proteinase K for 20 min at room temperature. After being washed, slides were incubated with a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase. Positive staining was identified in the cell nuclei with DNA fragmentation under fluorescence microscopy and expressed as apoptotic cells per high-power field.

Flow cytometric analysis. Apoptosis in cultured cells was quantified by flow cytometry after double staining cells with annexin V-FITC and propidium iodide using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s protocol. Cells positive for annexin-V but negative for propidium iodide were considered to be apoptotic.

Western Blot Analysis

Kidney cortex and harvested cultured cells were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM EGTA, and 1% Triton X-100) with a protease inhibitor cocktail. The supernatants of tissue and cell lysates were extracted after centrifugation at 10,000 rpm for 15 min at 4°C. The protein concentration was measured with the Bradford protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein from lysates were loaded and separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane. The blots were probed overnight at 4°C with primary antibodies respectively directed against proteins including E-cadherin, α-SMA, HSP72, GAPDH, and β-actin followed by the respective horseradish peroxidase-linked secondary antibody. Horseradish peroxidase activity was visualized by an enhanced chemiluminescence system. Densitometric quantification was performed with the Image analysis program (FluorChemTM 8900, Alpha Inotech).

Statistical Analyses

The data are expressed as means ± SE. Analysis was performed with standard statistical software (SPSS for Windows, version 11.0). Comparison among groups was made with one-way ANOVA followed by the Student-Newman-Keuls test. A P value <0.05 was considered as statistically significant.

Fig. 1. Induction of heat shock protein (HSP) expression in vivo and in vitro by geranylgeranylacetone (GGA). A and B: dose- and time-dependent expression of HSP 72 kDa (HSP72) after GGA was analyzed by Western blotting. Graphic presentation is shown of renal HSP72 protein abundances in different groups as indicated. Relative HSP72 level is reported after normalization with GAPDH. Values in baseline were set as 1.0. Values are means ± SE; n = 5, *P < 0.05 vs. baseline. C: immunohistochemistry in GGA-treated animal revealed HSP72 expression primarily in the inner stripe of the outer medulla of normal kidney. Original magnification ×100. D: Western blot showed an increase of HSP72 protein expression in rats after GGA administration compared with vehicle alone. In contrast, GGA treatment did not change the protein levels of HSP90, HSP60, or HSP27. E: quantitative analysis of the expression levels of HSPs in vehicle (filled bars) and GGA (open bars) treatment groups. Relative HSP72 levels were calculated and are expressed as fold-induction over vehicle after normalization with GAPDH content. Value in the vehicle was set as 1.0. Values are means ± SE; n = 5. †P < 0.05 vs. vehicle. F: by Western blot analysis, GGA induced HSP72 overexpression in NRK52E cells at a dose of 100 μmol/l and further increased HSP72 level at a dose of 200 μmol/l GGA. G: with a dose of 200 μmol/l and 2-h exposure, GGA induced a transient increase in HSP72 expression in NRK52E cells, which peaked at 2–4 h, followed by a rapid decline by 6 h.
RESULTS

GGA Selectively Enhanced Expression of HSP72 In Vivo and In Vitro

The dose-dependent induction of HSP72 by GGA in the rat kidney was assessed 24 h after single oral administration of GGA (Fig. 1A). HSP72 expression increased significantly in a dose-dependent manner from a starting dose of 200 mg/kg to a maximal dose of 800 mg/kg. The time-dependent effect of GGA on the expression of HSP72 was also evaluated by administration of GGA with a single dose of 400 mg/kg (Fig. 1B). HSP72 expression in kidneys significantly increased from 12 h, peaked at 24 h, and then mildly decreased from the peak by 48 h. Since HSP72 expression was maximal at 24 h, a dose

Fig. 2. Effect of GGA treatment on renal tubular injury and interstitial fibrosis after unilateral ureteral obstruction (UUO). A: representative micrographs from different groups with either periodic acid-Schiff (PAS) or Masson’s trichrome stain revealed marked increase in tubulointerstitial fibrosis in UUO/Vehicle vs. sham control, and in contrast, there was significantly less tubulointerstitial fibrosis in UUO/GGA. Original magnification ×200. B and C: semiquantitative analysis of relative tubular injury and interstitial fibrosis scores, respectively. Values are means ± SE; n = 5. *P < 0.01 vs. sham. †P < 0.05 vs. vehicle.

Original magnification ×200.
of 400 mg/kg was given 24 h before and during the following experiments. In normal control rats without oral GGA, HSP72 expression was detected by immunohistochemical staining (data not shown). Oral administration of GGA markedly enhanced HSP72, staining in the whole kidney essentially with the same distribution as in normal kidneys (Fig. 1C). GGA significantly increased the expression of HSP72, but not HSP90, HSP60, or HSP27 in the cortex as evaluated by Western blot analysis, (Fig. 1, D and E). Exposure of NRK52E cells to GGA also significantly increased HSP72 content in a dose- and time-dependent manner (Fig. 1, F and G). HSP72 overexpression lasted 6 h after transient GGA exposure, whereas TGF-β-mediated EMT in NRK52E cells occurred between 48 and 72 h after incubation. Thus we used adenoviruses containing wild-type human HSP72 to increase HSP72 level in vitro and further observe the effect of HSP72 on TGF-β-induced renal EMT over time.

**GGA Attenuated Sclerotic Changes**

To evaluate the effect of GGA-induced HSP72 expression on renal fibrosis, tubular injury and interstitial matrix deposition were evaluated at day 7 after UUO. Compared with the sham-operated kidneys, the obstructed kidneys exhibited a dramatic tubular dilation and atrophy and widening of interstitial spaces illustrated in periodic acid-Schiff-stained sections. Obstructed kidneys with vehicle alone demonstrated marked TIF mostly in cortical areas, indicated by a positive blue color in Masson’s trichrome-stained sections. In contrast, obstructed kidneys from GGA-treated animals had less sclerotic damage (Fig. 2A). The tubular injury and TIF scores were reduced by 35.2 and 69.6% on day 7 after UUO, respectively (P < 0.05; Fig. 2, A–C). Immunofluorescence staining with anti-collagen I antibody revealed increased accumulation of collagen I in the interstitium (Fig. 2D), whereas obstructed kidneys with GGA-induced HSP72 expression showed a reduction in the interstitial volume expansion and collagen I accumulation (Fig. 2, D and E).

**GGA Suppressed In Vivo Renal EMT Marked by Retaining E-Cadherin and Inhibiting α-SMA**

Myofibroblasts play an important role in interstitial fibrosis. Therefore, the effects of GGA on the expression of both E-cadherin, an epithelial adhesion molecule, and α-SMA, a molecular hallmark of mature myofibroblasts, were examined. Compared with sham-operated animals, the level of HSP72 expression in UUO was elevated in the rat with vehicle alone and upregulated further in the GGA-treated rats (Fig. 3), which was associated with preserving E-cadherin expression and lowering α-SMA accumulation in the obstructed kidney at day 7 after UUO (Fig. 4A). Quantitative determination of Western blot analysis revealed that GGA retained E-cadherin expression by 41.8% and suppressed α-SMA expression by 46.5% in the obstructed kidneys (Fig. 4, B and C). The above-noted changes in the expression of both E-cadherin and α-SMA in EMT cells were observed and confirmed by immunofluorescence staining of renal tissue after UUO (Fig. 4D). Of note, the sham group revealed only arterial and arteriolar staining adjacent to the glomerulus, but no interstitial staining (Fig. 4D). In brief, HSP72 expression induced by GGA significantly reduced EMT associated with UUO (Fig. 4). These findings suggest that HSP72 targets tubular EMT, a key event in the pathogenesis of renal interstitial fibrosis.

**GGA Reduced Renal Tubular Apoptosis and Proliferation**

The protective effects of GGA on cell apoptosis and proliferation in the obstructed kidney at day 7 after UUO were then assessed by TUNEL staining. Compared with sham UUO, TUNEL-positive cells were increased in the obstructed kidney (Fig. 5, A and C). Most TUNEL-positive cells were proximal tubular epithelial cells, although small numbers of apoptotic cells were also observed in interstitial and endothelial cells. In contrast, GGA exposure significantly reduced the number of TUNEL-positive cells (Fig. 5, A and C). Similarly, GGA significantly decreased renal PCNA expression in the cortex of the kidney after UUO (Fig. 5, B and C).

**ZVAD-FMK Inhibited TGF-β1-Induced Apoptosis But Not EMT in NRK52E Cells**

In the intact animal, GGA exposure attenuated interstitial matrix protein deposition, cell apoptosis, and cell proliferation. To examine a potential mechanism by which HSP72 inhibits EMT, an in vitro model was developed in which NRK52E cells were exposed to TGF-β1, an established inducer of EMT.

Cells treated with TGF-β1 underwent both apoptosis (data not shown) and EMT in both dose- and time-dependent manners (Fig. 6, A and B). Immunofluorescence staining also confirmed that TGF-β1 treatment for 72 h induced a suppression of E-cadherin and a dramatic induction of α-SMA (Fig. 6C). These morphological changes are consistent with the features of EMT. In addition, TGF-β1 treatment for 72 h led to a 36.6% increase in apoptosis (Fig. 7).

To examine whether apoptosis is associated with the occurrence of an EMT, ZVAD-FMK, a cell-permeable pan-caspase inhibitor, was used. Flow cytometric analysis revealed that preincubation with 50 μM ZVAD-FMK reduced apoptosis by up to 53.8% in the cells treated with TGF-β1 for 72 h (Fig. 7).
ZVAD-FMK preserved the levels of E-cadherin expression at the dose of 50 μM and above, but not at 25 μM (Fig. 8, A and B). Moreover, ZVAD-FMK did not suppress TGF-β1-induced expression of α-SMA (Fig. 8, A and C), a hallmark of EMT.

To further confirm the effect of ZVAD-FMK on EMT, confocal immunofluorescent microscopy was employed to determine whether ZVAD-FMK maintains E-cadherin at the cell surface and abolishes α-SMA expression in TGF-β1-treated cells. As shown in Fig. 8D, TGF-β1 treatment removed E-cadherin from the cell membrane and induced α-SMA expression. ZVAD-FMK treatment largely preserved E-cadherin at the cell periphery following TGF-β1 treatment. In contrast, ZVAD-FMK did not attenuate TGF-β1-induced expression of α-SMA. The immunofluorescent images were thus consistent with the Western blot data, indicating that ZVAD-FMK treatment preserved E-cadherin, but did not inhibit α-SMA expression in renal epithelial cells exposed to TGF-β1.

Fig. 4. Effect of GGA on E-cadherin and α-smooth muscle actin (SMA) expression after UUO. A: representative Western blot analysis showed the protein levels of E-cadherin and α-SMA from sham, vehicle, and GGA-treated rat kidneys after UUO. Numbers (1, 2, and 3) denote each individual animal within a given group. UUO showed a virtually complete block of E-cadherin expression and marked induction of α-SMA expression. Compared with vehicle treatment, GGA treatment preserved E-cadherin and lowered α-SMA expression in the obstructed kidney. B and C: graphic representation of E-cadherin and α-SMA protein levels in different groups, as indicated after normalization with GAPDH content. Values are means ± SE. *P < 0.05 vs. sham. †P < 0.05 vs. vehicle. D: representative pictures of E-cadherin (red staining) and α-SMA immunofluorescence (red staining) in different groups as indicated. Original magnification: ×200 (E-cadherin) and ×400 (α-SMA).
These results indicated that blocking tubular apoptosis via a caspase-dependent signal pathway could not prevent cells from TGF-β1-induced EMT in culture, suggesting possible separate underlying mechanism(s) for tubular apoptosis and EMT in chronic TIF.

**HSP72 Inhibited TGF-β1-Induced In Vitro EMT in NRK52E Cells**

Our previous studies demonstrated that HSP72 exerted anti-apoptotic effects in cultured renal proximal epithelial cells subjected to ATP depletion (17, 31, 41). Here, we explored whether HSP72 inhibits EMT in the manner independent of its antiapoptotic effects. Wild-type HSP72 was selectively expressed before TGF-β1 exposure by the infection of NRK52E cells with adenovirus encoding human HSP72. Adenoviral infection at 40 and 60 MOI resulted in a significant increase in steady-state HSP72 protein content (Fig. 9A). Compared with empty vector control (infected with GFP- and tTA-encoding adenoviruses), enhanced expression of HSP72 prevented cells from the TGF-β1-induced loss of E-cadherin expression and from a gain of α-SMA expression, as demonstrated by Western blot analysis (Fig. 9A, lanes 4 and 5). Of note, there are no significant differences in E-cadherin and α-SMA baseline expression levels between normal control and empty vector control cells (data not shown). In contrast to the results obtained using ZVAD-FMK (Fig. 9A, lane 3), increased HSP72 expression not only preserved E-cadherin expression but also inhibited α-SMA expression. Quantitative determination revealed that overexpression of HSP72 restored E-cadherin content by 47.2% and reduced α-SMA expression by 43.8% at 60 MOI of adenoviruses, compared with empty vector control (Fig. 9, B and C). This action of HSP72 also was dose dependent at MOI of adenovirus between 40 and 60.

To determine whether inhibiting TGF-β1-induced in vitro EMT was specifically due to an increase in HSP72 expression, an HSP72 siRNA knockdown approach was taken. Our studies showed that endogenous HSP72 was not detectable in NRK52E cells by Western blotting with a specific anti-HSP72 antibody (Fig. 9, A and E), even though a large amount of protein (80 μg/lane) was loaded. We first increased HSP72 expression by overexpression of an adenoviral vector encoding human HSP72 in NRK52E cells, and then knocked down HSP72 expression using siRNA in HSP72-overexpressing cells. As showed in Fig. 9D, transfection of NRK52E cells with HSP72-specific siRNA (20 nM) nearly completely abolished adenovirus-induced HSP72 protein expression, compared with the same concentration of siRNA controls. Knockdown of adenovirus-induced HSP72 protein expression blocked protective effects of HSP72 protein overexpression on TGF-β1-induced EMT (preserving E-cadherin content and inhibiting α-SMA expression) (Fig. 9, E–G).

We further investigated the effect of HSP72 siRNA on EMT using confocal immunofluorescent microscopy. As shown in Fig. 9H, after TGF-β1 treatment for 72 h, both control and HSP72 siRNA knockdown cells developed EMT, as shown by decreased E-cadherin expression levels and induction of α-SMA expression. In contrast, HSP72-overexpressing cells demonstrated resistance to TGF-β1-induced EMT. However, neither the mock transfection nor adenovirus infection control affected cell morphology and expression of E-cadherin and α-SMA (data not shown). Therefore, these findings support our...
hypothesis that HSP72 targets EMT directly by a mechanism(s) independent of its effect on tubular apoptosis.

DISCUSSION

This study demonstrates that GGA-induced HSP72 expression in the rat kidney after UUO decreased the number of α-SMA-positive myofibroblasts and reduced TIF and collagen I deposition. In addition, HSP72 expression significantly inhibited tubular cell apoptosis and reduced tubular cell proliferation in obstructed kidneys. In vitro experiments in NRK52E cells revealed that blocking the caspase-dependent apoptotic pathway prevented TGF-β1-induced apoptosis, but not EMT. It is of potential significance that HSP72 expression not only attenuated apoptosis, an established effect of this cytoprotective protein, but also inhibited EMT, a process that contributes to chronic fibrosis and the progressive loss of organ function.

The effect of HSP72 on EMT could be attributed, at least partly, to preserving E-cadherin and suppressing α-SMA expression both in vivo and in vitro. These results suggest that enhanced expression of HSP72 attenuates renal tubular apoptosis and TIF caused by ureteric obstruction. Our data shed new light on the mechanism of HSP72-mediated renoprotection in chronic progressive renal fibrosis.

In both rats and humans, sustained UUO gives rise to tubular cell injury and death and eventually causes the affected kidney to become fibrotic (15). In the present study, tubular apoptosis was markedly increased in the obstructed kidney compared with the sham-operated kidney. This is important, since renal tubular apoptosis positively correlates with subsequent tissue injury as demonstrated by Daemen et al. (4). These investigators reported that inhibition of apoptosis induced by ischemia-reperfusion prevented the early onset of not only renal apop-
tosis but also later development of inflammation and organ dysfunction. Their results indicate that tubular cell apoptosis is an early event that precedes overt fibrosis (22, 24, 32). According to this paradigm, it is clear that successful inhibition of initial apoptosis would be expected to limit renal fibrosis.

Previous studies on the protective effects of HSP72 against various insults have been mainly focused on models of acute injury. It has been shown that HSP72 can protect a variety of cells, including renal tubule cells, from thermal, toxic, and ATP depletion-mediated injury in vitro (31, 35, 47). Enhanced HSP72 expression also ameliorates myocardial, liver, brain, and renal damage induced by ischemia-reperfusion, cyclosporin, and endotoxin exposure in rats (9, 10, 26, 35). Our preliminary results (data not shown), as well as those reported by others, showed that after ureteric obstruction, increased HSP72 expression started at day 3, peaked at day 7, and was maintained over the following 14 days, which paralleled the progression of renal fibrosis (18, 37). However, endogenous HSP72 is inadequate to accomplish cytoprotection. In addition, the underlying mechanism by which HSP72 is elevated after UUO being not yet known, it is postulated that during stress HSP72 probably folds proteins into a configuration which can be refolded to a normal state rapidly after reversal of the injury. We therefore used a UUO model to evaluate the role of HSP72 in attenuating chronic renal damage.

GGA, an antiulcer drug, has been widely used in the clinical setting and is a specific inducer of HSP72 in rats (35, 39). GGA induces HSP72 expression through activation of heat shock factor 1 (HSF1), expression of HSP72 mRNA, and subsequent accumulation of HSP72 protein (11, 12, 27). Because GGA-induced HSP72 overexpression in vivo peaked at 24 h after administration of a single oral dose, the rats in our study were given GGA one day before UUO. Our study demonstrated that orally administered GGA markedly augmented the expression of HSP72, but not that of HSP27, HSP60, and HSP90 in the kidney, confirming its selectivity in our model. HSP72 may elicit its renoprotective activities by multiple mechanisms. One relevant finding in our study is that GGA-induced HSP72 expression decreased interstitial volume, /SMA expression, and collagen I deposition in obstructed kidneys. Given the fact that increased HSP72 expression can reduce or slow down fibrogenic processes, we assumed that delayed administration of GGA after occurrence of UUO would also be effective. Another interesting observation is that GGA may exert its beneficial effects by inhibiting tubular proliferation and apoptosis in the obstructed kidney (Fig. 5). Because HSP72 is an antiapoptotic and molecular chaperone, overexpression of HSP72 could limit initial tubular injury and thereby reduce the requirement of epithelial cell proliferation during repair. The protective effects of enhanced HSP72 expression on renal interstitial fibrosis and tubular apoptosis after UUO in rats indicate that HSP72 induction could be of potential therapeutic value for treating chronic kidney diseases in which both apoptosis and fibrosis have been implicated in disease progression.

TGF-β1 can induce concomitant cell apoptosis and EMT. TGF-β1-induced cell apoptosis is through a caspase-dependent pathway (14). Loss of E-cadherin on apoptosis has been described in other studies and is thought to result from the activation of caspases (8). EMT is also associated with a
decrease in E-cadherin protein levels. Collectively, both apoptosis and EMT contribute to the decrease or loss of E-cadherin content. Since TGF-β1-induced concomitant apoptosis and EMT are two mutually exclusive processes, our study demonstrated that preincubation with ZVAD-FMK preserved the content of E-cadherin, presumably due to its inhibition on the cleavage of E-cadherin resulting from apoptosis. However, ZVAD-FMK did not suppress TNF-β1-induced expression of α-SMA, the molecular hallmark of myofibroblast transformation and the morphological changes of EMT. This suggests that TGF-β1-induced EMT is independent of its effect on any caspase-mediated apoptotic response. Recent evidence supports the notion that TGF-β1 induces both EMT and apoptosis via a cell cycle-dependent mechanism in which apoptosis and EMT occur at G2/M and G1/S phases, respectively (6, 34, 48, 49). In addition, a different signaling mechanism(s) may underlie tubular EMT and apoptosis in chronic TIF. Signaling molecules, including Smad, Rho-A, p38, and Par-6, were reported to be involved in the regulation of EMT. On the other hand, the release of cytochrome c from the mitochondria is a crucial step in the intrinsic pathway of apoptosis in chronic TIF.

Fig. 8. Effect of ZVAD-FMK treatment on TGF-β1-induced epithelial-to-mesenchymal transition (EMT). A: following a 1-h preincubation with 0–150 μM ZVAD-FMK, NRK52E cells were treated with 10 ng/ml TGF-β1 for 72 h. ZVAD-FMK preserved E-cadherin expression with concentrations at 50 μM and above, but essentially could not block α-SMA expression. B and C: densitometric analysis of the effect of ZVAD-FMK on E-cadherin and α-SMA expression, corrected with GAPDH content. Values are means ± SE; n = 3/treatment. *P < 0.05 vs. control. †P < 0.05 vs. TGF-β1-treated cells without ZVAD-FMK.

D: EMT was examined by confocal microscopic images of E-cadherin and α-SMA staining in control and ZVAD-FMK treated cells following TGF-β1 exposure. Original magnification ×400.
HSP72 is an inducible cytoprotectant and antiapoptotic protein (1, 20, 33). It plays a pivotal role in cell survival by interfering with multiple checkpoints in the apoptotic pathway (2, 43). We employed cultured NRK52E cells in an in vitro EMT model to examine the renoprotective regulatory mechanisms of HSP72. Compared with the empty vector and exposure to a pan-caspase inhibitor, overexpression of HSP72 markedly inhibited EMT by preserving E-cadherin and pre-

**Fig. 9.** Effect of HSP72 on TGF-β1-induced EMT in vitro. HSP72 expression in cells was increased by infection with 2 doses of adenovirus encoding human HSP72 (AdvTR5/HSP70-GFP) in AdvCMV/tTA system, with or without 10 ng/ml TGF-β1 or 100 μM ZVAD-FMK. Adenovirus without encoding human HSP72 (AdvTR5/GFP) served as a negative control. In addition, cells were transfected with specific HSP72 small interfering RNA (siRNA). Cells treated with control siRNA were used as a control. A: Western blot showed increase in HSP72 associated with preserving of E-cadherin expression and suppressing of α-SMA expression in a dose-dependent manner in TGF-β1-treated cells. B and C: densitometric analysis of the effect of HSP72 expression on E-cadherin and α-SMA levels normalized with GAPDH content in cells treated as described in A. Values are means ± SE; n = 5/treatment. *P < 0.01 vs. negative control. †P < 0.05 vs. TGF-β1-treated cells without either ZVAD-FMK or HSP72 overexpression (bar 2). D: specificity of HSP72 siRNA was examined using transfection agent, control siRNA, and different doses of HSP72 siRNA. HSP72 expression was analyzed by Western blotting. E: EMT was assessed by Western blot analysis after transfection with HSP72 siRNA. F and G: E-cadherin and α-SMA content were analyzed quantitatively using a densitometer. Values are means ± SE; n = 3/treatment. *P < 0.01 vs. negative control. †P < 0.05 vs. TGF-β1-treated cells without either HSP72 overexpression or HSP72 siRNA (bar 2). H: EMT was examined by confocal microscopic images of E-cadherin and α-SMA staining in control, HSP72-overexpressing, and HSP72 siRNA cells following TGF-β1 exposure. Original magnification ×400.
venting α-SMA expression. Furthermore, HSP72-mediated EMT inhibition could be diminished by knockdown of HSP72 using HSP72-specific siRNA. These findings suggest that HSP72 attenuates TIF after UUO, at least in part, by concurrently inhibiting both tubular EMT and apoptosis. To our knowledge, the effect of HSP72 on EMT has not yet been reported, and its underlying mechanism(s) remains unknown. Since Smad3 can transduce signals of TGF-β from the cell cytosol to the nucleus (29), HSP72 may act as a molecular chaperone to prevent nuclear translocation of Smad3 (30). In support of this speculation, Knuesel et al. (16) recently demonstrated an interaction between HSP72 and Smad3 using communoprecipitation. Thus HSP72 may prevent nuclear Smad3 translocation and suppress tubular EMT by regulating the TGF-β/smad signaling pathway. Whether HSP72 inhibits EMT via additional mechanisms, such as TGF-β, fibroblast growth factor, and epidermal growth factor expression requires further investigation.

In summary, this is the first study to demonstrate that HSP72 significantly attenuates TIF and tubular apoptosis in kidneys subjected to ureteric obstruction. Although the precise mechanism remains to be determined, our results showed that HSP72, but not a pan-caspase inhibitor, inhibited tubular EMT in vitro, in response to TGF-β1. This suggests that HSP72 exerts an important role in chronic TIF by mechanisms independent of its effect on tubular apoptosis and may be a potential therapeutic agent for preventing progressive renal fibrosis in humans.

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