Albumin-induced epithelial-mesenchymal transition and ER stress are regulated through a common ROS-c-Src kinase-mTOR pathway: effect of imatinib mesylate

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TUBULOINTERSTITIAL INJURY has been recognized as a key process in the progression of renal disease. It has been well appreciated that urinary protein, particularly albumin, plays an essential role in the progression of tubulointerstitial injury (24). However, how urinary protein contributes to tubulointerstitial injury has not been completely understood.

The epithelial-mesenchymal transition (EMT) defines a phenotypic conversion of primary epithelial cells into mesenchymal cells, leading to morphological changes in fibroblastoid morphology, downregulation of epithelial marker proteins such as E-cadherin, and upregulation of mesenchymal markers such as α-smooth muscle actin (30). Accumulating evidence suggests that renal tubular epithelial cells can undergo EMT to become matrix-producing fibroblasts under pathological conditions and therefore participate in the pathogenesis of chronic renal diseases (11). EMT typically occurs in response to a number of environmental stresses and associated cytokine/growth factor stimuli. It has been reported that albumin is able to induce EMT in tubular cells (30). However, how albumin induces EMT has not been well defined.

Endoplasmic reticulum (ER) stress refers to physiological or pathological states that result in accumulation of misfolded proteins in the ER. Cells respond to ER stress by activating a series of integrative stress pathways termed the unfolded protein response (UPR). UPR either may be adaptive and promote cell survival, or if the ER stress is chronic or excessive, may lead to cell death. The major UPR signaling pathways are initiated by three protein sensors, activating transcription factor-6, inositol requiring-1α, and phosphorylated ERK (PERK). GRP78 serves as a master regulator of the UPR sensors. Eukaryotic translation initiation factor-2α (eIF2α) is phosphorylated by PERK, leading to reducing initiation AUG codon recognition (6).

It has been shown that albumin is able to induce ER stress and apoptosis in tubular epithelial cells (15, 17, 21).

Therefore, EMT and ER stress associated with chronic proteinuria may contribute to the development and progression of renal tubulointerstitial injury. However, information about the albumin-induced EMT and ER stress, especially on the activation of certain signaling molecules, still remains limited.

Reactive oxygen species (ROS) are known to function as second messengers for postreceptor signal transduction in many cell types. It has been reported that reabsorbed albumin by proximal tubular cells is able to perturb cell functions by ROS generation (10, 20, 32). Although albumin is known to signal through ROS, it is not clear yet whether ROS are involved in albumin-induced EMT and ER stress in renal tubular epithelial cells.

c-Src is a member of the Src tyrosine kinase family, which plays a role in signal transduction in response to many external stimuli, and its activity is under tight redox control (19, 36). ROS directly oxidize this enzyme, leading to promote auto-phosphorylation at Tyr416, which, in turn, leads to enhanced Src kinase activity (8, 38). c-Src kinase is known to be activated by albumin in tubular epithelial cells (3).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a pivotal role in mediating cell size and mass, proliferation, and survival. mTOR serves as a sensor and integrator of multiple stimuli induced by growth factors,
Fig. 1. Induction of epithelial-mesenchymal transition (EMT) by albumin. Proximal tubular cells were incubated with albumin (5 mg/ml) for indicated times (0–5 days). The morphological changes were examined using phase-contrast microscopy (A). Western blotting was performed. The bands for α-smooth muscle actin (α-SMA; B) and E-cadherin (C) were normalized to those for actin, respectively, for standardization. α-SMA mRNA expression was measured by real-time PCR (B). Results are means ± SE of 3–4 experiments expressed as n-fold increase over control. #P < 0.05 vs. day 0.

Fig. 2. Induction of endoplasmic reticulum (ER) stress by albumin. Proximal tubular cells were incubated with albumin (5 mg/ml) for indicated times (0–5 days). Western blotting was performed. The bands for GRP78 protein (A) and phosphorylated eukaryotic translation initiation factor-2α (p-eIF2α; B) were normalized to those for actin and total eIF2α, respectively, for standardization. GRP78 mRNA expression was measured by real-time PCR (A). Results are means ± SE of 3–4 experiments expressed as n-fold increase over control. #P < 0.05 vs. day 0.
nutrients, energy, or stress. mTOR has emerged as an important modulator of several forms of renal disease (16). It has been reported that ROS and Src kinase activity may regulate mTOR signaling (34, 39). Furthermore, mTOR is known to play a role in EMT (16).

Therefore, we postulated that ROS, c-Src kinase, and mTOR would act as upstream signaling molecules mediating both albumin-induced EMT and ER stress. We investigated whether albumin-induced EMT and ER stress were regulated through induction of ROS followed by activation of c-Src kinase and mTOR.

Platelet-derived growth factor (PDGF) is a pleiotropic growth factor that is originally isolated from human platelet-rich plasma. PDGF plays an important role in many renal diseases, and their levels are correlated with disease severity (33). Selective inhibition of PDGF action is considered a major target for specific and effective therapy for renal diseases.

Imatinib mesylate (Gleevec) is a potent inhibitor of PDGF receptor tyrosine kinase. Albumin is known to induce PDGF expression in tubular epithelial cells (5). Therefore, we examined the effect of imatinib mesylate on the albumin-induced EMT and ER stress.

Monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), transforming growth factor-β1 (TGF-β1) and collagen I (α1) are considered as important mediators in tubulointerstitial injury. We further examined the effect of imatinib on the albumin-induced mRNA expression of these mediators.

**MATERIALS AND METHODS**

**Reagents.** Imatinib mesylate (Gleevec) was obtained from Novartis (Basel, Switzerland). Bovine serum albumin [(A 4919, fraction V, low endotoxin, <0.1 ng/mg), N-acetylcysteine, and tiron were obtained from Sigma (St. Louis, MO). Anti-α-smooth muscle actin (α-SMA), E-cadherin, and GRP78 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Antibodies to total c-Src kinase, phosphospecific c-Src kinase, EMT, and collagen I (α1) were obtained from Merck (Darmstadt, Germany).

**Fig. 3. Involvement of reactive oxygen species (ROS) in albumin-induced EMT and ER stress.**

A: induction of ROS by albumin. B: inhibition of albumin-induced ROS by antioxidants and imatinib. C: suppression of albumin-induced EMT and ER stress by antioxidants. Proximal tubular cells were incubated with albumin (5 mg/ml) for indicated times (0–5 days). For examining the effect of antioxidants, PP2, rapamycin, and imatinib mesylate on ROS generation, cells were treated with albumin for 3 days, followed by addition of antioxidants [tiron (10 mM), N-acetylcysteine (20 mM), PP2 (25 μM), rapamycin (5 μM), and imatinib mesylate (2 μM)] for 2 days. Dichlorofluorescein (DCF)-sensitive cellular ROS were measured using confocal scanning microscopy and flow cytometry. Results are expressed as means channel fluorescence ± SE of 3 experiments. For examining the effect of antioxidants on albumin-induced EMT and ER stress, cells were treated with albumin for 3 days, followed by addition of antioxidants. Western blotting for α-SMA, E-cadherin, GRP78, and p-eIF2α was performed. #P < 0.05 vs. day 0 or control (con). ##P < 0.05 vs. albumin (alb).
eIF2α, phosphospecific eIF2α (Ser51), and horseradish peroxidase-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA). c-Src kinase inhibitor (PP2) and mTOR inhibitor (rapamycin) were obtained from Calbiochem (San Diego, CA).

Cell culture and conditioning. All experiments were performed using HK-2 cells (from The American Type Culture Collection), a proximal tubular cell line derived from a normal adult human kidney (26). Cells were grown with keratinocyte serum-free medium supplemented with EGF (5 ng/ml) and bovine pituitary extract (40 μg/ml). Cells were incubated to confluence, after which they were incubated with serum-free medium for 24 h. Then, the medium was changed and cells were incubated with BSA (5 mg/ml) for various time periods (1, 3, and 5 days). The concentration of albumin used in our experiment was based on the previous studies (21, 37). For examining the effect of imatinib, cells were treated with albumin for 3 days, followed by addition of imatinib mesylate (0.2–2 μM) for 2 days. Concentration of imatinib mesylate used in this study was comparable to the dose that was reported in cultured mesangial cells (9).

Assessment of cell morphology. The morphological changes were examined using phase-contrast microscopy.

Assay of intracellular ROS. Intracellular ROS production was detected by confocal scanning microscopy using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA; Molecular Probes, Eugene, OR). CM-H2DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2′,7′-dichlorofluorescein (DCFH) and thereby trapped within the cells. In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were incubated in the dark with 5 μM CM-H2DCF-DA for 30 min. After washing three times, cells were examined with confocal scanning microscopy (excitation, 488 nm; emission, 515–540 nm).

To quantitate ROS generation, cells were resuspended and mean fluorescence intensity was measured using flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

Western blot analysis. An equal amount of protein from whole cell lysates was separated by 10% SDS-polyacrylamide gels and then transferred to nylon membranes. Membranes were incubated for 2 h with primary antibody, followed by peroxidase-conjugated secondary antibody. Antibody-antigen complexes were detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL). The band intensities were quantified using a GS-710 densitometer and QuantityOne software (Bio-Rad, Hercules, CA).

mRNA quantification by real-time PCR. Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). Reverse transcription

Fig. 4. Involvement of c-Src kinase in albumin-induced EMT and ER stress. A: induction of c-Src kinase by albumin. B: inhibition of albumin-induced EMT by c-Src kinase inhibitor. C: inhibition of albumin-induced ER stress by c-Src kinase inhibitor. D: suppression of c-Src kinase by antioxidants. Proximal tubular cells were incubated with albumin (5 mg/ml) for indicated times (0–5 days). For examining the effect of c-Src kinase inhibitor PP2 and antioxidants on albumin-induced EMT, ER stress, and c-Src kinase respectively, cells were incubated with albumin for 3 days, followed by addition of c-Src kinase inhibitor PP2 (25 μM) and antioxidants [tiron (10 mM), N-acetylcysteine (20 mM)] for 2 days. Western blotting was performed. The bands for α-SMA, E-cadherin, GRP78, and p-eIF2α were normalized to those for actin and total eIF2α, respectively, for standardization. Results are means ± SE of 3–4 experiments expressed as n-fold increase over control. #P < 0.05 vs. control. ##P < 0.05 vs. albumin.
was performed by high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocols. Quantitative real-time PCR was performed using an ABI PRISM 7000 (Applied Biosystems). Specific primers for human α-SMA, GRP78, MCP-1, VCAM-1, TGF-β1, collagen I (α1), and GAPDH were purchased from Applied Biosystems [assay ID Hs 00909449-m1 for α-SMA, Hs 0046350-g1 for GRP 78, Hs 00234140-m1 for MCP-1, Hs 01003369-m1 for VCAM-1, Hs 00998133-m1 for TGF-β1, Hs 01076777-m1 for collagen I (α1), and Hs 99999905-m1 for GAPDH].

Cycling conditions were 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 60°C for 1 min. Relative amounts of mRNA were normalized by GAPDH and calculated using the delta-delta method from threshold cycle numbers (18).

Statistical analysis. Data are expressed as means ± SE. A Kruskall-Wallis test was used for comparison of more than two groups, followed by a Mann-Whitney U-test for comparison using a microcomputer-assisted program with SPSS for Windows 10.0 (SPSS, Chicago, IL). A P value <0.05 was considered significant.

RESULTS

Albumin induced EMT in tubular epithelial cells. To evaluate albumin-induced EMT, we used three independent parameters: cell morphology, upregulation of α-SMA, and down-regulation of E-cadherin. Exposure of tubular cells to albumin (5 mg/ml) for up to 5 days caused phenotypic conversion in a time-dependent manner. The transformed cells lost the typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shaped, fibroblast-like morphology (Fig. 1A). Albumin induced an increase in α-SMA protein and mRNA in a time-dependent manner (Fig. 1B). Albumin decreased the expression of E-cadherin protein as well (Fig. 1C).

Albumin induced ER stress in tubular epithelial cells. To determine whether albumin induced ER stress, we examined the change of two ER stress biomarkers, upregulation of GRP78 protein and phosphorylation of eIF2α. Addition of albumin (5 mg/ml) induced an increase in GRP78 protein and mRNA in a time-dependent manner (Fig. 2A). Albumin induced phosphorylation of eIF2α as well (Fig. 2B).

Albumin-induced EMT and ER stress were mediated through induction of ROS. Albumin was found to induce ROS in a time-dependent manner for up to 5 days (Fig. 3A). As expected, antioxidants [tiron (10 mM), N-acetylcysteine (20 mM)] reduced albumin-induced intracellular ROS formation (Fig. 3B).

Fig. 5. Involvement of mammalian target of rapamycin (mTOR) in albumin-induced EMT and ER stress. A: induction of mTOR by albumin. B: inhibition of albumin-induced EMT by mTOR inhibitor. C: inhibition of albumin-induced ER stress by mTOR inhibitor. D: suppression of mTOR by c-Src kinase inhibitor. Proximal tubular cells were incubated with albumin (5 mg/ml) for indicated times (0–5 days). For examining the effect of mTOR inhibitor and c-Src kinase inhibitor on the albumin-induced EMT, ER stress and mTOR, respectively, cells were incubated with albumin for 3 days, followed by addition of mTOR inhibitor (rapamycin; 5 μM) or c-Src kinase inhibitor (PP2; 25 μM) for 2 days. Western blotting was performed. The bands for α-SMA, E-cadherin, GRP78, and p-mTOR were normalized to those for actin and total mTOR, respectively, for standardization. Results are means ± SE of 3–4 experiments expressed as n-fold increase over control. #P < 0.05 vs. control. ##P < 0.05 vs. albumin.
To determine whether albumin-induced EMT and ER stress were mediated through ROS, we examined the effect of antioxidants on albumin-induced EMT and ER stress. Tiron and N-acetylcysteine inhibited the albumin-induced upregulation of α-SMA and downregulation of E-cadherin. They also suppressed albumin-induced GRP78 and eIF2α (Fig. 3C).

Albumin-induced EMT and ER stress were mediated through induction of ROS, followed by activation of c-Src kinase. Albumin was found to induce phosphorylation of c-Src kinase in a time-dependent manner for up to 5 days (Fig. 4A). To determine whether albumin-induced EMT and ER stress were mediated through c-Src kinase, we examined the effect of c-Src kinase inhibitor PP2 (25 μM) on albumin-induced EMT and ER stress. PP2 inhibited the albumin-induced upregulation of α-SMA and downregulation of E-cadherin (Fig. 4B).

PP2 suppressed albumin-induced GRP78 and eIF2α as well (Fig. 4C). To examine whether induction of ROS was upstream of c-Src kinase, we examined the effect of antioxidants [tiron (10 mM), N-acetylcysteine (20 mM)] on the albumin-induced phosphorylation of c-Src kinase. Western blotting revealed that antioxidants inhibited albumin-induced phosphorylation of c-Src kinase (Fig. 4D). In addition, the c-Src kinase inhibitor PP2 had no significant effect on albumin-induced ROS generation (Fig. 3B), suggesting that albumin-induced EMT and ER stress were regulated through induction of ROS, followed by activation of c-Src kinase.

Albumin-induced EMT and ER stress were mediated through induction of ROS, followed by activation of upstream c-Src kinase and downstream mTOR. Albumin was found to induce phosphorylation of mTOR in a time-dependent manner for up to 5 days (Fig. 5A). To determine whether albumin-induced EMT and ER stress were mediated through mTOR, we examined the effect of the mTOR inhibitor rapamycin (5 μM) on albumin-induced EMT and ER stress. Rapamycin inhibited albumin-induced upregulation of α-SMA and downregulation of E-cadherin (Fig. 5B). Rapamycin suppressed albumin-induced GRP78 and eIF2α as well (Fig. 5C).

To examine whether activation of c-Src kinase was located upstream of mTOR, we examined the effect of the c-Src kinase inhibitor PP2 (25 μM) on the albumin-induced phosphorylation of mTOR. Western blotting revealed that PP2 suppressed the albumin-induced phosphorylation of mTOR (Fig. 5D). In addition, rapamycin had no significant effect on albumin-induced c-Src phosphorylation (data not shown) and ROS generation (Fig. 3B), suggesting that albumin-induced EMT was mediated through ROS, followed by activation of c-Src kinase and downstream mTOR.

To determine whether albumin-induced EMT and ER stress were mediated through induction of ROS, followed by activation of upstream c-Src kinase and downstream mTOR, proximal tubular cells were incubated with albumin for 3 days, followed by addition of various concentrations of imatinib mesylate (0.2–2 μM) for 2 days. Western blotting was performed. The bands for α-SMA, E-cadherin, GRP78, p-eIF2α, and p-c-Src kinase were normalized to those for actin, total eIF2α, and total c-Src kinase, respectively, for standardization. Results are means ± SE of 3–4 experiments expressed as n-fold increase over control. #P < 0.05 vs. control. ##P < 0.05 vs. albumin.
and ER stress were regulated through induction of ROS, followed by activation of upstream c-Src kinase and downstream mTOR.

Imatinib mesylate inhibited albumin-induced EMT and ER stress through inhibition of ROS and c-Src kinase. Imatinib mesylate was found to inhibit the albumin-induced upregulation of α-SMA and downregulation of E-cadherin in a dose-dependent manner (Fig. 6A). Imatinib suppressed albumin-induced GRP78 and eIF2α as well (Fig. 6B). In addition, imatinib suppressed albumin-induced ROS (Fig. 3B) and c-Src kinase (Fig. 6C).

Imatinib mesylate inhibited albumin-induced mRNA expression of MCP-1, VCAM-1, TGF-β1, and collagen I (α1). Real-time PCR revealed that imatinib mesylate inhibited the albumin-induced mRNA expression of MCP-1, VCAM-1, TGF-β1, and collagen I (α1) as well.

Urinary protein, particularly albumin, is being recognized as an important mediator of renal tubulointerstitial injury (24). However, the mechanisms underlying albumin-induced renal injury remain complex and multifactorial.

Emerging evidence suggests that tubular epithelial cells play a pivotal role in tubulointerstitial fibrosis through the process of EMT (23). It has been demonstrated that more than one-third of renal interstitial myofibroblasts are derived from renal tubular epithelium via EMT (11), suggesting that EMT is a major mechanism of tubulointerstitial fibrosis.

We found that albumin also induced EMT in tubular epithelial cells. These findings, together with other reports that proteinuria was one of the important mediators causing EMT (1, 8, 37), suggest that in chronic proteinuric diseases, tubular epithelial cells are able to undergo EMT and ER stress, which may lead to tubulointerstitial injury. However, the signaling pathway regulating both albumin-induced EMT and ER stress has not yet been revealed.

We found that ROS were involved in albumin-induced EMT. This finding has been supported by previous work showing that ROS are involved in aldosterone- and TGF-β-induced EMT in renal tubular epithelial cells (25, 41).

We found that ROS were also involved in albumin-induced ER stress. Similarly, it has been reported that ROS are involved in glucose-induced ER stress in endothelial cells (28).

c-Src kinase and mTOR are also implicated in EMT pathways in nonkidney cells such as tumor cells, epithelial cells, and peritumoral cells (2, 13, 40). However, whether c-Src kinase and mTOR are involved in albumin-induced ER stress in renal tubular epithelial cells has not yet been revealed.

We found that both albumin-induced EMT and albumin-induced ER stress were regulated through induction of ROS followed by activation of upstream c-Src kinase and downstream mTOR. These findings indicated that the ROS-c-Src kinase-mTOR pathway played a central role in the signaling pathway that linked albumin to EMT and ER stress. Therefore,
c-Src kinase and mTOR might provide a selective intervention site to protect the albumin-induced renal injury associated with EMT and ER stress.

In keeping with this notion, it has been suggested that inhibition of c-Src kinase and mTOR ameliorates poly cystic kidney disease (29, 31). Substantial evidence exists that the mTOR pathway plays an important role in the mechanisms underlying the progression of chronic kidney disease and its inhibitor, rapamycin, attenuates the renal injury (16).

Targeting EMT and/or ER stress has been suggested as one of the possible therapeutic approaches to tubulointerstitial injury.

Imatinib mesylate, a phenylaminopyrimidine derivative, is the prototypical inhibitor of tyrosine kinases, such as abl, c-kit, and the PDGF receptor (4). It has been approved for treatment of chronic myeloid leukemia and gastrointestinal stromal tumors. Accumulating evidence suggests that imatinib mesylate provides therapeutic benefits in animal models of kidney diseases, such as anti-glomerular basement membrane glomrulonephritis (12), diabetic nephropathy (14), lupus nephritis (42), mesangial proliferative glomerulonephritis (9), chronic allograft nephropathy (27), and unilateral obstructive nephropathy (35). These therapeutic effects of imatinib mesylate are considered the result of its main inhibitory action on PDGF, leading to a reduction of glomerular cell proliferation and extracellular matrix accumulation.

We found that imatinib mesylate also suppressed the albumin-induced EMT and ER stress in tubular epithelial cells via inhibition of ROS and c-Src kinase. In support for this finding, imatinib mesylate reduced the interstitial SMA-positive myofibroblast in a animal model of lupus (42).

It has been well appreciated that mediators such as MCP-1, VCAM-1, TGF-β1, and collagen I (α1) contribute to tubulointerstitial injury. We found that imatinib mesylate inhibited the albumin-induced mRNA expression of these mediators. These data suggested that imatinib mesylate might be beneficial in attenuating the albumin-induced tubular injury.

The present study has some limitations. The albumin concentration of 5.0 mg/ml used in our study may not appropriately reflect the real proteinuric state in human diseases since the real concentration of proteins in the proximal tubular ultrafiltrate in human proteinuric renal diseases is unknown. However, with regard to this issue, there has been a report suggesting that a protein concentration of 5.0 mg/ml is comparable to levels which may be expected in the proximal tubular ultrafiltrate in nephrotic syndrome (37).

In addition, this is a relatively short-term study which examines the acute effects of albumin over a few days in an in vitro culture model. Therefore, further studies are needed to establish the in vivo counterparts of our findings in the setting of chronic proteinuria.

In conclusion, our study provides further clues regarding therapeutic approaches to inhibition of the signal transduction pathways involved in both albumin-induced EMT and ER stress and also provides data supporting the concept of a beneficial effect of imatinib mesylate on renal disease.

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REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


