Mitochondria Are the Major Targets in Albumin-Induced Apoptosis in Proximal Tubule Cells

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Nephrotic-range proteinuria is considered a poor prognostic factor. A correlation between tubulointerstitial injury and the degree of proteinuria is well established. In an attempt to explain the tubular atrophy that is observed in advanced glomerulonephritides, this study investigated apoptotic mechanisms in cultured human proximal tubule cells (HKC-8) that were exposed to endotoxin-free albumin (5, 10, and 20 mg/ml). Apoptosis was detected by Hoechst 33342; annexin staining; and assays for caspases 3, 8, and 9. The apoptotic effect of albumin was maximal at 10 mg/ml albumin, and necrosis prevailed in cells that were incubated with 20 mg/ml. Increase in caspase-9 and -3 activity was observed starting at 6 and maximally at 16 to 24 h. The proapoptotic Bcl-2 protein Bax was upregulated at 6 h, associated with translocation of cytochrome-c from mitochondria to cytosol and alteration in the mitochondrial membrane potential. Production of reactive oxygen species (ROS) was significant at 6 h but declined at 16 and 24 h. Treatment with ROS scavenger dimethylthiourea or antioxidant N-acetylcysteine did not alleviate caspase-3 production. Pan protein kinase C inhibitor bisindolylmaleimide-1 protected the cells from apoptosis. It is concluded that albumin induces apoptosis in human proximal tubule cells by stimulating mitochondrial apoptotic pathway independent of ROS production.

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Most of the glomerular diseases have the potential to progress into renal insufficiency unless maneuvers to halt the progression are instituted at an early stage. Proteinuria is considered a hallmark of glomerular diseases. Clinical studies that analyzed the outcome of glomerular diseases such as IgA nephropathy, FSGS, and membranoproliferative glomerulonephritis have shown nephrotic-range proteinuria (>2.5 g/d) to represent a poor prognostic factor (1–3). Therefore, a reduction in the degree of proteinuria has been a goal of treatment and used as a parameter to monitor the response to therapy. The adverse effect of proteinuria has been linked to its potential to stimulate production of proinflammatory and profibrotic molecules by the proximal tubule cells, contributing to tubulointerstitial inflammation and fibrosis (4–6).

Albumin represents the major protein in nephrotic urine. Studies have focused on the consequences of high albumin exposure in the kidney, particularly proximal tubule cells. For this purpose, animal and *in vitro* models of albumin overload have been used. In the animal models of albumin overload, intraperitoneal albumin injections have resulted in significant interstitial inflammation and fibrosis. In *in vitro* studies of albumin overload, proximal tubule cells that were exposed to excessive amounts of albumin, representing the nephrotic urine, have displayed upregulation of profibrotic cytokines such as IL-1, IL-8, monocyte chemoattractant protein, TGF-β1, endothelin-1, and transcriptional factor NF-κB (7–9).

Apoptosis, previously described as a physiologic mechanism of eliminating unwanted cells, is now implicated in pathologic conditions such as ischemic and nephrotoxic injury, obstructive nephropathy, and polycystic kidney disease (10–13). Apoptosis is triggered by two main pathways: The intrinsic pathway and the extrinsic pathway. Specific proteases now numbered up to 14 have been identified as initiator and effector caspases that mediate apoptotic pathways (14,15). The extrinsic pathway is stimulated when integral membrane death receptors Fas (CD95) or TNF receptor 1 are activated by apoptotic stimuli. This interaction gives rise to the recruitment of Fas-associated death domain (FADD) and cleavage of procaspase-8 to caspase-8. Mitochondria also mediate apoptosis, and this pathway is triggered by various stimuli, such as Ca^{2+} and increased free radical production. Mitochondrial apoptosis is initiated mainly by translocation of Bax, a proapoptotic protein that belongs to the Bcl-2 family, to the mitochondrial membrane, followed by release of cytochrome-c and activation of caspase-9 (16,17). In our previous studies, we showed the presence of tubule cell apoptosis in correlation with the degree of proteinuria in kidney biopsy specimens of patients with FSGS. An association between progression and tubular apoptosis was also identified (18). In an *in vitro* model of albumin overload, high concentrations of albumin mimicking the nephrotic milieu, proximal and distal tubule cells underwent apoptosis (19).

Tubular atrophy and tubulointerstitial fibrosis are well-described features of proteinuric states. We speculated that tubule

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cell apoptosis may lead to morphologic changes and loss of tubular epithelial cells, resulting in tubular atrophy. Therefore, identifying the downstream signaling pathway after albumin uptake is crucial to understanding the albumin-induced injury in the tubule cells. We hypothesized that albumin-induced tubule cell apoptosis precedes tubular atrophy. In this study, we investigated the apoptotic response and the responsible pathways that resulted from albumin exposure in human proximal tubule cells. In previous studies, incubation of human proximal tubule cells with albumin resulted in the increased production of reactive oxygen species (ROS) and activation of NFκ-B in relation to free radicals (7,20). Oxidative stress and ROS-mediated tubule cell apoptosis have also been implicated in ischemic kidney injury, diabetic nephropathy, and oxalate-associated nephrotoxicity (21–23). We therefore investigated ROS production in response to albumin exposure and its role in albumin-induced apoptosis.

Materials and Methods

Cell Culture System and Incubation

HKC-8 cells are an immortalized human kidney proximal tubule cell line (24) and were courtesy of Dr. L. Racusen (Johns Hopkins Medical Center, Baltimore, MD). They were grown in DMEM/F-12 (Life Technologies, Gaithersburg, MD) with 5% FBS supplemented with penicillin (100 mg/ml) and streptomycin (100 mg/ml). The medium was changed to serum-free medium 24 h before incubation with endotoxin-free human serum albumin (A5843; Sigma, St. Louis, MO) at 70% confluence. Various concentrations of albumin (5, 10, and 20 mg/ml) were used. For examination of the specificity of albumin, HKC-8 cells were also incubated with 10 mg/ml transferrin (T8158; Sigma) and IgG (56834; Sigma).

Detection of Apoptosis

Hoechst 33342 Staining. Microscopic detection of apoptosis was carried out on adherent cells after treatment with 10 μg/ml Hoechst 33342 for 15 min at 37°C. Slides were visualized under ultraviolet filters. Cells with apoptotic nuclei were counted in at least five different fields and expressed as percentage of total cells counted ($n = 500$ cells).

Annexin V Staining. We used the annexin V–FITC cell membrane–labeling assay (Annexin V Kit; BD Biosciences, San Jose, CA). Cells that were grown on coverslips were incubated with annexin V–FITC antibody for 15 min at room temperature in the dark and visualized by fluorescence microscopy.

Caspase-3, -8, and -9 Assays

Caspase-3, -8, and -9 activity assays were performed by use of the BDApoAlert caspase-8 colorimetric assay and the caspase-3 and caspase-9 fluorescence assay kits, respectively (BD Biosciences). Cells (1×10^6) were incubated in cell lysis buffer and centrifuged at 14,000 rpm, and the supernatants were incubated with IETD-AFC (specific substrate for caspase-8), DEVD-AFC (specific substrate for caspase-3), or LEHD-AMC (specific substrate for caspase-9) at 37°C for 1 h. The activity was assayed by use of a spectrophotometer or a fluorometer. The specificity of changes in caspase activity was confirmed by the addition of caspase-3 inhibitor (AcDEVD-CHO, 5 μg; BD Biosciences) and caspase-9 inhibitor (Z-LEHD-FMK, 5 μg; BD Biosciences).

Caspase-9 Inhibition Experiments. Cells that were grown in T25 flasks were pretreated with 20 μM caspase-9 inhibitor (Z-LEHD-FMK; BD Biosciences) 30 min before and during albumin incubation. For investigation of the effect of antioxidants, cells were pretreated with dimethylthiourea (DMTU; 10 mM), N-acetylcysteine (NAC; 10 mM), and bisindolylmaleimide-1 (BIM; 1 μM) 30 min before and during the albumin incubation. Caspase-3 activity was determined after 24 h of albumin incubation.

Measurement of Cell Viability. After cells were harvested, they were incubated in 4% trypan blue solution and counted under light microscopy. Cells that failed to exclude trypan blue were considered as nonviable and expressed as a percentage of total cells counted.

Mitochondrial Membrane Potential Determination

The integrity of mitochondrial membrane potential ($\Delta\psi$) was measured by JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazolylcarbocyanine iodide; T-3168; Molecular Probes, Eugene, OR), a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (527 nm) to red (590 nm). With normal mitochondrial function, $\Delta\psi$ is high and red fluorescence is predominant. After injury to mitochondria, $\Delta\psi$ is reduced and an increase in green fluorescence is observed. HKC-8 cells were grown on cover slides. After incubation with albumin, cells were washed and incubated with JC-1 dye at the concentration of 10 μg/ml in DMEM/F-12 with 5% FBS for 10 min at 37°C. The presence of depolarized mitochondria was identified by fluorescence microscopy as diffuse green fluorescence replaces red aggregates.

Detection of Intracellular ROS

Oxidation-sensitive DCFH-DA dye (C369; Molecular Probes) was used to determine intracellular production of ROS. 2′, 7′-Dichlorofluorescein (DCF) is a nonfluorescent dye until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell, yielding the fluorescence product of DCF. The cells were loaded with DCFH-DA at a final concentration of 10 μM and incubated at 37°C for 30 min. Final fluorescence was measured by Elite flow cytometer at 490 nm excitation and 526 nm of emission. The results were expressed as mean fluorescence intensity.

Determination of Cytochrome-c and Bax Expression

For immunofluorescence, cells were treated with 10 mg/ml albumin for 24 h and incubated in 25 nm of mitotracker dye (Mitotracker Red 580; Molecular Probes) at 37°C for 10 min. Slides were washed with PBS^{+Mg+Ca} and fixed in 4% formaldehyde and incubated in antibodies to cytochrome-c (1:400) and Bax-NT (1:250; both from Upstate, Lake Placid, NY). Slides were incubated with FITC-labeled secondary antibodies and visualized under rhodamine and fluorescein filters.

For Western blots, cells were lysed in ice-cold mitochondrial buffer (0.3 M mannitol, 0.1% BSA, 0.2 mM EDTA, 10 mM Hepes [pH 7.4]) with protease inhibitor cocktail on ice for 20 min and homogenized with a Dounce homogenizer. Cells were centrifuged at $1000 \times g$ at 4°C, and the supernatant was removed and centrifuged at $14{,}000 \times g$ for 20 min. The supernatant that contained the cytosolic fragment and the pellet that contained the mitochondria were loaded in each lane, and the proteins were separated on a 12% gel and electrotransferred to nitrocellulose membranes. Total protein was measured by the BCA protein assay (Pierce Chemical, Rockford, IL). Blots were incubated with anti–cytochrome-c (Upstate) and Bax-NT (Upstate) antibody overnight. Expression of proteins was normalized for actin and Cox-IV (Abcam, Boston, MA).

Statistical Analyses

Results of three or more experiments were expressed as mean ± SD. The statistical difference between paired groups was assessed by Wilcoxon signed ranks test, and the unpaired groups were assessed by

Mann Whitney *U* test. The density of Western blot bands was evaluated by the use of Sigma software. $P < 0.05$ was considered significant.

Results

Albumin Exposure Causes Apoptosis in Human Proximal Tubule Cells

The presence of apoptosis was tested with multiple methods. Annexin staining demonstrated early signs of apoptosis at the plasma membrane, evidenced by externalization of phosphatidylserine. At all concentrations, albumin induced apoptotic changes at the plasma membrane; the maximum effect was visualized at 10 mg/ml concentration (Figure 1A). Nuclear condensation represents the changes in the DNA that are detected during late apoptosis (Figure 1A). Hoechst 33342 staining showed an increase in apoptosis in a concentration-dependent manner. A total of 16.05 ± 4.13% of cells that were treated with 10 mg/ml had nuclear changes in comparison with 0.62 ± 0.59% of control cells ($P < 0.05$). No additional apoptosis was demonstrated at the 20-mg/ml concentration. In contrast, higher concentration of albumin induced necrosis as evidenced by higher number of trypan-positive cells. Quantification of percentage of nonviable cells revealed 12.77 ± 1.28% necrotic or late apoptotic cells at 20 mg/ml of albumin concentration in comparison with 5.33 ± 1.63% in the control group ($P = 0.05$; Figure 1B).

Albumin-Induced Apoptosis Is Mediated via *Caspase-9 Pathway*

To identify the specific pathway that is responsible for albumin-induced apoptosis, we analyzed the cells for caspase-3, -8, and -9 activity at 24 h. Caspase activity was expressed as time fold increase in comparison with the control cells. Caspase-3 is the terminal cysteine protease that initiates the execution phase of apoptosis. Caspase-3 activity was significant at a concentration of 5 mg/ml of albumin, maximizing at 10 mg/ml. Caspase-3 activity was 2.66 times higher in cells that were treated with 10 mg/ml in comparison with controls. Albumin-induced mitochondria mediated apoptosis in human proximal tubule cells as evidenced by increased caspase-9 activity starting at a concentration of 5 mg/ml albumin. At 10 mg/ml, apoptotic activity was 3.6-fold higher than controls. Extrinsic apoptotic pathway involving caspase-8 was not activated (Figure 2). On the basis of these experiments, the optimal proapoptotic concentration of albumin was determined to be 10 mg/ml, and this concentration was used for the subsequent experiments.

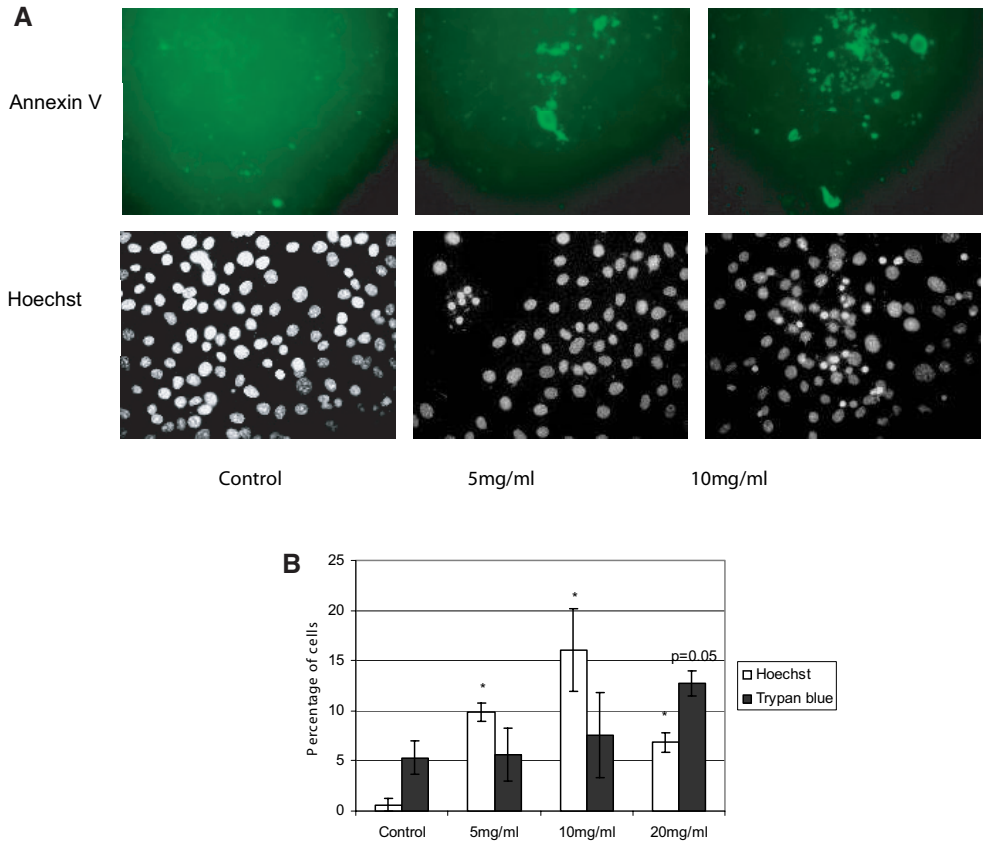

Figure 1. HKC-8 cells that were grown on the slides were incubated with various concentrations of albumin for 24 h. Albumin incubation resulted in externalization of phosphatidylserine, an early marker of apoptosis. Hoechst 33342 staining showed nuclear condensation with albumin incubation at 5- and 10-mg/ml concentrations ($n = 3$; A). Quantification of apoptosis and necrosis revealed increase in the percentage of condensed nuclei in a concentration-dependent manner. Albumin concentration of 20 mg/ml caused necrosis and late apoptosis ($n = 3$; B). *$P < 0.05$.

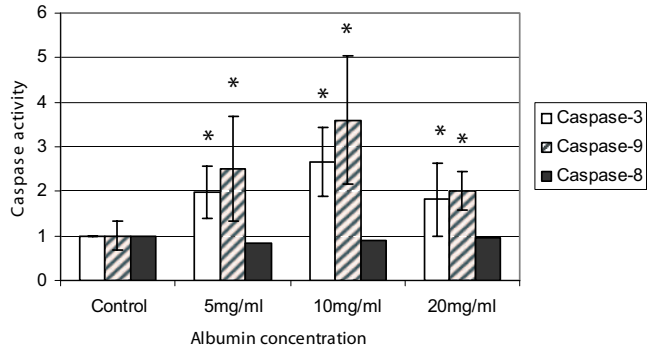

Figure 2. Relative increase in caspase activity in comparison with control cells. Caspase-9 and caspase-3 activity was significantly elevated; no significant change was observed in caspase-8 activity. The maximum apoptotic effect was observed at 10 mg/ml of albumin concentration ($n = 6$). *$P < 0.05$.

Time Course of Albumin-Induced Apoptosis

Cells were incubated with albumin (10 mg/ml) for various durations. Caspase-9 activity was apparent as early as 6 h and maximal at 16 h. Pretreatment with the specific caspase-9 inhibitor Z-LEHD FMK completely abolished the apoptosis (Figure 3A).

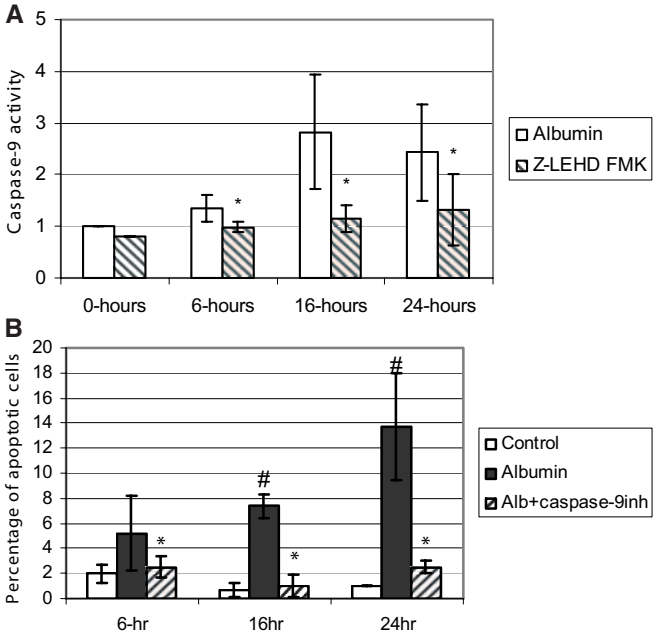

Figure 3. (A) Caspase-9 activity was determined after HKC-8 cells were incubated with 10 mg/ml albumin for various time periods. Caspase-9 activity started at 6 h continued for 16 and 24 h. Treatment with a caspase-9 inhibitor Z-LEHD FMK ameliorated the apoptotic effect. Control cells that were treated with caspase-9 inhibitor displayed some decrease in caspase-9 activity, but this was not statistically significant ($n = 3$; *$P < 0.05$. (B) Nuclear changes as a result of apoptosis were evident at 16 and 24 h of albumin incubation ($^{\#}P < 0.05$). Treatment with caspase-9 inhibitor resulted in statistically significant improvement in the number of apoptotic nuclei ($n = 3$; *$P < 0.05$).

Quantification of apoptotic cells by Hoechst 33342 staining revealed 5.2 ± 2.96, 7.38 ± 0.93 ($P < 0.05$) and 13.71 ± 4.26% ($P < 0.05$) apoptotic cells at 6, 16, and 24 h, respectively. This apoptotic effect was inhibited with 20 μM caspase-9 inhibitor Z-LEHD-FMK (Figure 3B).

Albumin-Induced Apoptosis Is Associated with Changes in the $\Delta\psi$

Under physiologic conditions, mitochondria remain in a hyperpolarized state, maintained by proton and pH gradients across the inner membrane. This produces the $\Delta\psi$. JC-1 accumulates in the mitochondria in the presence of negative charge across the mitochondrial membrane. Disturbance in the $\Delta\psi$ causes diffuse dissipation of green fluorescent dye in the cytoplasm. Alterations in the $\Delta\psi$ coincided with the initiation of apoptosis at 6 h (Figure 4).

Albumin-Induced Apoptosis Is Associated with Translocation of Bax and Cytochrome-c

Studies have shown a key role of proapoptotic protein Bax stimulating the alterations at the mitochondrial membrane and release of cytochrome-c from the mitochondria. We studied the translocation of proteins by immunofluorescence and Western blotting. Apoptotic nuclei were identified by Hoechst 33342 staining. In the control cells, cytochrome-c co-localized with mitotracker, indicating its mitochondrial location. Induction of apoptosis resulted in the translocation of cytochrome-c to the cytoplasm after incubation with albumin for 24 h (Figure 5). Bax was present as punctuate staining throughout the cytoplasm in the control cells. During tubular apoptosis, a significant fraction of Bax was found to redistribute from cytosol to mitochondria, co-localizing with mitotracker staining (Figure 6).

Western blotting of the proteins that were isolated from the mitochondria revealed the translocation of Bax starting as early as 6 h and maximizing at 16 and 24 h in correlation with the apoptotic response (Figures 7 and 8). Quantification of the protein expression showed that Bax expression in the mitochondrial lysates was up to 1.7-fold within 6 h of albumin incubation. Upregulation of Bax protein in the cytoplasm also suggests that the translocation from cytoplasm to mitochondria represents not only a shift of the molecule but also increased production of Bax during albumin exposure.

Cytochrome-c resides in the mitochondrial membrane under normal conditions. With induction of apoptosis and alterations in the mitochondrial membrane, cytochrome-c translocated from the mitochondrial membrane to the cytoplasm, leading to diminished cytochrome-c levels in the mitochondrial lysates starting at 6 h. Cytochrome-c expression in the cytoplasm was 1.5- and 2.8-fold by 6 and 24 h of albumin incubation, respectively (Figures 7 and 8).

Albumin Triggers ROS Production

To identify free radical production as a potential mechanism for albumin-induced apoptosis, we measured ROS levels using a fluorescence probe. The membrane-permeable DCFH-DA enters the cell and produces a fluorescence signal after intracellular oxidation by ROS. Albumin exposure stimulated ROS

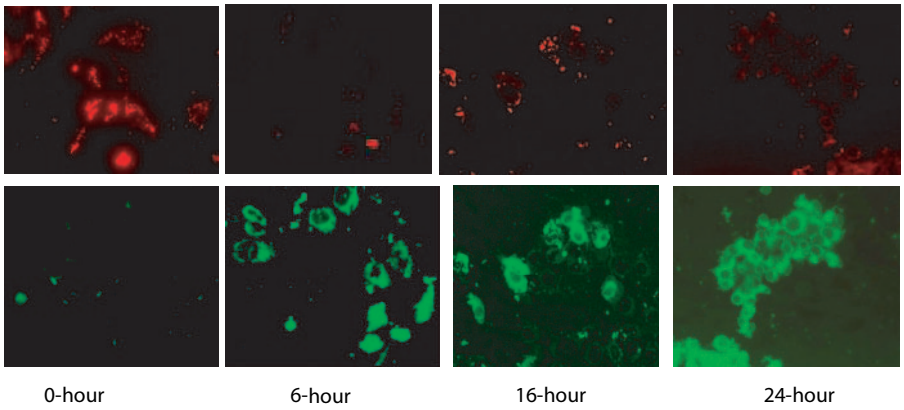

Figure 4. Demonstration of the time course of the changes in mitochondrial membrane potential ($\Delta\psi$). Alterations in the $\Delta\psi$ started at 6 h, maximizing at 16 to 24 h. JC-1 formed red aggregates in the presence of negative $\Delta\psi$. Disturbance in the $\Delta\psi$ resulted in appearance of green fluorescence throughout the cytoplasm ($n = 3$).

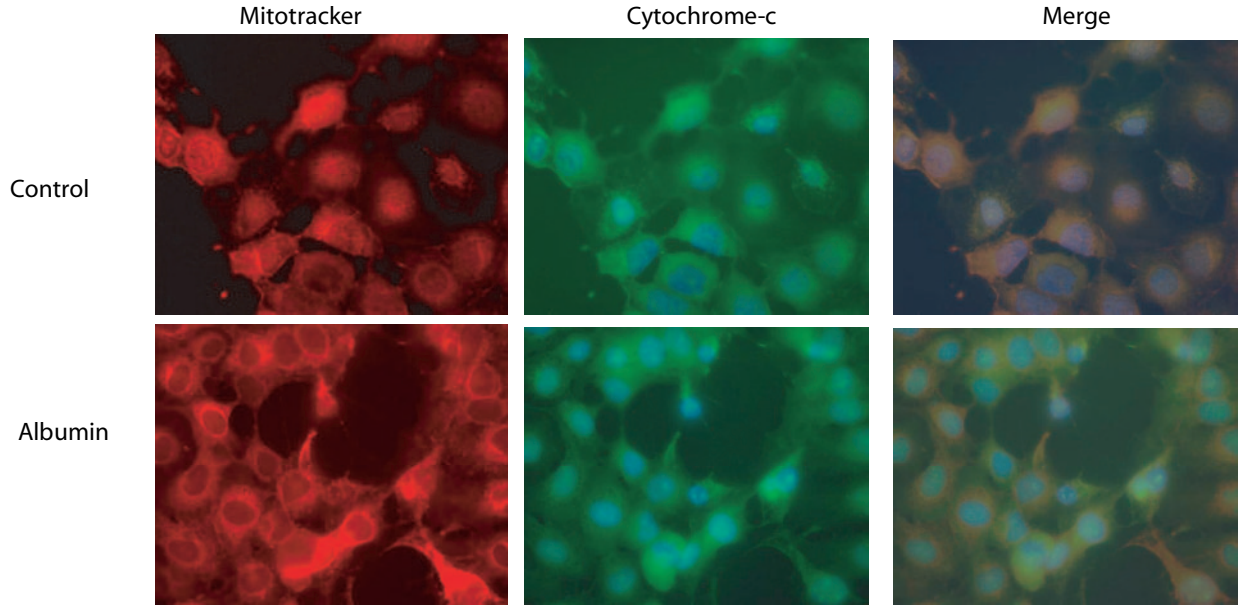

Figure 5. Immunofluorescence staining of cytochrome-c after 24 h of incubation in 10 mg/ml albumin. Cytochrome-c co-localized with mitotracker, demonstrating typical perinuclear staining characteristic of mitochondria. With the induction of apoptosis, cytochrome-c translocated to the cytoplasm. Nuclei were co-stained with Hoechst 33342 to identify the condensation ($n = 3$).

production at 6 h; however, at 16 and 24 h, the level of free radicals declined and reached a plateau. Fluorescence intensity peaked to 1.9 ± 0.82 from 0.4 ± 0.27 (4.8-fold increase) in 6 h of albumin exposure (Figure 9). At later time points, ROS levels were almost back to baseline.

Apoptotic Effect Is Unique to Albumin

Most glomerular diseases are characterized by nonselective proteinuria. To examine the potential apoptotic effect of other proteins that exist in the nephrotic urine, we incubated HKC-8 cells with 10 mg/ml transferrin and IgG for 6, 16, and 24 h. No significant increase in caspase-3 activity was detected in proximal tubule cells at any time point, demonstrating that tubular apoptosis is unique to albumin (Figure 10).

Albumin-Induced Apoptosis Is Inhibited by Caspase-9 Inhibitor and BIM-1 but not by DMTU or NAC

HKC-8 cells were incubated with various inhibitors to elucidate the mechanism of albumin-induced apoptosis. Preincubation with the specific caspase-9 inhibitor (Z-LEDH-FMK) inhibited caspase-3 activity, confirming the involvement of caspase-9 in albumin-induced apoptosis. Preincubation of cells with antioxidant NAC (10 mM) and DMTU (10 mM) did not abolish the caspase-3 activity, excluding the role of free radicals in apoptosis, although they inhibited ROS production significantly (data not shown). BIM-1 (1 μM), a pan protein kinase C (PKC) inhibitor, blocked the caspase-3 activity (Figure 11). BIM-1 but not DMTU and NAC inhibited the caspase-9 activity in HKC-8 cells that were treated with albumin for 24 h (data not

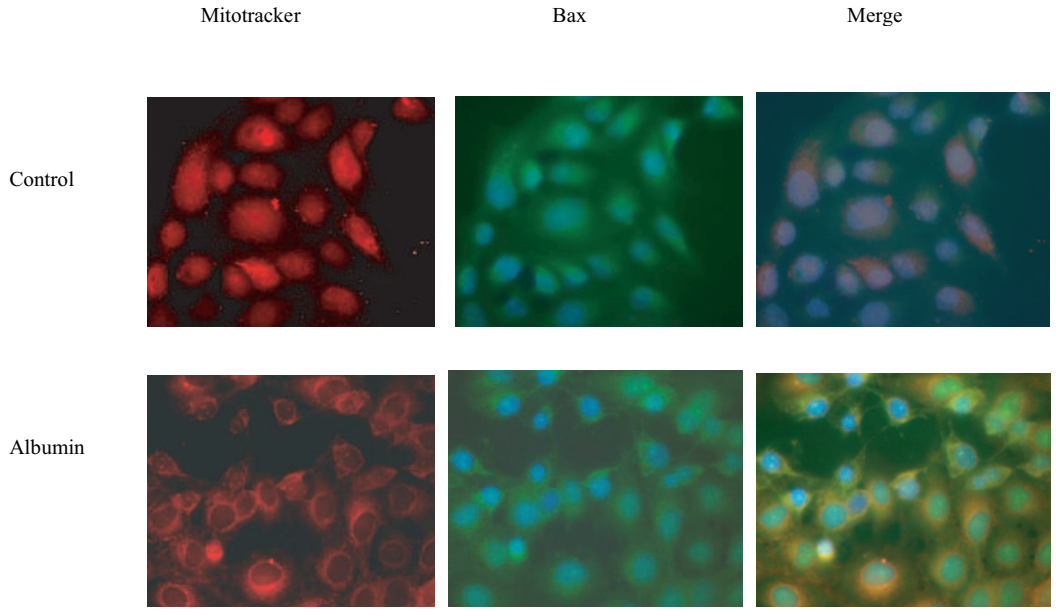

Figure 6. There is very little expression of proapoptotic Bax locating in the cytoplasm, evidenced by the green fluorescence in control cells. With induction of apoptosis, Bax expression is upregulated, and it translocates to mitochondria, merging with mitotracker. Nuclear changes secondary to apoptosis were displayed with Hoechst 33342 staining ($n = 3$).

shown). BIM-1 also decreased the percentage of apoptotic cells determined by Hoechst 33342 staining (1.53 ± 0.57 *versus* 16.05 ± 4.13, without BIM-1; $P < 0.05$).

Discussion

Nephrotic-range proteinuria is the hallmark of glomerular disease with poor prognosis. A body of evidence accumulated from *in vivo* and *in vitro* protein overload systems indicates that albumin induces inflammatory and fibrogenic response in human proteinuric states. Tubule cell apoptosis induced by albumin has been proposed as a potential mechanism to explain the extensive tubular atrophy reported in chronic glomerular diseases (18,19).

An earlier study by Benigni *et al.* (25) showed a significant increase in the atrophic and apoptotic proximal tubule cells in correlation to proteinuria in passive Heymann nephritis, and this effect was inhibited by angiotensin-converting enzyme inhibitor administration. As *in vivo* evidence, we have shown the presence of both proximal and distal tubular apoptosis on kidney biopsy specimens of patients with FSGS. There was a significant correlation between the degree of proteinuria and the number of apoptotic cells. Apoptosis was an independent predictor of ESRD. Examination of proliferation by proliferating cell nuclear antigen staining has demonstrated predominance of apoptosis over proliferation in kidney epithelial cells (18). Albumin exposure was shown to cause a mitogenic effect in wild-type opossum kidney cells *via* phosphatidylinositol 3-kinase, and this was inhibited by wortmannin and Δp85 expression (26). In a previous study, we also demonstrated the predominance of proliferation within the first 6 h of albumin incubation in human proximal tubule cells by the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cytotoxicity/proliferation assay. However, significant cytotoxicity was observed beyond 6 h, corresponding to occurrence of apoptosis (19).

In this study, we have shown that albumin exposure results in human proximal tubule cell apoptosis *via* caspase-9–mediated mitochondrial pathway. This apoptotic effect starts as early as 6 h and peaks at 24 h. This is the first study to show the induction of early apoptosis by albumin in human proximal tubule epithelial cells. Our previous observations in LLC-PK1 indicated the involvement of caspase-8–Fas-FADD through the receptor mediated extrinsic apoptotic pathway (27). We believe that the inconsistency between these two findings may be explained by the difference in origin, metabolism, and energy requirement of the cell types. It is also a possibility that albumin-induced apoptosis follows two phases: An early apoptosis that is mediated by the mitochondrial pathway and a late apoptosis that involves Fas-FADD–caspase-8. As we demonstrated in our previous study, Fas-induced apoptosis requires 3 d of incubation with a higher 20-mg/ml concentration of albumin. In the cell culture system used in this study, albumin concentration as low as 5 mg/ml resulted in apoptosis, with the optimal effect noted at 10 mg/ml. We examined mitochondria as a major target of albumin-induced apoptosis. The alterations in the $\Delta\psi$ that occurred at 6 h and coincided with translocation of Bax to the mitochondria and cytochrome-c to the cytoplasm were observed in this study. At 6 h, both caspase-3 and caspase-9 activity increased, peaking at 16 to 24 h. Previous studies have shown the involvement of Bcl-2 proteins in mitochondrial apoptosis. Members of the Bcl-2 family such as Bcl-2 and Bcl-XL inhibit apoptosis, but Bax and Bak stimulate apoptosis (28,29). Bax plays a pivotal role in inducing mitochondria-mediated apoptosis on glomerular disease and ischemic

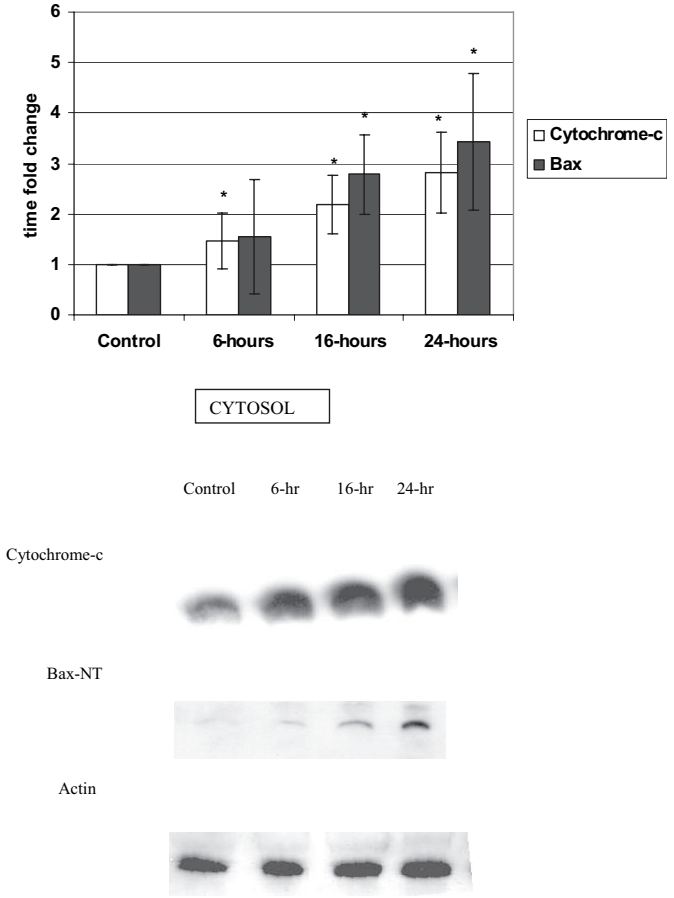

Figure 7. Western blot analysis of the proteins that are involved in albumin-induced apoptosis. Cytoplasmic extracts revealed upregulation of cytochrome-c and Bax expression starting at 6 h. Densitometric analysis of the proteins normalized for actin showed significant upregulation of both proteins in the cytoplasm ($n = 4$; $^*P < 0.05$).

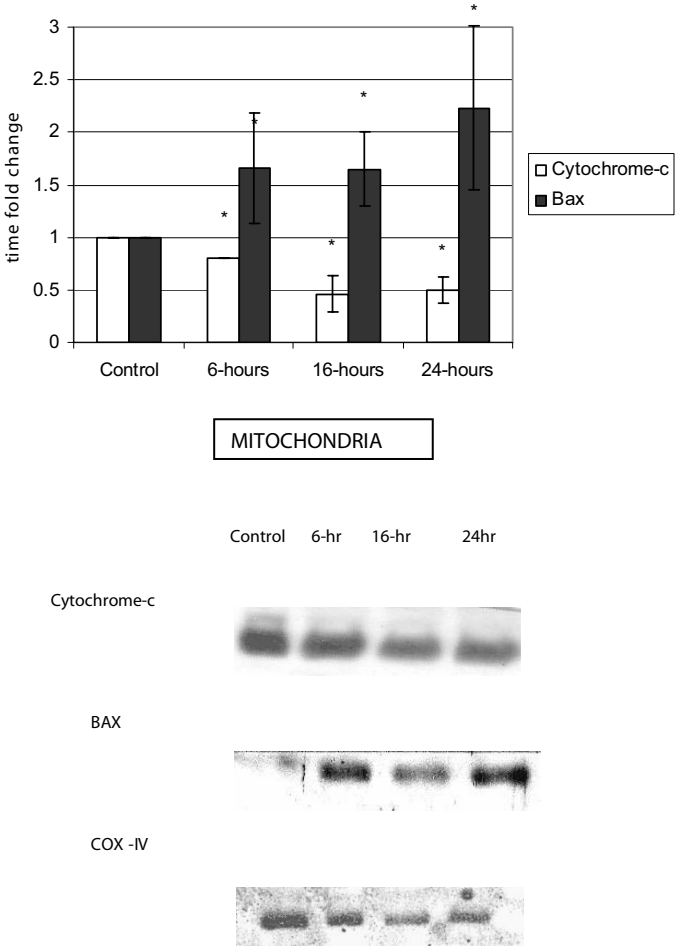

Figure 8. Bax was upregulated in the mitochondrial isolates, whereas cytochrome-c was downregulated, representing translocation of Bax to mitochondria and release of cytochrome-c from mitochondria to cytoplasm. Cox-IV was used as housekeeping protein. Quantification of mitochondrial Bax and cytochrome-c expression normalized for Cox-IV showed a significant increase in Bax and a decrease in cytochrome-c starting at 6 h ($n = 3$; $^*P < 0.05$).

and toxic kidney injury. Under physiologic conditions, Bax resides in the cytoplasm. With induction of apoptosis, Bax translocates to mitochondria and disrupts the $\Delta\psi$. This leads to cytochrome-c release, activation of caspase-9, and subsequent cell death (30–33). Studies have suggested a role of Bax in the pore-forming unit of mitochondrial outer membrane (34). In fact, incubation of isolated mitochondria with Bax protein results in cytochrome-c release (35). We did not study the expression of proapoptotic proteins earlier than 6 h, but it is conceivable to postulate that Bax translocation initiates the alterations in $\Delta\psi$.

Mitochondria are a major source of ROS production in the cells, and overproduction may tilt the balance toward cell death. However, involvement of ROS in apoptosis in the kidney has been controversial. Increased ROS was implicated in apoptotic cell death in the kidney in response to high glucose, ischemia/reperfusion, and cisplatin toxicity, but free radical formation was also proposed to be associated with apoptosis rather than being a causative agent (21,22,36,37). Generation of ROS has been linked to NF-κB and PKC activation in proximal tubule cells after albumin exposure. This pathway was proposed to lead to upregulation of inflammatory molecules by stimulating the transcriptional factors, and antioxidants DMTU and pyrrolidine dithiocarbamate ameliorated this response (7). We demonstrated the increased free radical production at 6 h of albumin exposure, and this effect subsided at 16 and 24 h. The time course of the elevated ROS production did not correlate with the onset of apoptosis. The peak ROS production at 6 h of incubation may represent an acute response, and cells may transition into a steady state. We postulate that albumin-induced apoptosis may occur as an early event independent of ROS production, or ROS may be involved in the initiation phase of apoptosis but not required for the execution (38). Diminished ROS production at the peak time of apoptosis and lack of protective effect by ROS scavengers DMTU and antioxidant NAC argues against oxidative injury as a sole cause of albumin-induced apoptosis.

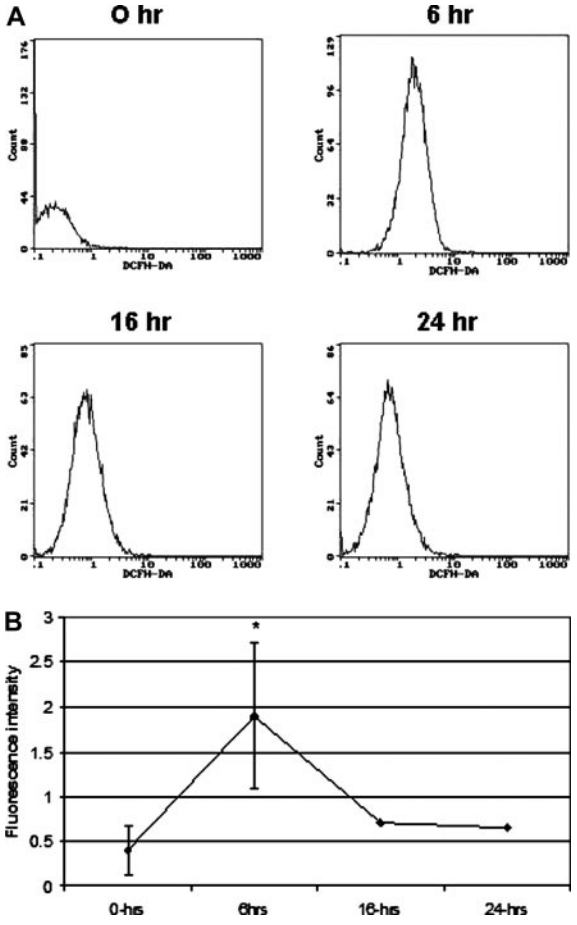

Figure 9. Determination of reactive oxygen species (ROS) production by DCFH-DA. In 6 h, a significant increase (4.8-fold) in the fluorescence intensity of ROS was observed. This effect declined at 16 and 24 h of albumin incubation ($n = 3$).

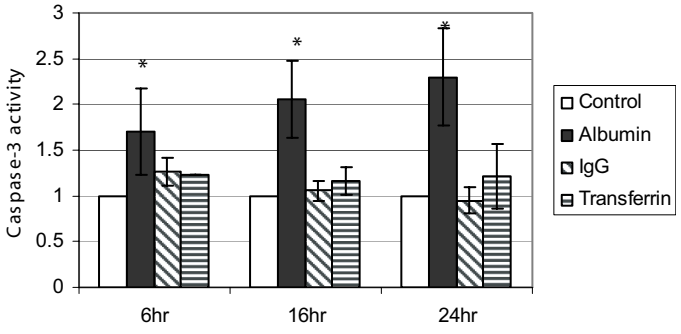

Figure 10. For examination of the specificity of albumin-induced apoptosis, human proximal tubule cells were incubated with transferrin and IgG at different time points. Transferrin (10 mg/ml) and IgG (10 mg/ml) did not cause any changes in caspase-3 activation ($n = 3$).

The pan PKC inhibitor BIM-1 study was shown to block albumin uptake in OK cells by inhibiting PKC-α (39). Rottlerin, a potent PKC-δ inhibitor, did not ameliorate apoptosis in our model (data not shown). Therefore, it is likely that the anti-

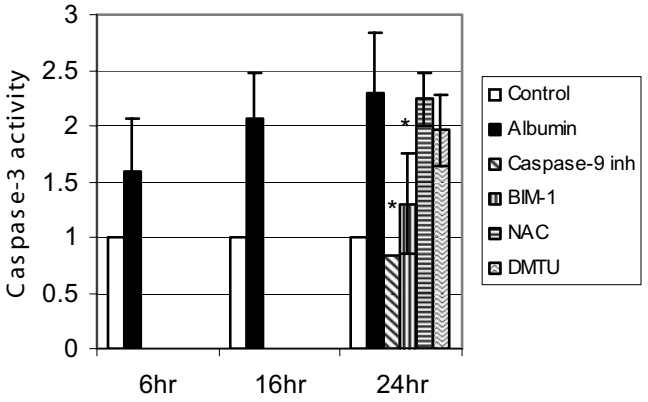

Figure 11. The effect of inhibitors on apoptosis. Caspase-3 activity was inhibited by caspase-9 inhibitor and bisindolylmaleimide-1 (BIM-1; 1 μM). Treatment with hydroxyl radical scavenger dimethylthiourea (DMTU; 10 mM) and antioxidant N-acetylcysteine (NAC; 10 mM) did not have any effect on the apoptotic response. BIM-1 incubation ameliorated apoptosis.

apoptotic effect of BIM-1 is related to decreased albumin uptake or inhibition of PKC-α–mediated apoptosis (39,40).

We also investigated the specificity of the apoptotic effect of albumin on proximal tubule cells. In a previous study, high molecular weight proteins (100 to 40 kD) rather than the albumin-rich fraction were found to induce tubular cell apoptosis at 24 to 48 and 72 h (41). Our findings failed to show apoptotic effect of other proteins that exist in the nephrotic milieu, such as IgG and transferrin, but proved that apoptosis was unique to albumin. Our experimental design may enable us to detect early apoptosis *via* the mitochondrial pathway, and albumin may be the responsible protein from this apoptotic effect.

In this study, we showed the involvement of mitochondrial proteins in albumin-induced apoptosis in human proximal tubule cells. The role of albumin-bound fatty acids *via* peroxisome proliferator–activated receptor γ (PPAR-γ) was implicated in albumin-induced tubule cell apoptosis. Overexpression of PPAR-γ and activation of PPAR-γ by the specific agonist PGJ_2 resulted in apoptosis, emphasizing the importance of lipid-lowering agents in management of glomerular diseases (42). The upstream intracellular molecular signaling pathway that initiates albumin-induced tubule cell apoptosis still remains to be investigated. Because caspase activation, which is a relatively late step in apoptosis, started as early as 6 h, the activation of apoptotic proteins at earlier stages and the proximal events that were initiated with albumin uptake still need to be elucidated. A recent study showed involvement of phosphatidylinositol 3-kinase/PKB pathway in albumin-induced apoptosis of LLC-PK1 cells. In this study, a relationship between megalin expression and PKB activity was demonstrated. At low concentrations, albumin was protective, but higher concentrations inhibited the megalin–PKB activity and Bad phosphorylation, resulting in apoptosis (43). On the basis of this study, it is conceivable to propose that endocytic pathways, particularly megalin, may play an important role in intracellular signal transduction that dictates cell survival or death.

Conclusion

Our study demonstrates tubule cell apoptosis as a result of early molecular interactions that activate mitochondrial apoptotic pathways. It is likely that tubule cell apoptosis may act as the precursor of tubular atrophy by causing cellular loss and morphologic changes in the remnant tubule cells. It is therefore important to examine the intracellular signaling cascade after uptake of albumin that culminates in apoptosis. Identification of the activated molecular cascades may provide development of novel interventions to prevent or decelerate the progression of glomerular diseases.

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Disclosures

None.

References

1. Cameron JS, Turner DR, Ogg CS, Chantler C, Williams DG: The long term prognosis of patients with focal glomerular sclerosis. *Clin Nephrol* 10: 213–218, 1978
2. Cameron JS, Turner DR, Heaton J, Williams G, Ogg CS, Chantler C, Haycock GB, Hicks J: Idiopathic mesangiocapillary glomerulonephritis: Comparison of types I and II in children and adults and long term prognosis. *Am J Med* 74: 175–192, 1983
3. Wyatt RJ, Kritchevsky SB, Woodford SY, Miller PM, Roy S 3rd, Holland NH, Jackson E, Bishof N: Ig A nephropathy: Long-term prognosis for pediatric patients. *J Pediatr* 127: 913–919, 1995
4. Eddy AA, McCulloch L, Liu E, Adams J: A relationship between proteinuria and acute tubulointerstitial disease in rats with experimental nephrotic syndrome. *Am J Pathol* 138: 1111–1123, 1991
5. Jones CL, Buch S, Post L, McCulloch L, Liu E, Eddy A: Renal extracellular matrix accumulation in acute puromycin aminonucleoside nephrosis. *Am J Pathol* 141: 1381–1396, 1992
6. Eddy AA, Giachelli CM: Renal expression of genes that promote interstitial inflammation and fibrosis in rats with protein-overload proteinuria. *Kidney Int* 47: 1546–1557, 1995
7. Morigi M, Macconi D, Zoja C, Donadelli SB, Zanchi C, Glulardi M, Remuzzi G: Protein overload induced NF-kappaB activation in proximal tubular cells requires H2O2 through a PKC-dependent pathway. *J Am Soc Nephrol* 13: 1179–1189, 2002
8. Zoja C, Morigi M, Figliuzzi M, Bruzzi I, Oldroyd S, Benigni A, Ronco P, Remuzzi G: Proximal tubular cell synthesis and secretion of endothelin-1 on challenge with albumin and other proteins. *Am J Kidney Dis* 26: 934–941, 1995
9. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC: Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 8: 1537–1545, 1997
10. Wiegele G, Brandis M, Zimmerhackl LB: Apoptosis and necrosis during ischemia in renal tubular cells (LLC-PK1 and MDCK). *Nephrol Dial Transplant* 13: 1158–1167, 1996
11. Healy E, Dempsey M, Lally C, Ryan MP: Apoptosis and necrosis: Mechanisms of cell death induced by cyclosporine A in renal proximal tubular cell line. *Kidney Int* 54: 1955–1966, 1998
12. Cummings BS, Schnellmann RG: Cisplatin-induced renal cell apoptosis: Caspase 3-dependent and independent pathways. *J Pharmacol Exp Ther* 302: 8–17, 2002
13. Tao Y, Kim J, Stanley M, He Z, Faubel S, Schrier RW, Edelstein CL: Pathways of caspase-mediated apoptosis in autosomal-dominant polycystic kidney disease. *Kidney Int* 67: 909–919, 2005
14. Thornberry NA, Lazebnik Y: Caspases: Enemies within. *Science* 281: 1312–1316, 1998
15. Ashkenazi A, Dixit VM: Death receptors: Signaling and modulation. *Science* 281: 1305–1308, 1998
16. Kim R, Emi M, Tanabe K: Role of mitochondria as the gardens of cell death. *Cancer Chemother Pharmacol* 57: 545–553, 2005
17. Wolter KG, Hsu Y, Smith CL, Nechushtan A, Xi XG, Youle RJ: Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 139: 1281–1292, 1997
18. Erkan E, Garcia CD, Patterson LT, Mishra J, Mitsnefes MM, Kaskel FJ, Devarajan P: Induction of renal tubular cell apoptosis in focal segmental glomerulosclerosis. *J Am Soc Nephrol* 16: 398–407, 2005
19. Erkan E, Devarajan P, Schwartz GJ: Apoptotic response to albumin overload: Proximal vs. distal/collecting tubule cells. *Am J Nephrol* 25: 121–131, 2005
20. Tang S, Leung JCK, Abe K, Chan KW, Chan LYY, Chan LYY, Chan TM, Lai KN: Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *J Clin Invest* 111: 515–527, 2003
21. Chieng CT, Lee PH, Chen CF, Ma MC, Lai MK, Hsu SM: De novo demonstration and co-localization of free radical production and apoptosis formation in rat kidney subjected to ischemia/reperfusion. *J Am Soc Nephrol* 12: 973–982, 2001
22. Allen DA, Harwood S, Varagunam M, Raftery MJ, Yaqoob MM: High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *FASEB J* 17: 908–910, 2003
23. Cao LC, Honeyman TW, Cooney R, Kennington L, Scheid CR, Jonassen JA: Mitochondrial dysfunction is a primary event in renal cell oxalate toxicity. *Kidney Int* 66: 1890–1900, 2004
24. Racusen LC, Monteil C, Sgrignoli A, Lucskay M, Marouillat S, Rhim JGS, Morin JP: Cell lines with extended in vitro growth potential from human renal proximal tubule: Characterization, response to inducers, and comparison with established cell lines. *J Lab Clin Med* 129: 318–329, 1996
25. Benigni A, Gagliardini E, Remuzzi A, Corna D, Remuzzi G: Angiotensin-converting enzyme inhibition prevents glomerular-tubule disconnection and atrophy in passive Heymann nephritis, an effect not observed with a calcium antagonist. *Am J Pathol* 159: 1743–1750, 2001
26. Dixon R, Brunskill NJ: Activation of mitogenic pathways by albumin in kidney proximal tubule epithelial cells: Implications for the pathophysiology of proteinuric states. *J Am Soc Nephrol* 10: 1487–1497, 1999

27. Erkan E, DeLeon M, Devarajan P: Albumin overload induces apoptosis in LLC-PK1 cells. *Am J Physiol Renal Physiol* 280: F1107–F1114, 2001
28. Pawlowski J, Kraft AS: Bax-induced apoptotic cell death. *Proc Natl Acad Sci U S A* 95: 4997–5002, 1998
29. Reed JC: Double identity for proteins of the Bcl-2 family. *Nature* 387: 773–776, 1997
30. Lee RH, Song JM, Park MY, Kang SK, Kim YK, Jung JS: Cisplatin-induced apoptosis by translocation of endogenous Bax in mouse collecting duct cells. *Biochem Pharmacol* 62: 1013–1023, 2001
31. Yoshimura A, Uda S, Inui K, Nemoto T, Sugenoya Y, Sharif S, Yokota N, Watanebe S, Ideura T: Expression of bcl-2 and bax in glomerular disease. *Nephrol Dial Transplant* 14: 55–57, 1999
32. Rawat S, Gray C, Johnson TS, Raftery AT, El Nahas AM, Haylor J: Apoptosis and expression of Bcl-2 and bax in cyclosporine-induced experimental renal fibrosis. *Transplant Proc* 35: 187–188, 2003
33. Saikumar P, Dong Z, Patel Y, Hall K, Hopfer U, Weinberg JM: Role of hypoxia-induced bax translocation and cytochrome c release in reoxygenation injury. *Oncogene* 17: 3401–3415, 1998
34. Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G, Korsmeyer SJ: Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc Natl Acad Sci U S A* 94: 11357–11362, 1997
35. Jurgensmeier J, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC: Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* 95: 4997–5002, 1998
36. Baek SM, Kwon CH, Kim JH, Woo JS, Jung JS, Kim YK: Differential roles of hydrogen peroxide and hydroxyl radical in cisplatin-induced cell death in renal proximal tubular epithelial cells. *J Lab Clin Med* 142: 178–186, 2003
37. Kruidering M, Water BVD, Heer DE, Mulder GJ, Nagelkerke JF: Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: Mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory chain. *J Pharmacol Exp Ther* 280: 638–649, 1997
38. Jacobson MD: Reactive oxygen species and programmed cell death. *Trends Biochem Sci* 21: 83–86, 1996
39. Hryciw DH, Pollock CA, Poronnik P: PKC-alpha-mediated remodeling of the actin cytoskeleton is involved in constitutive albumin uptake by proximal tubule cells. *Am J Physiol Renal Physiol* 288: F1227–F1235, 2005
40. Nowak G: Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells. *J Biol Chem* 277: 43377–43388, 2002
41. Morais C, Westhuyzen J, Metharom P, Healy H: High molecular weight plasma proteins induce apoptosis and Fas/FasL expression in human proximal tubular cells. *Nephrol Dial Transplant* 20: 50–58, 2005
42. Arici M, Chana R, Lewington A, Brown J, Brunskill NJ: Stimulation of proximal tubular cell apoptosis by albumin bound fatty acids mediated by peroxisome proliferator activated receptor-gamma. *J Am Soc Nephrol* 14: 17–27, 2003
43. Caruso-Neves C, Pinheiro AAS, Cai H, Menezes-Souza J, Guggino WB: PKB and megalin determine the survival or death of renal proximal tubule cells. *Proc Natl Acad Sci U S A* 103: 18810–18815, 2006