Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells

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Chronic proteinuria appears to be a key factor in tubulointerstitial damage. Recent studies have emphasized a pathogenic role of endoplasmic reticulum (ER) stress which is induced by the accumulation of misfolded proteins in ER, extracellular stress, etc. In the present study, we investigated ER stress and ER stress-induced apoptosis in proximal tubular cells (PTCs). Immortalized rat PTCs (IRPTCs) were cultured with bovine serum albumin (BSA). The viability of IRPTCs decreased proportionately with BSA overload in a time-dependent manner. Quantitative real-time polymerase chain reaction analysis revealed that 40 mg/ml BSA increases mRNA of ER stress markers by 7.7- and 4.6-fold (glucose-regulated protein 78 (GRP78) and oxygen-regulated protein 150 (ORP150), respectively) as compared to control. The increased expression of ORP150 and GRP78 in IRPTCs with albumin overload was detected by Western blot and immunofluorescence study. These *in vitro* observations were supported by *in vivo* studies, which demonstrated that ER stress proteins were upregulated at PTCs in experimental proteinuric rats. Furthermore, increased ER stress-induced apoptosis and activation of caspase-12 were observed in IRPTCs with albumin overload and kidneys of experimental proteinuric rats. We confirmed that apoptotic cell death was attenuated by co-incubation with caspase-3 inhibitor or calpain inhibitors. These results indicate that the ER stress-induced apoptosis pathway contributed to the insult of tubular cells by proteinuria. In conclusion, renal tubular cells exposed to high protein load suffer from ER stress. ER stress may subsequently lead to tubular damage by activation of caspase-12.

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Chronic proteinuria is now considered to play an essential role in progression of tubulointerstitial damage which is considered as a final common pathway to end-stage renal disease.[1–3] In healthy human beings, a small amount of proteins filtered by the glomerulus are reabsorbed by proximal tubular cells (PTCs). However, enhanced protein traffic through the glomerular capillary, whatever the cause is, leads to an increased concentration of proteins in the proximal tubular lumen. When PTCs are exposed to excessive amounts of protein, a variety of harmful responses are initiated in PTCs, including an increased synthesis of endothelin-1,[4] RANTES (regulated upon activation normal T-cell expressed and secreted),[5] monocyte chemoattractant protein 1,[6–8] complement components,[9–13] and osteopontin.[14] Previous studies also suggested that a transcription factor such as nuclear factor-κB plays an important role in the pathogenesis of tubulointerstitial damage with proteinuria.[5,15,16] As a consequence of proteinuria, tubular cells become apoptotic, leading to eventual tubulointerstitial fibrosis.[17] Apoptotic pathways are activated by a diverse array of extrinsic and intrinsic signals. The endoplasmic reticulum (ER) regulates protein synthesis, folding and trafficking, as well as cellular responses to stress, and intracellular calcium (Ca^{2+}) levels.[18–20] Recent reports emphasize the importance of imbalance between so-called client proteins and the folding of proteins in the ER as a cause of apoptosis.[21] A threat to equilibrium between the load of nascent client proteins synthesized and the capacity of ER is referred to as ER stress. Alterations in Ca^{2+} homeostasis and accumulation of unfolded proteins in the ER cause ER stress.[22,23] Caspase-12 plays an essential role in the ER stress-induced apoptosis pathway.[21] Activation of caspase-12 by ER stress is mediated by calpain,[24] which is activated by Ca^{2+} elevation. The evidence that ER stress is pathogenic in certain neurodegenerative diseases[25] and renal injury[26,27] is accumulating. Furthermore, a variety of toxic insults, including that induced by Ca^{2+} ionophores, inhibitors of glycosylation, chemical toxins, and oxidative stress, all can cause ER stress and ultimately lead to cell death. However, a role of ER stress in proteinuria-induced tubular cell death remains unknown. The present study was designed

to delineate how ER stress induces apoptosis in PTCs exposed to protein overload.

RESULTS

Albumin overload decreased cell viability of cultured PTCs

In order to mimic the *in vivo* conditions of PTCs exposed to proteinuria, we employed albumin overload. To evaluate that albumin overload damages immortalized rat PTCs (IRPTCs), we performed lactate dehydrogenase (LDH) release assays to evaluate cell viability after exposure to high albumin concentrations. Indeed, cell viability was decreased in a dose-dependent manner when cells were incubated with bovine serum albumin (BSA) (Figure 1a). Subsequently, IRPTCs were incubated with 40 mg/ml BSA for various incubation times. The ratio of LDH release increased in a time-dependent manner (Figure 1b). Similar results were observed by utilizing albumin derived from human serum instead of BSA (data not shown).

Real-time quantitative PCR analysis revealed ER stress in PTC treated with albumin

To determine whether albumin overload induces ER stress in IRPTCs, we evaluated changes in mRNA levels of representative ER stress proteins using quantitative real-time polymerase chain reaction (PCR). Upregulation of mRNAs of two of these proteins, oxygen-regulated protein (ORP150) and glucose-regulated protein (GRP78), occurred in a time-dependent manner (Figure 2). IRPTCs were also incubated with various concentrations of albumin (0, 10, 20, 40 mg/ml) for 24 h, and a significant increase in GRP78 and ORP150 mRNA was observed in cells exposed to 40 mg/ml BSA for 24 h (7.7 ± 0.92-fold ($P < 0.005$) and 4.6 ± 1.2-fold increase ($P < 0.05$), respectively) as compared to cells cultured in serum-free media at the same time point (data of other doses are not shown).

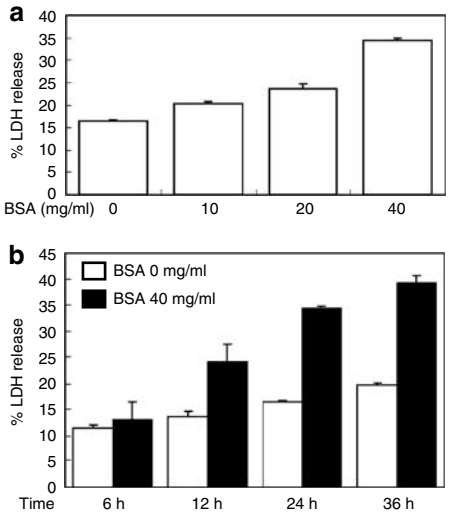

Figure 1 | The viability of IRPTCs was dependent on concentrations of BSA and incubation time. (**a**) IRPTC was incubated with 0, 10, 20, 40 mg/ml BSA for 24 h. Dead cells were significantly increased in a dose-dependent manner. (**b**) IRPTC was incubated with or without 40 mg/ml BSA for 6, 12, 24, 36 h. Dead cells were increased in a time-dependent manner.

Evaluation of protein expression of ORP150 and GRP78

We evaluated the expression of the representative ER stress proteins at the protein level (Figure 3a–d). Immunofluorescence studies revealed constitutive GRP78 expression in control cells (Figure 3a), which was increased by albumin exposure (Figure 3b). Baseline expression of ORP150 (Figure 3c) was also increased by albumin overload (Figure 3d). Furthermore, the subcellular distribution of GRP78 or ORP150 was expanded by BSA exposure (Figure 3b and d). The expression of GRP78 was colocalized with that of ER resident protein, calnexin (Figure 3e). Western blot analysis followed by densitometry revealed that albumin overload significantly upregulated expression levels of GRP78 and ORP150 (Figure 3f and g).

Expression pattern of ORP150 and GRP78 in normal and PAN nephropathy model

Next, we examined ER stress in an animal model of massive proteinuria, puromycin aminonucleoside (PAN) nephropathy,[28] and compared expression of various ER stress proteins in the kidneys of these animals to those of normal control rats. As shown in Figure 4a, GRP78 was expressed in normal kidneys; immunohistochemical analysis using tubule-specific markers identified that expression of GRP78 was limited in distal tubules under baseline conditions. On the other hand, ORP150 was not detected in normal kidneys (Figure 4d). In PAN nephropathy, expression of GRP78 was not only observed in distal tubules but in proximal tubules, and the expression of ORP150 was now present in distal and proximal tubules (Figure 4g–l). These results suggest that ER stress was induced in proximal tubules in association with the massive proteinuria induced in this model.

Caspase-3 inhibitor suppressed albumin-induced cell death

To explore the pathophysiology of cell death, we examined whether apoptosis occurred in IRPTCs incubated with BSA. Staining with Hoechst33342 revealed nuclear shrinkage and condensation, which suggest that albumin overload induced

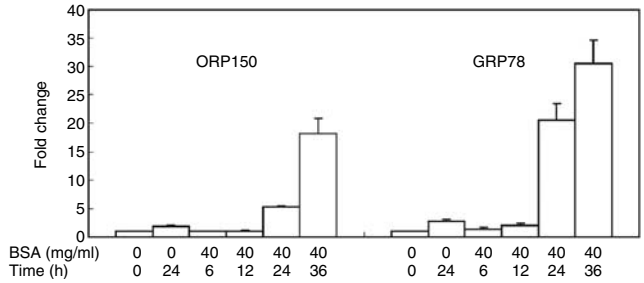

Figure 2 | mRNA expression of ORP150 and GRP78 was increased by incubation with BSA. IRPTC was incubated with 40 mg/ml BSA for 6, 12, 24, 36 h. mRNA of ORP150 and GRP78 was increased in a time-dependent manner. mRNA of GRP78 was increased 30-fold compared to normal control (0 h).