Title
Participation of Endoplasmic Reticulum Stress in the Pathogenesis of Spontaneous Glomerulosclerosis - Role of Intra-Renal Angiotensin System

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Endoplasmic reticulum (ER) is the site of synthesis, folding, assembly, and degradation of proteins. Disruption of ER function leads to ER stress, which is marked by accumulation of unfolded proteins in the ER lumen. Detection of unfolded proteins by the ER membrane receptors triggers the "unfolded protein response (UPR)" designed to restore ER function via activation of the adaptive/cytoprotective responses. Failure of UPR or persistent stress triggers activation of ER stress-mediated apoptotic pathway. Several in vivo and in vitro studies have demonstrated the association of ER stress with glomerular diseases. Imai rats develop progressive glomerulosclerosis (GS), which is associated with oxidative stress, inflammation and activation of intra-renal angiotensin system, and can be prevented by AT-1 receptor blockade (ARB). Since persistent oxidative and inflammatory stresses trigger ER stress-induced apoptosis and tissue injury, we hypothesized that kidneys in the Imai rats may exhibit failure of the adaptive and activation of the apoptotic ER stress responses, which could be prevented by ARB. To this end 10-week old Imai rats were randomized to untreated and ARB-treated groups and observed for 24 weeks. At age 34 weeks, untreated rats showed heavy proteinuria, azotemia, advanced GS, impaired ER stress adaptive/cytoprotective responses (depletion of UPR-mediating proteins), and activation of ER stress apoptotic responses. ARB treatment attenuated GS, suppressed intra-renal oxidative stress, restored ER-associated adaptive/cytoprotective system, and prevented the ER stress mediated apoptotic response in this model. Thus, progressive GS in Imai rats is accompanied by activation of ER stress-associated apoptosis, which can be prevented by ARB. (Translational Research 2012; -:1–10)

Abbreviations: ER = endoplasmic reticulum; UPR = unfolded protein response; GS = glomerulosclerosis; AT-1 = angiotensin II receptor type 1; ARB = angiotensin receptor-1 blockade; GRP94 = glucose-regulated protein 94; GRP78 = glucose-regulated protein 78, GRP78 also known as BiP; PERK = double stranded RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase; ATF6 = activating transcription factor 6; IRE1 = inositol-requiring enzyme 1; eIF2α = eukaryotic translation initiation factor-2α; XBP1 = X-box-binding protein-1; Ask1 = apoptosis-signal-regulating kinase-1; NFXB = nuclear factor kappa B; Bcl2 = B-cell lymphoma2 family of proteins; FSGS = focal segmental glomerulosclerosis; Nrf2 = nuclear factor-erythroid-2-related factor 2; BAX = B-cell lymphoma 22 family of proteins; NOSRATOLA D. VAZIRI
AT A GLANCE COMMENTARY

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Background

Progressive glomerulosclerosis (GS) is associated with oxidative stress, inflammation, and activation of intra-renal angiotensin system in Imai rats, and can be prevented by AT-1 receptor blockade (ARB). Since persistent oxidative and inflammatory stresses trigger endoplasmic reticulum (ER) stress-induced apoptosis and tissue injury, we hypothesized that kidneys in the Imai rats may exhibit failure of the adaptive and activation of the apoptotic ER stress responses that could be prevented by ARB.

Translational Significance

Restoration of ER-associated adaptive/cytoprotective system and prevention of ER stress mediated apoptotic responses reveals another salutary effect of ARB treatment in progressive GS.

The endoplasmic reticulum (ER) consists of a membranous network that is contiguous with the nuclear envelope and extends throughout the cytoplasm. It serves as the principal site of synthesis, folding, assembly, and degradation of secreted membrane-bound, and certain organelle-targeted proteins, production of steroids, cholesterol, and other lipids and the major intracellular reservoir of calcium. Newly synthesized proteins are released into the lumen of ER wherein ER-resident enzymes and chaperones mediate their covalent modification and correct folded conformation. In the ER lumen, peptidyl-prolyl isomerase catalyzes protein folding, glycosidases and mannosidases mediate protein glycosylation and classical chaperones such as glucose-regulated proteins (GRP) 94 and GRP78 (BiP), and lectin-like chaperones, such as calnexin and calreticulin, maintain their proper folding states.

Proper functioning of the ER is critical for the cell function and survival. Conditions that disrupt ER function result in ER stress, which is marked by accumulation and aggregation of unfolded proteins in the ER lumen. Accumulation of the unfolded proteins is detected by ER membrane receptors, which trigger an adaptive/cytoprotective response termed “unfolded protein response (UPR)” to restore normal ER function and cell survival via transmission of signals to the nucleus and cytoplasm. The UPR represents a concerted and complex cellular response mediated by 3 ER membrane receptors including double stranded RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). At resting condition, these ER stress receptors are held in an inactive state by the ER chaperone, GRP78. Accumulation of unfolded proteins triggers the UPR by promoting dissociation and activation of these receptors from GRP78. Once released, ATF6 migrates to the Golgi apparatus where it is activated via cleavage by site-1 and site-2 proteases. It then migrates to the nucleus to promote transcription of ER chaperones and enzymes involved in protein folding, maturation, and secretion. Simultaneously, PERK is activated via its homodimerization and transphosphorylation. This allows PERK to phosphorylate the eukaryotic translation initiation factor-2α subunit (eIF2α), which by lowering the initiation AUG codon recognition helps to slow the translation rate, thereby reducing the protein load on the damaged ER. Finally, IRE1 undergoes autophosphorylation and activation of its endoribonuclease activity, which by cleaving X-box-binding protein-1 (XBP1) mRNA and changing its reading frame, yields a potent transcriptional activator. Spliced XBP1, in turn, works via transmission of signals to the nucleus to promote transcription of ER enzymes and chaperones (Fig 1).

The UPR is an adaptive/cytoprotective response designed to reduce accumulation of unfolded proteins and restore ER function and cell survival. However, failure of UPR and/or persistence of stress trigger the activation of the ER stress-induced apoptotic responses. Several apoptotic mediators have been recognized in relation to the ER stress. They include apoptosis-signal-regulating kinase-1 (Ask1), nuclear factor kappa B (NFκB), IRE1, and B-cell lymphoma 2 family of protein (Bcl2) (Fig 1). There is increasing evidence for the role of ER stress in the pathogenesis of diverse illnesses including kidney diseases. ER stress is present in glomerular cells from the animal models of membranous nephropathy and membranoproliferative glomerulonephritis. Development of proteinuria in animals with
puromycin aminonucleoside-induced nephrosis is associated with upregulation of podocyte GRP78. In addition induction of familial focal segmental glomerulosclerosis (FSGS) in the mice (by expression of α-actinin-4K256E transgene in podocytes) results in expression of ER stress markers and proapoptotic proteins. Moreover, markers of ER stress have been identified in the renal biopsy specimens from patients with various inflammatory and noninflammatory glomerulopathies. These studies, among others, point to the roles of ER stress in the pathophysiology of kidney disease.

The Imai rats develop heavy proteinuria, hyperlipidemia, and progressive FSGS at 6 to 8 weeks of age culminating in end-stage renal disease and death by age 8 to 9 months. Imai rats were originally derived from the mating of a male Sprague-Dawley rat that exhibited spontaneous renal disease and hyperlipidemia while consuming a low-fat diet. In an earlier study, we found that progression of renal disease in this model is accompanied by activation of intra-renal angiotensin system, oxidative stress, inflammation, and impaired activation of nuclear factor-erythroid-2-related factor 2 (Nrf2), which is the master regulator of genes encoding...
many antioxidant and phase II detoxifying enzymes. Long-term angiotensin receptor-1 blockade (ARB) therapy attenuated intra-renal oxidative stress and inflammation, restored Nr2 activity, and prevented nephropathy in the treated animals pointing to the role of intra-renal angiotensin system in the pathogenesis of oxidative stress, inflammation, and nephropathy in this model.22 Endoplasmic reticulum (ER) is highly susceptible to the effects of cellular redox state and as such persistent oxidative stress can interfere with the ER function and trigger ER stress. The present study was conducted to test the hypothesis that progression of glomerulosclerosis in this model may be associated with the failure of the ER-stress-mediated adaptive response and activation of ER stress-induced apoptotic pathway. We further predicted that amelioration of nephropathy with ARB may restore ER function.

METHODS

Study groups. Male Imai and Sprague Dawley rats were obtained from Takeda Clinical Industries, (Osaka, Japan). The 10-week-old Imai rats were randomized to ARB-treated (olmesartan, 10 mg/kg/day by gastric gavage for 24 weeks) or vehicle-treated groups. The Sprague-Dawley rats served as controls. The given dosage of olmesartan (Sankyo Pharmaceutical Inc., Tokyo, Japan) was chosen based on earlier studies which had demonstrated optimal renoprotective effects of this agent in the rat.23 The animals were fed regular rat chow and water ad libitum. Arterial blood pressure was determined by tail cuff plethysmography as detailed in previous studies.24 Timed urine collections were obtained using metabolic cages. At the conclusion of the observation period, the animals were placed in metabolic cages for a 24-h urine collection that was used for measurement of creatinine and protein concentrations. They were then anesthetized with intra-peritoneal injection of pentobarital, 50 mg/kg and euthanized by exsanguinations using cardiac puncture. Kidneys were immediately harvested and stored at -70°F until processed. Blood was collected and plasma was separated and used for measurement of creatinine, urea, albumin, cholesterol, and triglyceride concentrations. Plasma creatinine, urea nitrogen, cholesterol, albumin, and triglycerides were measured by Synchro CX3 autoanalyzer (Beckman Instruments, Fullerton, Calif). Urine protein was quantified by a kit purchased from Wako Pure Chemical Industries (Tokyo, Japan). Proteinuria was determined in 24-h urine collections and creatinine clearance was calculated using standard formula.

The study protocol was approved by the Animal Care and Ethical Committee of the Saga Medical School, Saga, Japan.

Preparation of kidney homogenates and nuclear extracts. All solutions, tubes, and centrifuges were maintained at 0-4°C. The nuclear extract was prepared as described previously.25 Briefly, 100 mg of kidney cortex was homogenized using a glass-Teflon homogenizer in 0.5 mL buffer A containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM PMSF, 1 μM pepstatin, and 1 mM P-aminobenzamidine using a tissue homogenizer. Homogenates were kept on ice for 15 min and then 125 μL of a 10% Nonidet p40 (NP 40) solution was added and mixed for 15 s, and the mixture was centrifuged for 2 min at 12,000 rpm. The supernatant containing cytosolic proteins was collected. The pelleted nuclei were washed once with 200 μL of buffer A plus 25 μL of 10% NP 40, centrifuged, then suspended in 50 μL of buffer B (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 10% (v/v) glycerol), mixed for 20 min, and centrifuged for 5 min at 12,000 rpm. The supernatant containing nuclear proteins was stored at ~8°C. The protein concentrations in tissue homogenates and nuclear extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif). Target proteins in the cytoplasmic and/or nuclear fractions of the kidney tissue were measured by Western blot analysis using the following antibodies: Rabbit antibodies against rat GRP 78, PERK, Phospho-PERK (Thr980), IRE1, eIF2α, Akt, Phospho-Akt (Thr308), Bcl2, Phospho-Bcl2 (Ser70), Phospho-ASK1 (Thr845), and Bcl-2-associated X protein (BAX) were purchased from Cell Signaling (Denver, Colo). Antibodies against Nr2, NFX P65 and Histone H1 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). ASK1, cyclophilin B, GAPDH, LC3 and β-actin antibodies (Sigma-Aldrich, St. Louis, Mo), and ATF6 antibody (Abcam, San Francisco, Calif) were purchased from cited sources.

Briefly, aliquots containing 50 μg proteins were fractionated on 8 and 4% to 20% tris-glycine gel (Novex, San Diego, Calif) at 120 V for 2 h and transferred to a hybond-ECL membrane (Amersham Life Science, Arlington Heights, Ill). The membrane was incubated for 1 h in blocking buffer (1 X TBS, 0.05% Tween-20 and 5% nonfat milk) and then overnight in the same buffer containing the given antibodies. The membrane was washed 3 times for 5 min in 1 X TBS, 0.05% Tween-20 before a 2-h incubation in a buffer (1 X TBS, 0.05% Tween-20 and 3% nonfat milk) containing horseradish peroxidase-linked anti-rabbit IgG and antimouse IgG (Amersham Life Science) at 1:10,000 dilution. The membrane was washed 4 times and developed by autoradiography using the ECL chemiluminescent agents (Amersham Life Science). Beta-actin,...
GAPDH, and histone H1 were used as housekeeping proteins against which expressions of the proteins of interest were normalized.

**Data analysis.** Analysis of variance (ANOVA), multiple range tests, and regression analysis were used in statistical analysis of the data. Data are presented as mean ± SD. *P* values less than 0.05 were considered significant.

**RESULTS**

**General data.** Data are summarized in the Table. As expected, plasma creatinine, blood urea nitrogen, urine protein excretion, and arterial pressure were significantly elevated whereas creatinine clearance and plasma albumin were significantly reduced in the untreated Imai rats. These changes were associated with decreased body weight and increased kidney weight in the untreated Imai rats compared with the normal control rats. Long-term ARB administration prevented these abnormalities.

**ER stress-associated adaptive/cytoprotective system.** Data are shown in Figs. 2 and 3. Among proteins that mediate ER stress-induced adaptive/cytoprotective responses, the abundance of GRP78, PERK, ATF6, and eIF2α was significantly lower in the kidneys of the untreated Imai rats than those found in the normal control group. This was accompanied by decreased nuclear and elevated cytoplasmic abundance of Nrf2, pointing to its impaired activity in the untreated Imai rats’ kidneys. By means of upregulation of the genes encoding many antioxidants and phase II detoxifying enzymes and other molecules, activation of Nrf2 contributes to the ER stress-associated adaptive/cytoprotective responses. Several kinases including PERK and phosphorylated Akt facilitate activation and translocation of Nrf2 to the nucleus and consequent transcription of its targeted genes. In fact the abundance of PERK, phosphorylated PERK, and phosphorylated Akt was significantly reduced in the kidneys of the untreated Imai rats, a phenomenon that can in part account for the impaired activation of Nrf2.

The reduction of phosphorylated Akt in the kidneys of the untreated animals was accompanied by increased Akt abundance highlighting the defect in Akt phosphorylation. The protein abundance of cyclophilin B, which is a key factor in protein folding and serves as a major mediator of the ER stress-induced adaptive/cytoprotective response, was significantly reduced in the kidneys of the untreated Imai rats. Long-term ARB therapy prevented the above abnormalities.

**ER stress-associated apoptotic pathway and autophagy.** Data are shown in Figs. 4 and 5. ASK1, BAX, IRE1, and NFκB are among the main mediators of ER stress-induced apoptotic responses. The protein abundance of these proteins was significantly higher in the kidney tissue of untreated Imai rats than those found in the normal control group. IRE1 appears to have both prosurvival and proapoptotic properties. In early stages of ER stress, IRE1 participates in the ER stress adaptive/cytoprotective responses; but in presence of persistent ER stress, IRE1 facilitates apoptosis by promoting the synthesis and activation of apoptotic proteins such as ASK1. In fact, protein abundance of IRE1 was significantly elevated in the kidneys of the untreated Imai rats. This was accompanied by increased abundance and activation of the proapoptotic proteins, phosphorylated ASK1 and phosphorylated Bcl2.

Phosphorylation of Bcl2 via activated c-Jun N-terminal kinase (JNK), which is a downstream MAP kinase of IRE1, participates in the ER stress-induced apoptotic responses. The protein abundance of ASK1, promotes apoptosis by obviating its prosurvival activity.

ER stress has been shown to trigger autophagy in the kidney and other tissues. In fact the observed activation of the ER stress-induced apoptotic pathway in
the kidneys of our untreated Imai rats was accompanied by marked upregulation of LC3, which is a well-known marker of autophagy.

**DISCUSSION**

Progression of renal disease in the Imai rats is associated with activation of intra-renal angiotensin system, upregulation of oxidative and inflammatory cascades, and downregulation of antioxidant and cytoprotective systems leading to oxidative stress and inflammation in the renal tissue.22 Alteration of cellular redox state can profoundly affect the ER function and lead to accumulation of unfolded proteins and ER stress, which if persists, can culminate in apoptotic cell death. Oxidative stress and inflammation in the untreated Imai rat kidney was associated with the impaired ER stress adaptive/cytoprotective responses and activation of ER stress apoptotic responses. In fact, kidneys in the untreated animals showed marked reduction of PERK and phosphorylated PERK, eIF2α, ATF6, GRP78, nuclear Nrf2, phosphorylated Akt, and cyclophilin B but, significant elevation of IRE1, ASK1, phosphorylated ASK1, BAX, NFκB phosphorylated Bcl2, and LC3.

As noted earlier, ATF6 and PERK play crucial roles in mediating UPR. Activation of PERK through homodimerization and transphosphorylation enables it to phosphorylate the eIF2α and, consequently, reduce protein load on a damaged ER via lowering the general rate of translation. In addition, once activated, ATF6 promotes transcription of ER chaperones and enzymes involved in protein folding, maturation, and secretion, thereby reducing the unfolded protein load. The kidneys in the untreated Imai rats showed marked reductions of PERK, phosphorylated PERK, eIF2α, and ATF6 abundance. The reduction of these important mediators of UPR reflects the failure of the ER stress-mediated adaptive response in this model. In addition, the observed reduction of cyclophilin B, which facilitates protein folding via its peptidyl-prolyl isomerase activity, further contributes to the failure of the ER adaptive response in this model.29

In an earlier study, we found marked reduction of nuclear translocation of Nrf2 and downregulation of the antioxidant and cytoprotective byproducts of its target genes in the kidneys of Imai rats.22 In confirmation of the latter study, we found marked reduction of nuclear Nrf2 content in the untreated Imai rat kidneys. This was accompanied by significant increase in cytoplasmic Nrf2 abundance pointing to impaired activation of Nrf2 as the primary problem in this case. The impairment of the ER stress response shown here may contribute to the defective activation of Nrf2 in this model. Nrf2 is held in the cytoplasm as an inactive complex bound to the
repressor molecule, Keap1 (Kelch-like ECH-associated protein 1). Separation of Nrf2 from Keap1 is critical for its activation (nuclear translocation). This process depends on either phosphorylation of Nrf2 and/or oxidative or covalent modification of thiols in cysteine residue of Keap1.30,31 By means of phosphorylation of Nrf2, several upstream kinases including PERK can activate Nrf2 to induce production of antioxidant and cytoprotective molecules.32 This phenomenon represents an important component of adaptive/cytoprotective responses of ER stress to maintain the cellular redox homeostasis.33 Thus, the observed reduction in PERK abundance and activity may have contributed to the impaired Nrf2 activation in this model. In addition to PERK, phosphorylated Akt can activate Nrf2 and thereby participate in the ER stress-associated prosurvival response.34 Prolonged ER stress has been shown to suppress phosphorylation of Akt and thereby promote apoptosis.35 Consistent with these findings, phosphorylated Akt abundance was markedly reduced in the kidneys of our untreated Imai rats. The observed reduction of phosphorylated Akt reveals another mechanism for the impaired Nrf2 activation and the failure of the ER stress-mediated adaptive/cytoprotective responses in this model. Long-term treatment with ARB restored expression of PERK, phosphorylated PERK, phosphorylated Akt, and Nrf2 activity.

Activation of the IRE1 appears to have both prosurvival and proapoptotic properties. On the one hand, activation of its endonuclease function by ER stress results in removal of a 26-nucleotide from X binding protein 1 (XBP1) mRNA and generation of a frame shift splice variant (sXBP), which avidly induces expression of ER chaperones and P58IPK.36,37 The ER chaperones and P58IPK, in turn, contribute to normalization of cell function and survival by increasing ER protein folding and relieving protein translational block at the conclusion of ER stress. However, activation of IRE1 in the face of persistent ER stress can facilitate apoptosis by promoting the synthesis of proapoptotic proteins.38 Accordingly, the severity and duration of ER stress determines the success or failure of the ER stress response.39,40 In addition to participating in the ER stress-induced apoptosis, IRE1 has been shown to promote autophagy in the eukaryotic cells.26

The kidneys in our untreated Imai rats showed a marked increase in IRE1 abundance, which was associated with marked elevation of ASK1, phosphorylated
ASK1, phosphorylated Bcl2, and LC3. IRE1 has been shown to induce apoptosis through recruitment of ASK1. ASK1 is a mitogen activated protein kinase kinase (MAPKKK) that relays stress signals to downstream MAP kinases including JNK and p38.41,42

The role of ASK1 in mediating apoptosis is supported by the observations that overexpression of ASK1 induces apoptosis in several cell types and the neurons from ASK1-/- mice are resistant to lethal ER stress.9 It is of note that once activated, JNK can phosphorylate Bcl2 and, thereby obviate its prosurvival activity.43 In addition to promoting ASK1-mediated apoptosis, IRE1 activates NFκB, which can accentuate ER stress-associated tissue damage and inflammation.10 In fact upregulation of IRE1 in the kidneys of our untreated Imai rats was accompanied by activation of NFκB. Thus, upregulation of IRE1, ASK1, phosphorylated ASK1, and phosphorylated Bcl2 in the kidneys of the untreated Imai rats was accompanied by activation of NFκB. Upregulation of LC3 was also prevented with ARB administration. This observation points to the central role of pathologic activation of intra-renal angiotensin system in the defective ER stress response and autophagy in this model. Hypertension and proteinuria have been shown to induce ER stress.44-46 Activation of

**Fig 4.** Representative western blots and group data depicting the abundance of proteins mediating ER stress-induced apoptotic response (IRE1, ASK1, p-ASK1, p-Bcl2, BAX, and NFκB) in the control group and in the untreated Imai and ARB-treated Imai groups. n = 6 in each group. Data are presented as mean ± SD. **P < 0.01 and ***P < 0.001 vs control group.

**Fig 5.** Representative Western blots and group data depicting the abundance of LC3 protein in the control group and in the untreated Imai and ARB-treated Imai groups. n = 6 in each group. Data are presented as mean ± SD. **P < 0.01 vs control group.
intra-renal angiotensin system in the diseased kidney contributes to progression of renal disease by promoting oxidative stress, inflammation, hypertension, and proteinuria. Therefore, prevention/attenuation of oxidative stress, inflammation, hypertension, and proteinuria in our ARB-treated animals could have collectively contributed to alleviation of ER stress in this model.

In conclusion, advanced glomerulosclerosis in the Imai rats is associated with conspicuous impairment and failure of ER stress adaptive/cytoprotective responses and activation of ER stress apoptotic responses. Long-term administration of ARB preserved renal function and structure, restored normal ER function, and prevented activation of ER stress. These findings point to the important role of activation of intra-renal angiotensin system in the defective ER stress response in this model.

All authors have read the journal’s policy on disclosure of potential conflicts of interest and have no conflict of interest to declare.

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