Preconditioning with Endoplasmic Reticulum Stress Ameliorates Mesangioproliferative Glomerulonephritis

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ABSTRACT

Accumulating evidence suggests a pathophysiologic role of endoplasmic reticulum (ER) stress in kidney disease. This study investigated the potential of therapeutic approaches targeting ER stress in the anti-Thy1 model of mesangioproliferative glomerulonephritis in rats. Immunohistochemistry and Western blotting showed a time-dependent increase in the expression of the ER stress–inducible chaperones glucose-regulated protein 78 (GRP78) and oxygen-related protein 150 in isolated glomeruli, especially in the glomerular epithelial cells and mesangial cells, after induction of anti-Thy1 nephritis. For evaluation of whether preconditioning with ER stress ameliorates the severity of disease, rats were pretreated with a subnephritogenic dose of the ER stress inducer tunicamycin or thapsigargin for 4 d before disease was induced. Although preconditioning with ER stress had no effect on the degree of disease induction, it strongly ameliorated the manifestations of disease, evidenced by marked reductions in microaneurysm formation, mesangial proliferation, and adhesion of Bowman’s capsule to the glomerular tuft. This improvement in histologic damage was associated with reduced proteinuria (39.4 ± 10.5 versus 126.1 ± 18.1 mg/d; P < 0.01) and with attenuated increases in glucose-regulated protein 78 and oxygen-related protein 150 expression. Of note, pretreatment with tunicamycin or thapsigargin decreased the excessive ER stress–induced intracellular signaling observed in anti-Thy1 nephritis. In conclusion, preconditioning with ER stress ameliorates the severity of disease in rats with anti-Thy1 nephritis. These findings suggest the possibility of therapeutic approaches targeting ER stress in mesangioproliferative glomerulonephritis.


The endoplasmic reticulum (ER) fulfills multiple cellular functions, including the regulation of protein synthesis, folding and trafficking, and cellular responses to stress. Owing to its role in protein folding and transport, the ER is also rich in Ca2+-dependent molecular chaperones, such as glucose-regulated protein 78 (GRP78), GRP94, and calreticulin, which stabilize protein-folding intermediates.1,2 A wide variety of disturbances cause the accumulation of unfolded or malfolded proteins in the ER, in turn leading to ER stress.3,4 Perturbation of normal ER function induces an evolutionarily conserved cell stress response, the unfolded protein response (UPR), which is initially aimed at ameliorating the damage but can eventually trigger cell death if ER dysfunction is severe or prolonged.

The initial purpose of the UPR is to facilitate adaptation to the changing environment and re-establish normal ER function.3–7 One UPR pathway enhances protein-folding capacity by acti-
vating the transcription of UPR target genes such as ER chaperones, including GRP78. A second pathway decreases the influx of new proteins into the ER by reducing the frequency of initiation of mRNA translation. When adaptation fails, however, excessive or prolonged ER stress triggers cell suicide, usually in the form of apoptosis, representing a last resort of multicellular organisms to the dispensation of dysfunctional cells.7

GRP78, also referred to as BiP, is a central regulator of ER function.1 The N-termini of these transmembrane ER proteins are normally folded by GRP78, preventing their aggregation. When unfolded proteins accumulate in the ER, GRP78 releases these transmembrane ER proteins, allowing their aggregation and thereby launching the UPR.

The UPR pathway that regulates translation is the protein kinase R-like ER kinase (PERK) pathway. PERK is a Ser/Thr protein kinase; the catalytic domain shares substantial homology with other kinases of the eukaryotic initiation factor 2α (eIF2α) family.6 PERK phosphorylates and inactivates eIF2α, thereby shutting off mRNA translation globally and reducing the protein load on the ER.8,9

ER stress is associated with a range of diseases, including ischemia/reperfusion injury, neuronal degeneration, and diabetes.10–12 Accumulating evidence, including our previous studies, suggests a pathophysiologic role of ER stress in the kidney,13–19 the details of which remain unclear. Here, we investigated whether therapeutic approaches targeting ER stress might be effective against renal disease using a model of mesangial proliferative glomerulonephritis in rats.1

RESULTS

ER Stress Was Induced in Rats with Anti-Thy1 Nephritis

To determine whether ER stress occurs during the progression of mesangial proliferative glomerulonephritis, we assessed changes in the expression levels of GRP78, an ER stress–inducible chaperone, in the glomeruli of rats with anti-Thy1 nephritis. Immunohistochemical analysis showed that GRP78 expression was significantly increased in glomerular cells, especially glomerular epithelial and mesangial cells, in rats with anti-Thy1 nephritis compared with control rats at all time points examined (Figure 1). This finding of increased GRP78 expression in anti-Thy1 nephritis was supported by quantitative analysis using computer-assisted morphometry (Figure 2A). GRP78 expression was notably higher at day 7 after induction of anti-Thy1 nephritis, which corresponds to the stage of mesangial hypercellularity, than at day 1, the early onset of glomerulonephritis. Similar results were obtained with Western blot analysis followed by quantitative densitometry using isolated glomeruli of rats with anti-Thy1 nephritis (Figure 2B).

To confirm the induction of ER stress in anti-Thy1 nephritis, we also examined the expression of a second ER stress–inducible chaperone, oxygen-related protein 150 (ORP150). Immunohistochemical analysis using anti-ORP150 antibody also demonstrated an increase in ORP150 expression in these rats (Figures 1 and 2).

As an additional confirmation, rats that were administered an injection of an irrelevant mouse IgG, which induced neither morphologic nor functional damages, showed no changes in glomerular expression levels of GRP78 or ORP150 (Figure 1). Quantitative immunohistochemical analysis demonstrated that areas positive for ER stress–inducible chaperones in rats treated with an irrelevant mouse IgG were statistically equivalent to those in untreated control rats (1067 ± 427 versus 1062 ± 420 for GRP78 [P = 0.98]; 4664 ± 2298 versus 6133 ± 1956 pixels² for ORP150 [P = 0.28]).

Anti-Thy1 Nephritis Manifestations Were Improved by Preconditioning with ER Stress

On the basis of our observation of ER stress in rats with anti-Thy1 nephritis, we hypothesized that ER stress may contribute to the progression of mesangiproliferative glomerulonephritis. To verify this, we assessed whether preconditioning with ER stress prevented disease progression using two ER stress inducers, tunicamycin and thapsigargin, at subnephritogenic dosages.

Tunicamycin or thapsigargin was injected intraperitoneally into the rats 4 d before induction of anti-Thy1 nephritis. Al-
though the concentrations used (tunicamycin 0.3 mg/kg, thapsigargin 0.2 mg/kg) did not affect renal pathology or functions in healthy control rats (Figures 3 and 4), expression levels of GRP78 and ORP150 as estimated by computer-assisted quantitative morphometry were higher in the tunicamycin- or thapsigargin-treated rats than in nontreated rats (GRP78 2.8 ± 0.9-fold and 4.4 ± 1.8-fold, respectively; ORP150 2.6 ± 0.8-fold and 3.1 ± 0.9-fold, respectively; P < 0.001 for each; Figures 5 and 6).

It is interesting that preconditioning with ER stress produced a dramatic improvement in disease manifestations (Figure 3). At day 3 of anti-Thy1 nephritis with tunicamycin or thapsigargin pretreatment, the percentage of glomeruli displaying microaneurysms (0.5 and 1.4%, respectively) was markedly reduced compared with that in nonpretreated rats with anti-Thy1 nephritis (8.0%; P < 0.05), and subsequent mesangial proliferation was ameliorated at day 7. The adhesion of Bowman’s capsule to the tuft observed in the control rats (51.0%) was also dramatically decreased by tunicamycin or thapsigargin pretreatment (5.0 and 11.1%, respectively; P < 0.05).

To quantify these glomerular changes, we performed computer-assisted morphometry to measure glomerular size and glomerular cell number. Results confirmed that preconditioning with ER stress ameliorated these increases in glomerular size and glomerular cell number (Figure 3). Furthermore, this histologic improvement was associated with a decrease in proteinuria (Figure 4).

Importantly, the protective effect of tunicamycin or thapsigargin against the glomerular damage induced by anti-Thy1 nephritis...
antibody was associated with the modulation of expression levels of GRP78 and ORP150 (Figures 5 and 6). Although control levels were significantly increased as the disease progressed, expression was suppressed by tunicamycin or thapsigargin pretreatment and remained relatively stable throughout the experimental period.

For elimination of the possibility that the ER stress inducers directly affected the binding of anti-Thy1 antibody to mesangial cells thereby to abolish disease induction, binding of anti-Thy1 antibody to mesangial cells of rats with anti-Thy1 nephritis with or without tunicamycin or thapsigargin pretreatment was compared by immunofluorescence microscopy. Pathogenic anti-Thy1 antibody was detected in the mesangial area in rats with nephritis both with and without ER stress preconditioning at day 7, with no difference seen in binding area among groups as estimated by computer-assisted quantitative morphometry (1.0 ± 0.17, 0.92 ± 0.08, and 0.94 ± 0.1-fold positive area in nonpretreated, tunicamycin-pretreated, and thapsigargin-pretreated rats with anti-Thy1 nephritis, respectively; n = 3 for each; P > 0.05; Figure 7).

We then investigated the local effect of preconditioning with ER stress by administration of a low dosage of tunicamycin (0.06 mg/kg body wt) directly into the right kidney via the renal artery before the induction of anti-Thy1 nephritis. Local administration of tunicamycin also showed preconditioning effects, with significant suppression of the increases in glomerular size and glomerular hypercellularity induced by anti-Thy1 nephritis (glomerular size of the right [tunicamycin-treated] and left [untreated] kidney 564,071.1 ± 99,800.0 and 697,100.7 ± 68,278.0 pixels², respectively [P < 0.05]; glomerular cell number of the right [tunicamycin-treated] and left [untreated] kidney 73.3 ± 11.8 and 88.9 ± 14.8, respectively [P < 0.05]). This improvement of these manifestations tended to be consistent with the decreased number of adhesions of Bowman’s capsule to the tuft (25.2 ± 3.4 and 31.1 ± 2.3% in the right [tunicamycin-treated] and left [untreated] kidney of rats with anti-Thy1 nephritis, respectively) but did not reach statistical significance (P = 0.06).

To determine whether the protection was indeed a preconditioning phenomenon, we treated rats that had anti-Thy1 nephritis with tunicamycin after the induction of disease. Results showed no improvement in disease manifestations when tunicamycin was injected at 1 h after induction (glomerular size in

Figure 4. Improvement in anti-Thy1 nephritis by preconditioning with ER stress was associated with a decrease in proteinuria. Urine samples were collected from control rats and rats with anti-Thy1 nephritis (day 7) pretreated with or without tunicamycin or thapsigargin (n = 5 for each). The histologic improvement in anti-Thy1 nephritis by this pretreatment was associated with a significant decrease in proteinuria. *P < 0.05 versus control rats without pretreatment; #P < 0.05 versus rats with anti-Thy1 nephritis without pretreatment.

Figure 5. Preconditioning with ER stress modulated GRP78 expression induced by anti-Thy1 nephritis. Immunohistochemical analysis for detection of GRP78 (A) followed by quantitative analysis using computer-assisted morphometry (B) was performed in the kidney of nondisease control rats and rats with anti-Thy1 nephritis (at day 3 or day 7) with or without ER stress preconditioning. Preconditioning was induced by tunicamycin or thapsigargin injection 4 d before the induction of anti-Thy1 nephritis. Each renal paraffin-embedded section of five rats derived from each group was examined, and averages were expressed as means ± SD (B). Preconditioning with ER stress significantly enhanced basal GRP78 expression in the kidney of nondisease control rats and suppressed the remarkable increase in GRP78 expression observed in rats with anti-Thy1 nephritis. *P < 0.05 versus control rats without pretreatment; #P < 0.05 versus tunicamycin-pretreated rats with anti-Thy1 nephritis at day 3. Magnification, ×400.
rats with anti-Thy1 nephritis treated with and without tunicamycin and respectively $P < 0.96$; glomerular cell number in rats with anti-Thy1 nephritis treated with and without tunicamycin 100.2 ± 10.2 and 105.1 ± 16.9, respectively $[P = 0.55])$.

**ER Stress–Inducible Signal Transduction Pathway in Rats with Anti-Thy1 Nephritis Was Modulated by Preconditioning with ER Stress**

In addition to the induction of expression of ER chaperones such as GRP78, ER stress is known to induce the shutdown of protein translation for cell survival. This shutdown of protein synthesis is initiated by the activation of eIF2α kinase (PERK), which subsequently phosphorylates and inactivates eIF2α. We therefore evaluated the status of this signal transduction pathway in the glomeruli of rats with anti-Thy1 nephritis treated with and without tunicamycin and without ER stress preconditioning.

As expected, activation of PERK and subsequent phosphorylation of eIF2α were barely observed in isolated glomeruli of control rats without disease. This signal transduction pathway was enhanced by tunicamycin or thapsigargin treatment alone (Figure 8). It is interesting that activation of PERK followed by phosphorylation of eIF2α was significantly augmented in rats with anti-Thy1 nephritis at day 7, and tunicamycin or thapsigargin pretreatment led to the amelioration of further phosphorylation induced by anti-Thy1 nephritis ($P < 0.05$).

**DISCUSSION**

In this study, we showed that ER stress including the induction of ER stress–inducible chaperones and signal transduction was induced in rats with anti-Thy1 nephritis. To our knowledge, this is the first report of the involvement of ER stress in a model of mesangial injury. Previous reports by our and other groups have emphasized a pathogenic role of ER stress in glomerular and tubular epithelial injury. Immunohistochemical analysis in this study showed upregulation of the ER chaperones GRP78 and ORP150 in the glomeruli of these rats. Although the molecular mechanisms by which ER stress is evoked in anti-Thy1 nephritis remain to be elucidated, one proposed explanation is that the mesangial injury is initiated by complement activation, which may induce ER stress in mesangial cells as it also does in podocytes.

We also investigated whether preconditioning with ER stress could improve disease manifestations in anti-Thy1 nephritis. Preconditioning was first identified in ischemic diseases from the finding that brief ischemic treatment before the subsequent insult induced a state of resistance to the loss of blood supply by initiating a cascade of biochemical events, which allows for the upregulation of the cellular protective genes in the tissue. A subsequent report described a successful pharmaceutical approach to preconditioning against ischemia.
Given that overwhelming ER stress leads to eventual cell death via the activation of caspase 12, induction of ER stress as a therapeutic approach may sound paradoxical\(^2\). However, ER stress also induces the adaptive UPR, which includes the expression of ER chaperones, which aid in the folding of proteins, and shutdown of translation, which improves energy consumption efficiency. Among ER chaperones, GRP78 is a major player in the cellular protective response.\(^3\),\(^5\),\(^6\) Cells overexpressing GRP78 are resistant to conditions associated with ER stress.\(^27\) The subneprhotogenic dosages of tunicamycin and thapsigargin used for preconditioning in this study evoked the adaptive UPR without renal pathologic or functional effects in healthy control rats and ameliorated the severity of the disease manifestations of anti-Thy1 nephritis.

Both systemic and local preconditioning with ER stress decreased the extent of anti-Thy1 nephritis–induced UPR at the late stage (day 7), whereas it mildly enhanced basal UPR level in healthy control rats. This finding suggests that slight enhancement of ER stress before the insult maintains the ER function of glomerular cells and thereby modulates prolonged or excessive ER stress responses, which are pathogenic and not cellular protective. That these beneficial effects were due to pre- rather than postconditioning or other effects is emphasized by the finding that tunicamycin had no effect when given after the induction of anti-Thy1 nephritis.

Various studies support a protective role of the ER stress proteins’ induction. Overexpression of GRP78 or ORP150 in cultured tubular cells producing a mutant type of amyloid-β precursor protein suppressed the production of amyloid-β peptides, which play an important role in the pathogenesis of Alzheimer’s disease.\(^28\) Systemic overexpression of ORP150 improved insulin intolerance in a murine model of spontaneous diabetes.\(^29\) Furthermore, transgenic mice overexpressing ORP150 were more resistant to renal ischemia/reperfusion injury.\(^19\) Taken together, these studies along with our study suggest the benefits of preconditioning with ER stress, namely that preconditioning enhances the adaptive UPR to protect the cell more effectively against the pathogenic environment via the maintenance of ER function.

Recent studies using chemical compounds that target the ER stress pathway have emphasized the therapeutic potential of ER stress response augmentation. Among them, trans-4,5-dihydroxy-1,2-dithiane stimulated ER stress proteins and protected the proximal tubular epithelium against challenge with a nephrotoxic chemical.\(^30\) Screening for inhibitors of ER stress–induced neuronal death identified compounds that suppress protein phosphatases responsible for dephosphorylation of eIF2α on serine 51, thereby increasing the accumulation of phosphorylated eIF2α and providing protection from apoptosis induced by several inducers of ER stress.\(^31\) Another possible approach is the use of chemical chaperones: Sodium 4-phenylbutyrate, for example, can act as a chemical chaperone by reducing the load of proteins retained in ER and has been proved effective in models of cerebral ischemia\(^32\) and Fabry disease.\(^33\)

In conclusion, anti-Thy1 nephritis is associated with ER stress and the subsequent induction of UPR for cell survival, such as an increase in the expression of ER chaperones GRP78 and ORP150, and activation of signal transduction pathways to shutdown translation. Preconditioning with ER stress ameliorated the severity of the manifestations of anti-Thy1 nephritis.
These findings suggest the possibility of therapeutic approaches targeting ER stress in mesangioproliferative glomerulonephritis.

CONCISE METHODS

Rats with Anti-Thy1 Glomerulonephritis
All animal studies conformed to the principles of the Guide for Animal Experimentation at the University of Tokyo. Male Wistar rats (150 g; Nippon Bio-Supp. Center, Tokyo, Japan) received a single intravenous injection of mouse monoclonal IgG against rat Thy1.1 (OX-7, 1.2 mg/kg body wt), and the kidney was obtained at 1, 3, or 7 d thereafter. For collection of urine, experimental rats were housed in metabolic cages for 24 h. As a negative control, nonspecific irrelevant mouse IgG purified from the sera of Balb/c mice was used in place of OX-7.

Pretreatment with tunicamycin or thapsigargin (tunicamycin 0.3 mg/kg body wt, thapsigargin 0.2 mg/kg body; Sigma-Aldrich, St. Louis, MO) was performed by intraperitoneal injection at 4 d before injection of anti-Thy1 antibody. In some experiments, tunicamycin was given at 1 h after injection of anti-Thy1 antibody. Local administration of tunicamycin was carried out by direct injection of tunicamycin into the right kidney via the renal artery at 4 d before the induction of anti-Thy1 nephritis, as described previously.34

Renal Histology
For light microscopic analysis, kidneys were fixed in 10% neutral-buffered formaldehyde, embedded in paraffin, sectioned at a 3-μm thickness, and stained with periodic acid–Schiff. Quantitative analysis of glomerular damage was performed by computer-assisted morphometry. Each staining picture was scanned using a 3CCD camera (Olympus Optical Co., Tokyo, Japan), and the glomerular tuft areas of glomeruli were estimated in 200 randomly selected glomeruli in a blinded manner. The tuft were estimated in 200 randomly selected glomeruli in a blinded manner.

Immunochemistry

Immunohistochemistry

GRP78 and ORP150 as markers of ER stress were detected using renal tissue sections (3 μm) fixed with methyl Carnoy solution. Each section was first incubated with goat polyclonal anti-GRP78 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-ORP150 antibody35 (5 μg/ml purified IgG fraction; donated by Prof. Satoshi Ogawa) followed by biotinylated rabbit anti-goat IgG (1:800; DakoCytomation, Kyoto, Japan) or goat anti-rabbit IgG (1:200; Zymed Laboratories, South San Francisco, CA). Development was performed with peroxidase-conjugated avidin (Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan). Finally, the sections were counterstained with hematoxylin. Quantification of positive staining area for GRP78 was assessed in a blinded manner by computer-assisted morphometry using NIH ImageJ software.

Immunofluorescence Studies

For evaluation of the degree of disease induction in rats with anti-Thy1 nephritis with or without tunicamycin pretreatment, binding of the mouse anti-Thy1 antibody OX-7 to mesangial cells was detected by immunofluorescence microscopy. Renal frozen sections (5 μm) were fixed with cold acetone/methanol (1:1) at −20°C for 10 min, reacted with Alexa 546-conjugated goat anti-mouse IgG (1:100 dilution; Molecular Probes, Carlsbad, CA), and analyzed in a single setting to minimize effects as a result of variations in tissue processing or in the intensity of the ultraviolet light source. Quantification of the positive staining area in 20 glomeruli for each slide was assessed in a blinded manner by computer-assisted morphometry utilizing NIH ImageJ software.

Western Blot Analysis

Isolated glomeruli were obtained from the experimental rats using a conventional sieving method17 and homogenized in a sample buffer (0.33 M Tris–HCl [pH 6.8], 10% SDS, 36% glycerol, 5% β-mercaptoethanol, and 0.012% bromophenol blue). The obtained test samples (50 μg of proteins for each) were electrophoresed on SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ).

For detection of GRP78 protein, goat anti-GRP78 polyclonal antibody (Santa Cruz Biotechnology) was used as the first antibody and horseradish peroxidase–conjugated donkey anti-goat IgG polyclonal antibody (Santa Cruz Biotechnology) as the second. For detection of ER stress–inducible cellular signaling (e.g., phosphorylation of eIF2α or activation of eIF2α kinase 3 [PERK]), rabbit antibodies to phospho-eIF2α (Ser 51; Cell Signaling Technology, Danvers, MA), eIF2α, and PERK (Santa Cruz Biotechnology) was used as the second antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG polyclonal antibody (Bio-Rad Laboratories, Hercules, CA) as the second antibody. Rabbit anti-actin polyclonal antibody (Sigma-Aldrich) was also used as the first antibody. The bands were detected by the enhanced chemiluminescence system (Amersham Biosciences), and quantitative densitometry using NIH ImageJ software was performed.

Statistical Analyses

Data are expressed as means ± SD. ANOVA was used to evaluate the statistical significance of various differences. If the analysis detected a significant difference, Scheffe test was used to compare the results from the experimental animals.

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