



Endoplasmic Reticulum Stress Causes Renal Epithelial Cell Disjunction

Dickhout JG*, Chahal J, Matthews A, Carlisle RE, Tat V and Safaa Naiel

Department of Medicine, Division of Nephrology, McMaster University and St. Joseph's Healthcare Hamilton, Hamilton, Ontario, Canada

Abstract

Endoplasmic reticulum (ER) stress results from the accumulation of misfolded proteins in the ER. ER stress is associated with acute kidney injury (AKI) of various causes, including two of the major causes of AKI, nephrotoxic drugs and renal ischemia. AKI is a pathology affecting approximately 15% of all hospital stays. When AKI occurs, it results in both significantly increased mortality and increased length of hospital stay. If the patient survives to leave the hospital, it increases the likelihood of developing chronic kidney disease 10-fold, end stage kidney disease 3-fold, and the incidence of premature death 2-fold. An important pathological feature of AKI is the disruption of epithelial cell junctions in the proximal tubule. This loss of epithelial cell junctions disrupts cell polarity preventing the reabsorption of ultrafiltrate components, including sodium, back into the circulation. We hypothesized that ER stress causes epithelial cell disjunction in human proximal tubular cells by trapping the junctional components in the ER and preventing them from reaching the cell surface. ER stress was induced by a variety of stressors including tunicamycin, thapsigargin, and A23187, all of which differ in their mechanism of action. We found that induction of ER stress, by any means, caused epithelial cell disjunction in the renal tubular epithelium. Disjunction was determined by the translocation of β -catenin, which is both a structural component of epithelial cell adherens junctions and a molecular chaperone, from the cell periphery to the perinuclear region. Co-localization experiments found β -catenin to be trapped within the ER.

Keywords: Endoplasmic reticulum stress; Epithelial junctions; β -catenin; Acute kidney injury

Introduction

The renal tubules are comprised of the renal epithelium. These are important structures in the kidney since they act to reabsorb the majority of material in the ultrafiltrate, thereby concentrating the urine for the disposal of metabolic wastes [1]. This process is mediated by active transport of solutes, and as such, the integrity of the renal epithelial cell junctions is critical in maintaining apical-basal polarity mediating this transport [2].

The proteins of the epithelial junctions are synthesized in the endoplasmic reticulum (ER), a key intracellular organelle dedicated to the synthesis, folding, maturation and transport of nascent proteins [3]. ER stress, a feature of many forms of kidney disease, results from the accumulation of misfolded proteins in the ER, which may be due to an unfavorable local environment for protein folding, or due to mutations in a protein that interferes with its proper folding [4]. The accumulation of these unfolded proteins in the ER activate an evolutionarily conserved cellular response known as the unfolded protein response (UPR) [5]. Protein folding chaperones, such as the glucose-regulated protein 78 (GRP78), help prevent the aggregation of unfolded proteins in the ER by aiding in their folding. These molecules are referred to as molecular chaperones. When ER stress is induced by drugs that disrupt protein folding in the ER, or pathophysiological states that result in protein misfolding, molecular chaperone expression is increased by the UPR to aid in protein folding [6]. Therefore, the level of expression of these molecular chaperones can act as a marker of ER stress. Since GRP78 is a well-established molecular chaperone, we utilized it as a marker of ER stress and UPR activation in our experiments [7].

ER stress can be induced by a variety of agents differing in their mechanisms of action. For our purposes, we used thapsigargin (Tg), tunicamycin (Tm), and A23187, as these have previously been shown to induce ER stress within renal tubular epithelial cells [8,9]. Tg is a Ca^{2+} ATPase inhibitor which raises cytosolic calcium concentration by blocking the ability of the cell to pump calcium into the ER, thus causing these stores to become depleted [10]. Tm induces ER stress by

preventing N-linked glycosylation in the ER, and has both antibiotic and antiviral properties [10]. Our group and several others have shown its effects on the kidney, including up regulated ER stress response proteins, extensive tubular interstitial damage at the cortico-medullary junction, and increased apoptosis [7,11-13]. The calcium ionophore A23187 has been shown to induce ER stress by disrupting calcium homeostasis [10].

Acute kidney injury (AKI) is a disease process with no known treatment that may be caused by ER stress [14]. AKI increases patient morbidity and mortality, is commonly found in the hospital setting, and is increasing in incidence [14]. Strategies to diagnose AKI in its early stages and interventions to prevent poor patient outcomes are urgently needed [15]. A major component of AKI is acute tubular necrosis (ATN) [7], which leads to nephron loss through epithelial cell disjunction. Therefore, preservation of epithelial cell junctions may prevent, or reduce, the severity of AKI.

Disturbances in ER homeostasis prevent proteins manufactured in the ER from attaining their proper tertiary structure, and from properly functioning within the cell. Such proteins include important junctional proteins such as E-cadherin and its specific chaperone β -catenin, which interact with the actin cytoskeleton to form the adherens junctions between epithelial cells [2]. β -catenin associating with E-cadherin allows its movement from the ER to the epithelial cell membrane [16].

***Corresponding author:** Jeffrey G Dickhout, Department of Medicine, Division of Nephrology, McMaster University and St. Joseph's Healthcare Hamilton, 50 Charlton Avenue East, Hamilton, ON, L8N 4A6, Canada, Tel: 905-522-1155 ext. 35334; Fax: 905-540-6589; E-mail: jdickhou@stjosham.on.ca

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As such, ER stress induction may interfere with the formation of the E-cadherin and β -catenin junctional complex, leading to epithelial cell disjunction in the renal tubular epithelium. Conditions responsible for AKI, such as nephrotoxic agents [4,7], or ischemia [17], induce such disturbances in ER homeostasis, thus they are potentially responsible for epithelial cell loss in AKI.

Therefore, we hypothesized that the induction of ER stress causes epithelial cell disjunction in proximal tubular epithelial cells. To test this hypothesis, we examined the epithelial cell junctions in HK-2 cells before and after they were treated with known ER stress inducers Tm, Tg, and A23187. We used the change in location of β -catenin and expression of ER stress marker GRP78 to assess disjunction amongst the epithelial cells.

Method

Cell culture

HK-2 cells were used as a model of renal tubular epithelium. These cells are human proximal tubular epithelial cells that have been transformed with human papilloma virus [18]. Cell growth and culture conditions were described previously [8]. Cells were allowed to form epithelial junctions at 100% confluence before experiments were performed.

Reagents

DMSO (Sigma-Aldrich) was used to dissolve non-water soluble reagents, and as a vehicle control. Tm, Tg, and A23187 were dissolved in DMSO. These reagents and 4',6'-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Paraformaldehyde was obtained as a 4% solution in phosphate buffered saline (BioLynx Inc; Brockville, Canada) and used for fixation.

Lactate dehydrogenase (LDH) assay

Cell injury of in vitro human proximal tubule epithelial cells was evaluated by a LDH release assay (Cytotoxicity Detection Kit, Roche). Percentage cell death was calculated as

$$\% \text{ Cell Death} = \frac{(\text{LDH release experiment} - \text{LDH concentration medium})}{(\text{LDH release cell lysis} - \text{LDH concentration medium})}$$

The LDH assay was performed in a medium containing 1% fetal bovine serum. LDH release total cell lysis was determined by lysing cells using 0.1% Triton X-100 in the medium, at the end of the experiment.

Gel electrophoresis

Total cell lysates were generated in 4X SDS lysis buffer with protease inhibitor cocktail added (complete Mini; Roche; Laval, Canada) as previously [8]. To produce equal protein loading, protein levels were determined using a Bio-Rad DC Protein Assay. Electrophoresis separation was performed in a 10% SDS-PAGE reducing gel (Bio-Rad), as described previously [19].

Western blotting

Western blotting was performed using nitrocellulose membranes, and developed with chemiluminescence reagent on high sensitivity X-ray film, as described previously [20].

Fluorescence microscopy

An Olympus IX81 microscope was used for fluorescence microscopy. HK-2 cells were grown on coverslips for

immunofluorescent experiments. Cells were stained using anti-GRP78 antibodies (1:100, sc-1050; Santa Cruz Biotechnology), and anti- β -catenin antibody (1:200, no. 2677; Cell Signaling). This was followed by the addition of species-specific secondary antibodies conjugated to an Alexa dye at 488- or 594-nm excitation (1:200; Invitrogen, Molecular Probes). The DNA-specific dye DAPI was used to label the nuclei of cells. PermaFluor (Thermo Scientific) was used to mount the coverslips on microscope slides.

Statistical analysis

Statistics were performed using one-way ANOVA with post-hoc comparisons using Bonferroni's correction for multiple comparisons of the mean at a 95% confidence level. Values indicate mean \pm standard error of the mean.

Results

Cell viability and ER stress induction

The percentage of cell death induced by the various ER stress inducers was determined using a LDH assay kit. The release of the intracellular enzyme LDH into the medium was used as a quantitative measurement of cell viability. LDH assay analysis demonstrated that the doses of the various ER stress inducers resulted in a significant increase in the percentage of cell death (Tm (4.1% \pm 0.2%), Tg (3.8% \pm 0.2%), and A23187 (3.9% \pm 0.3%)), as compared to vehicle (2.3% \pm 0.1%). However, when the cell membrane was intentionally disrupted (Lysis) using 0.1% Triton X-100, there was a dramatic, and significant increase in cell death (97.0% \pm 0.8%), as determined by the LDH assay (Figure 1). Thus, although ER stress inducers produced a significant increase in cell death, the cell death represented a small percentage of the total number of cells under study.

Western blot analysis showed a significant increase in the UPR marker, GRP78, at 24 hours in response to treatment with the ER stressor Tm, indicating ER stress induction (Figure 2A). Cells treated with Tg for 24 hours showed similar results demonstrating a significant increase in expression of the ER stress marker GRP78 (Figure 2B).

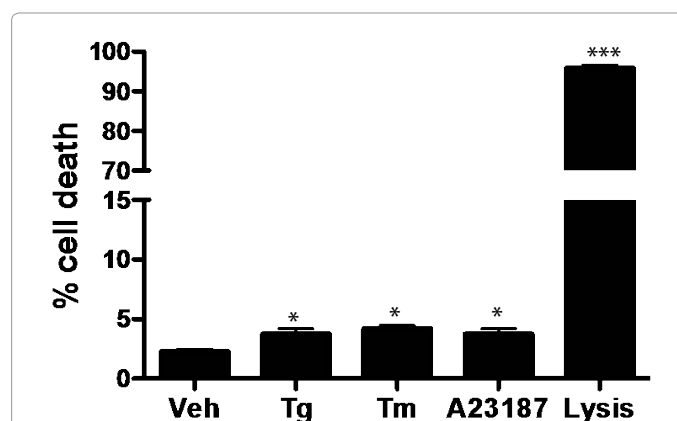
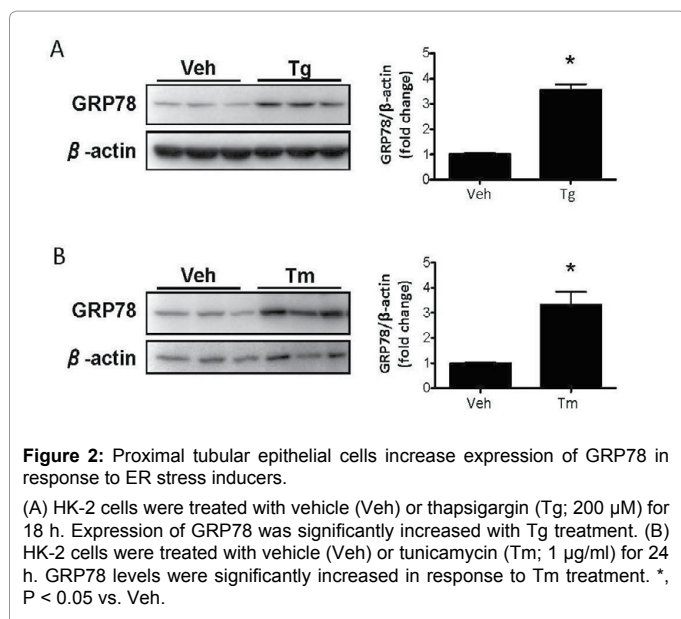


Figure 1: Percentage of cell death induced by various ER stressors.

HK-2 cells were treated with vehicle (Veh), thapsigargin (Tg), tunicamycin (Tm), A23187, and 0.1% Triton X-100 (Lysis) for 24 h. LDH release was measured as an indicator of cell toxicity. As intended, Triton X-100 caused 97% cell death. Vehicle had the least amount of cell death at 2.3%. The other ER stress inducers were slightly toxic at doses required to induce ER stress but resulted in relatively little LDH release in comparison to Lysis. Tg = 200 nM; Tm = 1 μ g/mL; A23187 = 5 μ M; Triton X-100 = 0.1% v/v. *, P < 0.05 vs. Veh. ***, P < 0.0001 vs. Veh.



ER stress disrupts renal epithelial cell junctions

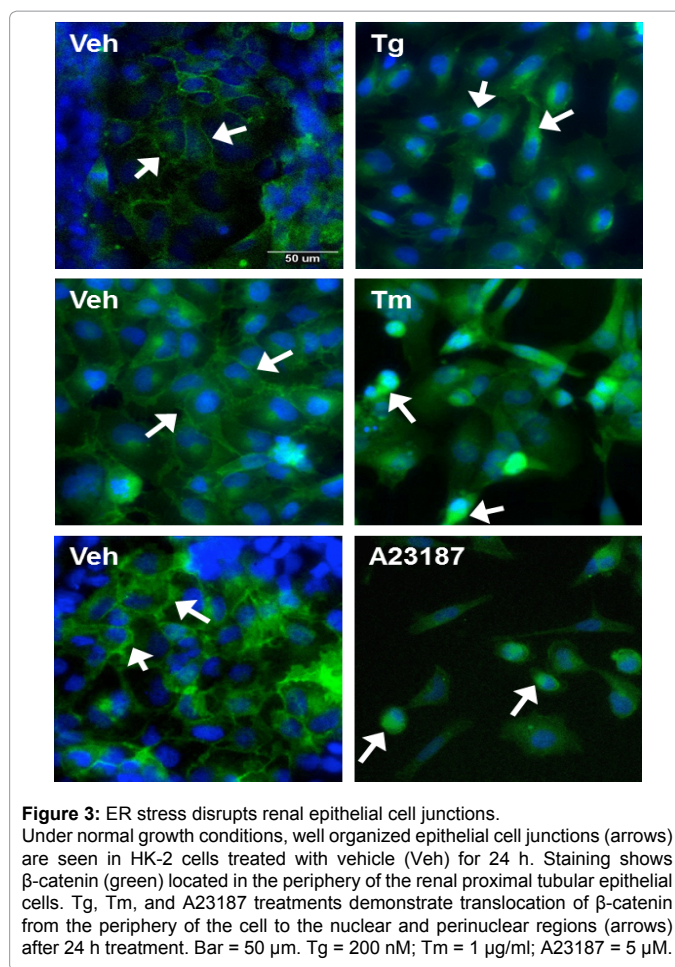
To demonstrate that ER stress causes epithelial cell disjunction, proximal tubular cells were treated for 24 hours with Tm, Tg, or A23187, and stained for β -catenin (green) and nuclei (blue). Renal proximal tubule epithelial cells treated with DMSO (vehicle) for 24 hours showed β -catenin near the periphery of the cell comprising well-organized epithelial cell junctions (Figure 3). Cells treated with Tg, Tm, or A23187 showed an accumulation of β -catenin in perinuclear region, with reduced levels of β -catenin in the periphery of the cell (Figure 3).

Co-localization of β -catenin with GRP78 in conditions of ER homeostasis and ER stress

GRP78 employs the KDEL amino acid sequence for ER retention since it is an ER luminal resident protein [21]. As such, GRP78 may be used as a physical marker of the sub-cellular location of the ER. We utilized confocal microscopy to determine the co-localization of β -catenin with GRP78 during periods of ER homeostasis and during periods of ER stress. Staining of β -catenin (green) is more abundant in the periphery of the cell with vehicle treatment in conditions of ER homeostasis (Figure 4). β -catenin is translocated to the perinuclear region of Tm-, Tg-, and A23187-treated cells, and is co-localized with GRP78 (red), producing yellow regions (Figure 4). Since the ER is located in the perinuclear region, GRP78 and β -catenin staining co-localization in the perinuclear region demonstrates the retention of this epithelial junction protein in the ER of proximal tubule cells undergoing ER stress. This appears to be the mechanism of ER stress mediated epithelial cell disjunction.

Discussion

In these studies, we demonstrated the effect of ER stress induction on epithelial cell adherens junctions. Renal proximal tubule epithelial cells undergo disjunction upon treatment with various ER stress inducers such as Tg, Tm, and A23187, all of which act through different mechanisms. Tg blocks the SERCA pump, Tm prevents the N-linked glycosylation of proteins [22], and A23187 is a calcium ionophore [23], all of which disrupt protein trafficking from the ER. Previous studies have been successful in using these agents for the purposes of inducing ER stress within cells [10].



ER stress is known to be a common feature of several renal pathologies [4,24]. The ER is an important organelle that provides a unique environment for normal protein synthesis and folding. Transmembrane, ER luminal resident, and secretory proteins are synthesized in the ER, including proximal tubular cell transporter proteins such as the Na^+ - K^+ -ATPase [4]. This protein is critical for the reabsorption of ultrafiltrate components, including sodium [25]. Other important proteins synthesized in the ER include junctional proteins such as E-cadherin and β -catenin [16], which help form adherens junctions between epithelial cells. Maintenance of the integrity of these cell-cell junctions is crucial towards proper kidney function since they help maintain epithelial cell polarity.

The accumulation of misfolded or mutant proteins results in ER stress, and activation of the UPR. The UPR is a coordinated adaptive response designed to improve cellular function and prevent cell death [26,27]. This is achieved through activation of the ER transmembrane sensors PERK, ATF6, and IRE-1 [4]. Under normal conditions, chaperone proteins such as β -catenin facilitate proper protein folding, and formation of junctional complexes between epithelial cells through their association with E-cadherin and the actin cytoskeleton [2]. During conditions of ER stress, β -catenin is released from its E-cadherin anchor in epithelial cell adherens junctions and accumulates in the cytoplasm [8,16]. Our findings demonstrate that ER stress-induced epithelial cell disjunction in renal proximal tubule cells is mediated by the retention of β -catenin in the ER. This result is consistent with a study by Ulianich et al [26], where thyroid epithelial cells exhibited loss of organized

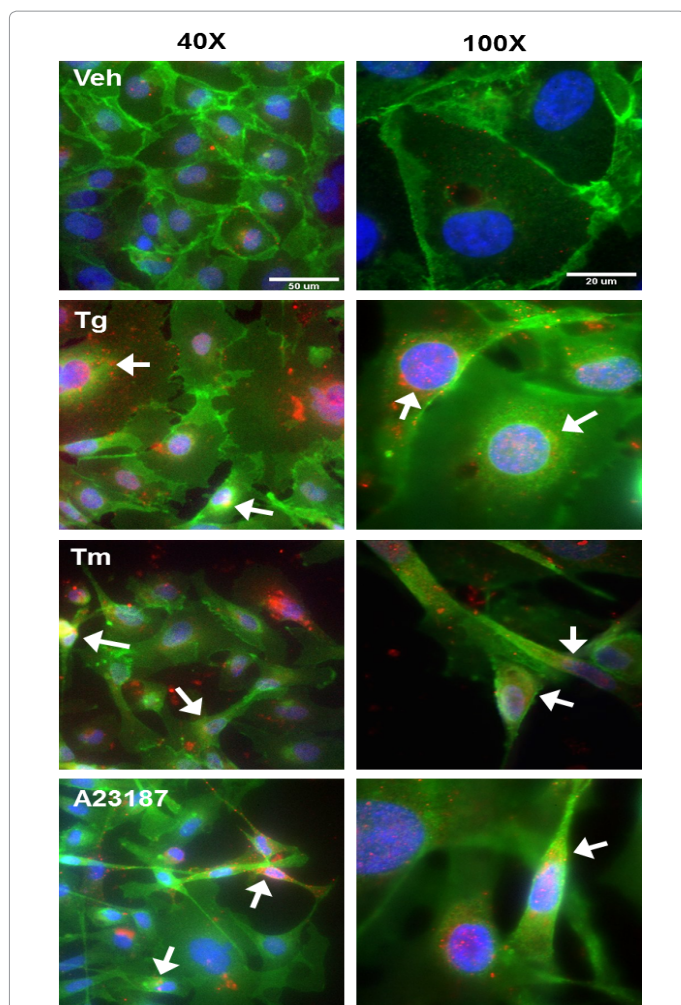


Figure 4: GRP78 expression and β -catenin co-localization in response to ER stress.

To determine the effect of ER stress on epithelial cell junctions, HK-2 cells were treated with vehicle (Veh), Tg, Tm, or A23187 for 24 h. The cells were stained for GRP78 (red), β -catenin (green) and cell nuclei with DAPI (blue). GRP78 upregulation was observed for Tg, Tm, and A23187. GRP78 and β -catenin co-localization indicate failure of junctional protein β -catenin to travel to cell periphery due to ER retention (yellow, arrows). 40X, Bar = 50 μ m. 100X, Bar = 20 μ m. Tg = 200 nM; Tm = 1 μ g/mL; A23187 = 5 μ M.

cell-cell junctions upon treatment with the ER stressors Tm, or Tg. In another study, expression of mutant surfactant protein C resulted in ER stress in type II alveolar epithelial cells. These cells were treated with Tm and showed prominent nuclear localization of β -catenin, as opposed to staining in the cell periphery, as found in vehicle-treated cells [28]. Taken together, available information suggests that disassembly of cell-cell junctions might be part of a common response by epithelial cells to significant or prolonged ER stress. Thus, the condition of ER stress results in epithelial cell disjunction, and loss of epithelial cell polarity.

We have determined, under conditions of ER stress, that β -catenin is retained in the ER. This prevents epithelial junction formation due to the loss of its function as a chaperone and its action to deliver E-cadherin to the epithelial cell membrane, which results in epithelial cell disjunction. These findings have implications in the pathology of AKI since epithelial cell disjunction and loss of cell polarity are related to a rapid and substantial decline in glomerular filtration rate associated with severe AKI. Treatments aimed at preventing epithelial

cell disjunction hold promise to alleviate the high morbidity and mortality associated with AKI.

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Disclosure

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