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Tunicamycin preserves intercellular junctions, cytoarchitecture, and cell–substratum interactions in ATP-depleted epithelial cells

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Abstract

Pretreatment with the nucleoside antibiotic tunicamycin was found to protect cultured renal epithelial cells in the face of ATP-depletion, in part by preserving junctional and cellular architecture. Tunicamycin pretreatment of Madin–Darby canine kidney cells not only preserved E-cadherin staining at the plasma membrane, but also inhibited ATP-depletion-mediated E-cadherin degradation. Electron microscopic analysis, together with the preservation of the staining patterns of the tight junction marker ZO-1, the apical/microvillar marker gp135, and basolateral marker Na/K-ATPase suggested that tunicamycin preserved the junctional complex and the polarized epithelial cell phenotype. Tunicamycin pretreatment also prevented reductions in the filamentous actin content of the cells, as well as preserving Golgi architecture. Moreover, a quantitative measure of cell adhesion demonstrated that tunicamycin pretreatment resulted in a fivefold increase in attachment of cells to the substratum (77% versus 16%). Thus, pretreatment with tunicamycin protects polarized epithelial cells from ischemic injury through the preservation of epithelial cell architecture, intercellular junctions, and cell–substratum interactions in the setting of intracellular ATP-depletion.

Keywords: Ischemia; ATP-depletion; MDCK Cells; Tight junctions; Adherens junctions; Cytoprotection

Kidney and gut epithelia serve as physical barriers between vascular and external compartments and regulate directional transport of specific molecules across these barriers. To accomplish this, epithelial cells have evolved a highly polarized structure with discrete apical and basolateral domains which is maintained in large part by the junctional complex characteristic of polarized epithelial cells. The tight junction (TJ) and the adherens junction (AJ) are key components of the junctional complex which function to connect epithelial cells with each other and to restrict lipids and proteins into apical and basolateral membrane domains. The resulting asymmetry of protein distribution provides the electrochemical gradients that drive solute transport across the epithelial cell layer. Under pathophysiologic conditions such as organ ischemia, reperfusion or toxic injury, epithelial function is perturbed via the disruption of intracellular junctions [1–6], protein polarization [1–3], the actin-based cytoskeleton [1,7], as well as protein synthetic mechanisms in the cytosol and endoplasmic reticulum [8,9]. Consequently, the epithelial cell layer is disrupted

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and the electrochemical gradient is compromised, contributing to the loss of organ function [10].

Although the precise mechanisms involved in the generation of the ischemic epithelial cell phenotype remain poorly understood, it does correlate well with cellular ATP-depletion [5,11–13]. The addition of agents that selectively inhibit glycolysis and/or mitochondrial oxidative phosphorylation to cell culture models has been used extensively to study the effect of cellular ATP-depletion [2,4,5,7,8,14,15]. For example, this model of short-term ATP-depletion and repletion has been used to investigate the behavior of proteins comprising the adherens junction (AJ) following ATP-depletion [2,6]. ATP-depletion was found to induce distinct biochemical lesions in the AJ, including rapid internalization of E-cadherin (the transmembrane protein of the AJ) [2], as well as the selective degradation of E-cadherin and disruption of the protein–protein interactions between E-cadherin and the catenins which comprise the AJ with prolonged insult [6]. Since the generation and maintenance of polarized epithelial cells is critically dependent upon E-cadherin-mediated cell–cell contact [16–20], the data suggest that alterations in the AJ, particularly in E-cadherin and its interactions with the cytoplasmic components of the AJ (the catenins), constitute a key lesion in epithelial ischemia.

Here, we have examined both the physical and biochemical properties of polarized epithelial cells (i.e., MDCK cells) subjected to chemical anoxia (ATP-depletion) following pretreatment with tunicamycin. Pretreatment of renal epithelial cells with tunicamycin, a nucleoside antibiotic which blocks N-linked glycosylation of secretory and transmembrane proteins in the endoplasmic reticulum (ER), has been found to confer a significant survival benefit to cells subjected to ATP-depletion [15] or oxidative injury [21]. The findings of this study indicate that the improved cell survival induced by tunicamycin pretreatment has a pleiotrophic effect leading not only to preservation of AJ integrity, but also the preservation of TJ integrity, the distribution of markers of epithelial polarity and cytoskeleton structure, as well as a reduction in the perturbation of cell–substratum interactions.

Materials and methods

Reagents and chemicals. MDCK cells were purchased from American Type Tissue Collection (Rockville, MD) and maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Herndon, VA) supplemented with 5% fetal calf serum and standard antibiotics. Tunicamycin was obtained from Calbiochem (La Jolla, CA). Antimycin A was obtained from Sigma (St. Louis, MO). Antibodies were obtained from the sources listed: anti-E-cadherin (RR-1, B. Gumbiner, University of Virginia, Charlottesville, VA), anti-α-, β-, γ-catenins, and GM130 (BD Transduction Laboratories, San Diego, CA), anti-ZO-1 (D. Goodenough, Harvard University, Boston, MA), anti-Na-K-ATPase (J. Lytton, University of Calgary, Alberta, Canada), anti-gp135 (G. Ojakian, SUNY, NY), and phalloidin–TRITC (Molecular Probes, Eugene, OR). The lactate dehydrogenase (LDH) cytotoxicity assay was from Boehringer–Mannheim (Indianapolis, IN). All other reagents used were of standard analytical grade.

Pretreatment with inducers of ER molecular chaperones. Confluent MDCK monolayers were rinsed twice in Dulbecco's phosphate-buffered saline (PBS) and incubated for 12 h in DMEM in the absence or presence of 1 μM tunicamycin. At the end of this period, the cells were thoroughly rinsed with PBS and incubated for an additional 3 h in standard DMEM with 5% fetal calf serum (recovery period).

Electron microscopy. MDCK cells grown to confluence on Lab-Tek Chamber slides (Nalge Nunc, Naperville, IL) were used for the experiments. The slides were fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4) overnight at 4 °C followed by 1% OsO_4 in 0.1 M Na-cacodylate buffer, pH 7.4. Subsequently dehydration was accomplished using a graded series of ethanol solutions followed by a 5 min treatment with propylene oxide and infiltration with epoxy resin (Scipoxy 812, Energy Beam Series, Agawam, MA). A thin layer of resin was placed into each well of the slide. After polymerization at 65 °C overnight, the embedded cells were removed as a monolayer and thin sections were cut at a 90° angle. The sections were picked up on 150 mesh maxtaform grids coated with 1% parlodion and counterstained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 80 kV using a Zeiss EM10B electron microscope.

Immunocytochemistry. Confluent MDCK monolayers grown on glass coverslips or transwell filters were used for the experiments. Monolayers were washed with PBS, either fixed in 100% methanol at −80 °C (ZO-1, Na/K-ATPase, gp135) or 4% paraformaldehyde (phalloidin, GM130, and E-cadherin), and permeabilized/blocked with PBS containing 0.05% Triton X-100 and 0.75% fish gelatin. The cells were then incubated with primary antibody diluted to the appropriate concentration for 1 h at room temperature. After several washes with PBS–Triton X-100, the cells were incubated with tetramethyl-rhodamine (TRITC) or fluorescein-isothiocyanate (FITC) coupled secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature, washed several times, and mounted in gelvatol (16% polyvinyl alcohol, 40 mM Tris–HCl (pH 8) in 60% v/v glycerol). Phalloidin staining of actin microfilaments was done by incubation of 4% paraformaldehyde fixed transwell filters in 0.01 mg/ml TRITC–phalloidin for 30 min followed by several washes. The coverslips/filters were viewed using a fluorescence microscope (Nikon) or a Zeiss LSM510 laser scanning confocal microscope equipped with an argon/krypton laser and Oil-DIC objectives. The images were scanned at 1024 × 1024 pixels in multi-tracking mode alternating the excitation of FITC and rhodamine. Images were processed in Photoshop (Adobe, San Jose, CA).

LDH assay. Confluent MDCK cell monolayers were grown in 96-well titer plates and pretreated with 1 μM tunicamycin for 3, 6, or 14 h or normal DMEM for controls before recovery (3 h) and ATP-depletion (6 h). Total cellular LDH was compared to LDH released into the medium due to cell death as described before [15]. Two hundred microliters of ATP-depleting medium was added to each 96-well. Cells in one half of the plate also received 0.1% Triton X-100 for permeabilization (total LDH). After ATP-depletion, the plate was centrifuged (1000 rpm, 10 min, 4 °C) before 100 μl of each well was pipetted into a fresh 96-well plate. LDH colorimetric determination solution (Boehringer–Mannheim) was added to each well and the plate was incubated at room temperature for 10 min. LDH content was determined on a microplate reader at 490 nm. LDH release was calculated from total LDH and LDH content in the treated samples.

ATP-depletion and measurement. To deplete cellular ATP following the recovery period, cells were rinsed twice in PBS and incubated for 0–6 h at 37 °C in the absence or presence of 10 μM antimycin A in PBS

supplemented with 1.5 mM $CaCl_2$ and 2 mM $MgCl_2$ as previously described [15]. Measurements of total cellular ATP content were performed with a luciferase-based ATP determination kit (Sigma, St. Louis, MO) as described previously [4,12].

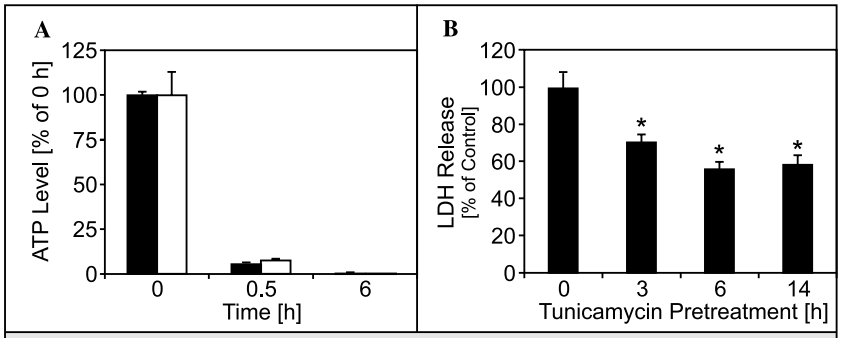
Cell–substratum adhesion assay. Confluent MDCK cell monolayers were grown in 6-well cell culture plates (Falkon, Lincoln Park, NJ). Four groups were compared: untreated monolayers, tunicamycin pretreated monolayers (1 μM, 12 h), ATP-depleted monolayers, and ATP-depleted monolayers that were also pretreated with tunicamycin. After incubation, the plates were rotated on a high frequency plate rotator (Hoefer, San Francisco, CA) for 10 min at a constant speed. After agitation the monolayers were washed with DMEM three times and each of the wells was examined by inverted phase contrast microscopy to determine the number of adherent cells. A cell was considered adherent if it was still part of the monolayer, connecting to the culture plate. The number of cells remaining on the culture plate was calculated as a percentage of the number of cells that adhered to the plate under control (untreated) conditions after agitation.

Western immunoblot analysis. Electrophoresed proteins were transferred to nitrocellulose filters (MSI, Westboro, MA) by electroblotting. Following blocking with 5% non-fat milk, primary antibodies were incubated for 2 h at room temperature. The blots were thoroughly washed with PBS/Tween (0.1%) and developed using HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) and Supersignal CL-horseradish peroxidase substrate (Pierce, Rockford, IL).

Results

In a recent study, we pretreated renal tubular cells in culture with tunicamycin, an agent thought to selectively upregulate the expression of ER chaperones, prior to subjecting the cells to ATP-depletion [15]. Confirming our prior observations [15], intracellular ATP levels in tunicamycin pretreated and untreated monolayers were similar after 30 and 360 min of ATP-depletion (5.5 ± 0.2% versus 7 ± 0.8% with tunicamycin, 30 min, Fig. 1A). ATP-depleted cells pretreated with tunicamycin demonstrated improved survival characteristics compared to those exposed to ATP-depletion without pretreatment (Fig. 1B). LDH release was significantly reduced in tunicamycin pretreated ATP-depleted monolayers (shown as a percentage of total cellular LDH) compared to non-pretreated monolayers (70.2% (3 h), 56.5% (6 h), and 58.3% (14 h) LDH release from total LDH, $n = 10$–16 samples per time point). To gain insight into the mechanism of this cytoprotection, we examined the effect of tunicamycin pretreatment followed by ATP-depletion on the structural, functional, and biochemical integrity of cell–cell interaction, cell membrane polarity, cytoskeletal architecture, and cell–substratum adhesion. Fig. 1C shows the scheme for the time course of tunicamycin pretreatment, recovery period, and ATP-depletion in this model.

A specific protein that has been studied under ATP-depletion is E-cadherin [6]. This transmembrane protein interacts with E-cadherin of adjacent epithelial cells via its extracellular domain. The interaction initiates cell–cell contact and helps catalyze the organization of epithelial cells into polarized tissues. The intracellular domain associates with cytoplasmic proteins such as catenins (α-, β-, and γ-catenin) and signaling molecules such as the heterotrimeric G-protein α subunit $G\alpha_{12}$ [22,23]. Immunocytochemical analysis of control cells demonstrated E-cadherin in the lateral membrane in a



C

Pretreatment 12 h	Recovery 3 h	ATP-Depletion 6 h	Recovery 6 h
1 mM Tunicamycin DMEM, 5% FCS	DMEM 5% FCS	10 mM Antimycin A PBS+	DMEM 5% FCS
Control DMEM, 5% FCS			

Fig. 1. (A) ATP content of ATP-depleted monolayers with (open columns) and without (closed columns) tunicamycin pretreatment. Control conditions were set as 100%. There was no statistical difference in the ATP content at 30 or 360 min of ATP-depletion ± tunicamycin pretreatment ($n = 4$ for each time point). (B) LDH release assay measuring MDCK cell death after ATP-depletion and different times of tunicamycin pretreatment. Data are expressed as means ± SEM. *Indicates statistical significance compared to control. (C) Scheme of the time course of tunicamycin pretreatment. Monolayers were incubated without (control) or with tunicamycin (1 μM) for 12 h. A recovery period of 3 h was followed by 6 h of ATP-depletion with antimycin A (10 μM) in PBS with calcium (1.5 mM) and magnesium (2 mM, PBS+).

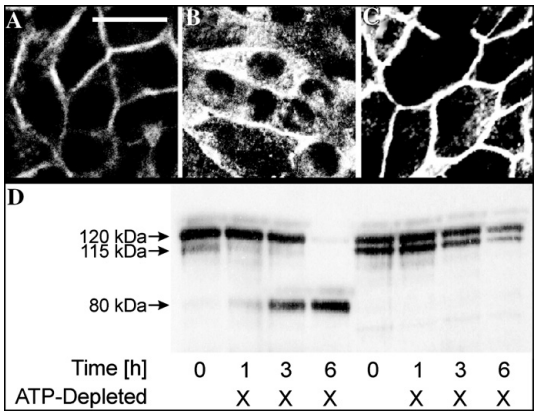


Fig. 2. (A–C) Confocal analysis of MDCK monolayers stained for the adherens junction protein E-cadherin was obtained under: (A) control conditions, (B) after 6 h of ATP-depletion, and (C) after tunicamycin pretreatment and 6 h of ATP-depletion. Bar 20 μm. (D) Western blot analysis of E-cadherin immunoreactivity in MDCK cells subjected to ATP-depletion for 0, 1, 3, and 6 h either in the absence (lanes 1–4) or presence (lanes 5–8) of tunicamycin pretreatment (1 μm, 12 h). The location of native E-cadherin (120 and 115 kDa) and its 80 kDa degradation fragment is indicated. ATP-depletion results in a progressive loss of native E-cadherin immunoreactivity (120 kDa) and the related increase of an 80 kDa degradation fragment.

continuous linear staining pattern with little evidence of internalization (Fig. 2A). After 6 h of ATP-depletion, E-cadherin staining occurred diffusely throughout most cells, suggesting cytosolic redistribution of this protein (Fig. 2B, see also [7]). Pretreatment with tunicamycin markedly reduced the loss of E-cadherin from the plasma membrane, and minimized intracellular accumulation (Fig. 2C). Western blot analysis of E-cadherin from MDCK cell lysates subjected to ATP-depletion showed a characteristic degradation pattern (Fig. 2D) [6]. Under control conditions, and after tunicamycin treatment, two E-cadherin immunoreactive bands appeared at approximately 120 and 115 kDa in molecular weight (Fig. 2D). The 115 kDa band probably corresponds to a less glycosylated variant [24]. Increasing time of ATP-depletion caused progressive loss of both mature forms of E-cadherin (115 and 120 kDa) and appearance of an 80 kDa degradation fragment (Fig. 2D). In contrast, tunicamycin pretreated cells showed minimal evidence of E-cadherin degradation after 3 h of ATP-depletion and only some loss from the 120 and 115 kDa bands after 6 h. After 6 h, this was associated with the emergence of trace amounts of the 80 kDa degradation fragment (Fig. 2D, right lane). One critical lesion in the generation of the ischemic epithelial phenotype is thought to involve the disassembly of the AJ secondary to E-cadherin degradation [6,10]. Without an intact AJ, maintenance of the TJ and other key processes such as apical–basolateral cell polarization and cell–cell interactions could fail to occur. By inhibiting ischemia-induced E-cadherin degradation, tunicamycin pretreatment may help maintain AJ integrity, preserve cellular architecture, and prevent initiation of downstream events that generate the ischemic epithelial phenotype. Stabilization of E-cadherin could also protect the functionally important association of E-cadherin with α- and β-catenin [6]. Retention of E-cadherin in the plasma membrane should result in stabilization of the AJ and preservation of the integrity of the epithelium.

In support of this notion, phase contrast microscopic examination of MDCK monolayers indicated that ATP-depletion resulted in progressive disruption of cell–cell contact, ultimately leading to cell loss (Figs. 3A–D, arrows). Tunicamycin pretreatment appeared to protect the monolayer in that, even after 6 h of ATP-depletion, no obvious cell loss was evident (Fig. 3D). The pretreated monolayers appeared similar to cells not subjected to ATP-depletion. This finding was confirmed by ultrastructural examination of the cells at the electron microscopic level. ATP-depleted MDCK cells in the absence of tunicamycin pretreatment showed striking changes along the lateral membrane (Fig. 3G) when compared to control monolayers (Figs. 3E and F). In most cell–cell junctions examined in ATP-depleted cells the apical region of the epithelial cell junction showed strong osmophilic staining with little paracellular space visible. These changes were either abolished or markedly reduced in cells pretreated with tunicamycin (Fig. 3H). Thus, not only did pretreatment with tunicamycin protect E-cadherin, it also appeared to preserve the junctional architecture of the polarized epithelial cells. Tunicamycin treatment alone (14 h) showed no obvious effect on the ultrastructure of the junctional complex (Figs. 3B and F).

Consistent with the electron microscopic changes, immunocytochemical studies of cells subjected to ATP-depletion showed a striking loss of cell–cell contact and TJ architecture (Fig. 4B) when compared with control monolayers (Fig. 4A). ZO-1 displayed characteristic separation of the tight junctions between two neighboring cells [4,5]. Also, some diffuse intracellular staining of ZO-1 was observed. Tunicamycin pretreatment preserved ZO-1 staining in MDCK cells subjected to 6 h of ATP-depletion (Fig. 4C). ZO-1 remained localized to the plasma membrane, with a continuous but jagged staining pattern and little evidence of internalization into the cell. Thus, despite severe ATP-depletion for 6 h, a time span after which cellular recovery is thought to be very unlikely [5], immunocytochemical analysis supported the EM finding that the junctional architecture was well preserved in MDCK cells pretreated with tunicamycin (Fig. 4C).

Tunicamycin pretreatment also appeared to partly preserve cellular polarity as determined by classical marker analysis of MDCK cells. The apical membrane

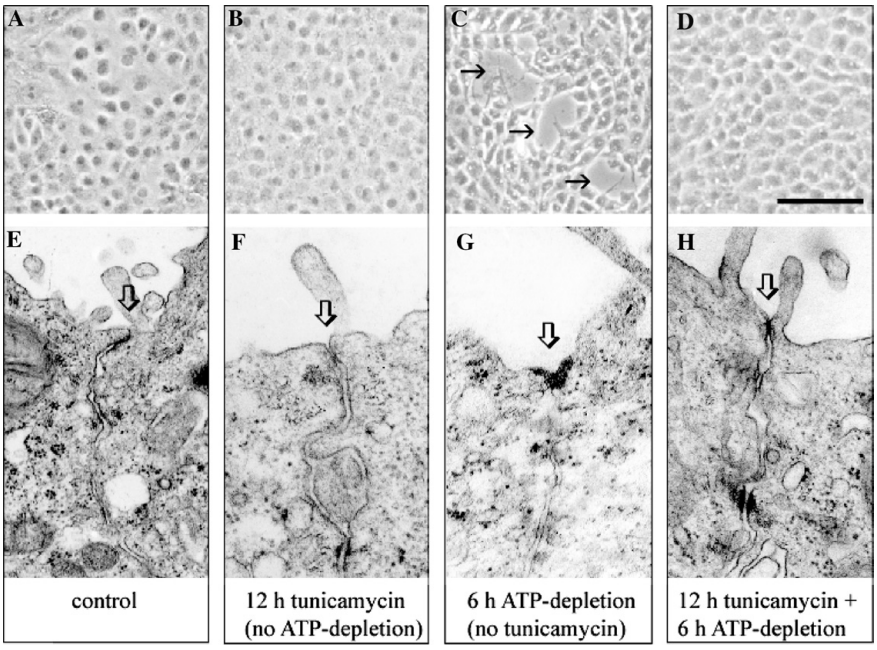


Fig. 3. Phase contrast microscopy (top panels; A–D) and electron micrographs (lower panels; E–H) of representative monolayers under the conditions of the model. Control (A,E), tunicamycin pretreated (B,F), ATP-depleted (C,G), and tunicamycin pretreated ATP-depleted (D,H) monolayers were photographed for phase contrast pictures at the end of the treatment period or fixed in modified Karnovsky's fixative for electron microscopy as detailed in 'Materials and methods.' Small arrows point to areas of cell loss, large arrows point to tight junctions. Bar, 100 μm (A, C, E, G). Electron micrograph magnification: 66,000×.

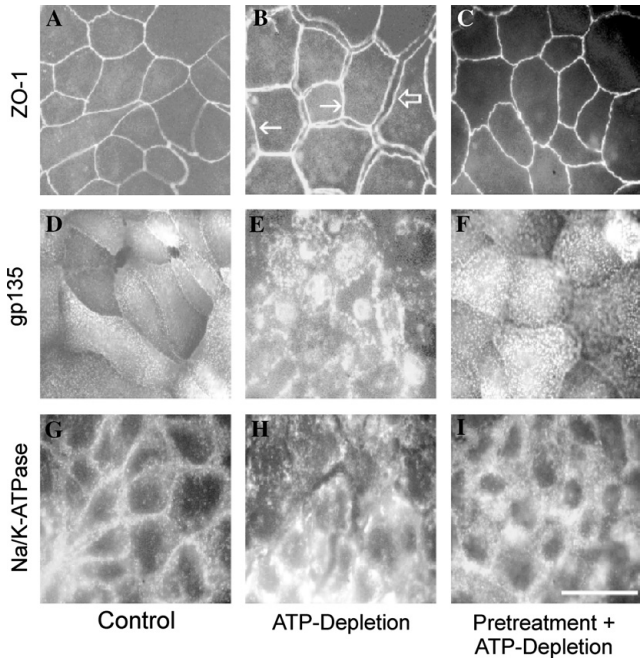


Fig. 4. Tunicamycin pretreatment partly preserves apical and basolateral protein distribution. Immunocytochemistry of representative MDCK cell monolayers on glass coverslips under the conditions of the model. Monolayers were stained for the tight junction marker ZO-1 (A–C), the microvillar protein gp135 (D–F), and basolateral Na/K-ATPase (G–I). The monolayers were untreated (control), ATP-depleted for 6 h or tunicamycin pretreated for 12 h and ATP-depleted, as indicated. Bar 20 μm. Note that cell–cell separation at the tight junction is evident in most areas (B, large arrow) whereas undisrupted ZO-1 staining can also be found (small arrows).

protein gp135 [25] and the basolaterally distributed Na/K-ATPase are useful markers of epithelial cell polarity. Immunocytochemical analysis of cells subjected to ATP-depletion demonstrated that gp135, which normally localizes to the microvillar tips of the apical membrane (Fig. 4D), was redistributed into the cell (Fig. 4E). Tunicamycin pretreatment preserved gp135 distribution in the apical membrane, although some intracellular aggregation of gp135 was evident in 15–20% of the cells (Fig. 4F). Na/K-ATPase had a basolateral staining pattern under control conditions (Fig. 4G). Similar to gp135, ATP-depletion resulted in a loss of the normal staining of Na/K-ATPase with an increase in intracellular staining (Fig. 4H). Pretreatment with tunicamycin enabled the cell to partly retain its basolateral plasma membrane Na/K-ATPase staining (Fig. 4I). Although the staining was less distinct than under control (non-ATP-depleted) conditions, it was also more intense than without tunicamycin treatment. Taken together, these findings suggested that tunicamycin pretreatment might partially protect cells from the loss of apical–basolateral polarity typically caused by ATP-depletion.

Cell–substratum interactions were examined using agitation experiments. ATP-depleted MDCK monolayers, with or without tunicamycin pretreatment, were agitated on a rotating shaker for 10 min. On gross morphological assessment, ATP-depleted monolayers showed a loss of a majority of the cells in the monolayer

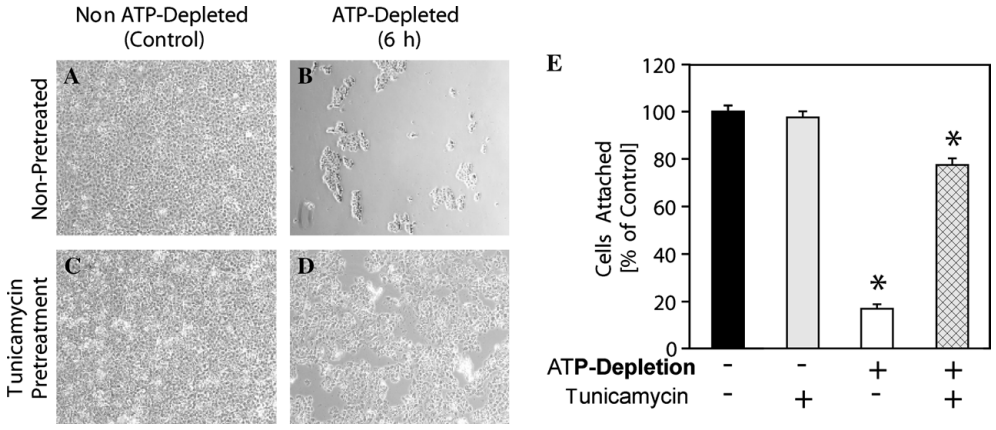


Fig. 5. Cell-substratum interactions are preserved by tunicamycin pretreatment. (A–D) Phase contrast microscopy of MDCK monolayers after 10 min of high speed rotation at 37 °C as indicated in 'Materials and methods' and treatment without (A,C) and with (B,D) ATP-depletion. Some monolayers were pretreated with 1 μM tunicamycin (C,D) while others were not (A,B). (E) Summary of the cell number attached to the culture dish after agitation. *Indicates statistical significance ($p \leqslant 0.05$).

(Fig. 5A), whereas those not exposed to ATP-depletion tolerated the treatment without significant cell loss, irrespective of tunicamycin pretreatment (Fig. 5C). Tunicamycin pretreatment, however, significantly protected MDCK cells from substratum detachment after ATP-depletion (Fig. 5D). Quantitative analysis of these images revealed that ATP-depletion led to retention of only 16 ± 2% of the cells, whereas tunicamycin pretreatment increased this value to 77 ± 4% (Fig. 5E, $n = 10$, $p \leqslant 0.05$). Tunicamycin alone, when compared to control cells, caused neither cell detachment nor increased cell adhesion (98 ± 3% versus 100 ± 2%, $n = 10$, p = not significant). These data suggest a functional stabilization of cell–substratum interaction following tunicamycin pretreatment. Tunicamycin treatment has been shown to reduce the glycosylation of integrin β1 subunits, resulting in stronger cell attachment to laminin-1 containing matrix [26]. The data presented here would appear to suggest a similar phenomenon is occurring in MDCK cells.

Others have shown that ATP-depletion leads to progressive collapse of the actin-based cytoskeleton with reduced ability to form actin stress fibers [5,27]. We assessed the effect of tunicamycin pretreatment on cytoskeletal disruption due to ATP-depletion using immunocytochemistry. Fig. 6 demonstrates that, after 6 h of ATP-depletion, there was a loss of filamentous actin staining intensity and structure (Fig. 6B) when compared to control (Fig. 6A). Only the cortical actin ring was still visible in ATP-depleted cells. Tunicamycin pretreatment lead to substantial preservation of the cytoskeletal architecture (Fig. 6C). Similarly, the Golgi matrix marker GM130 [28] showed vesicular staining in the center of the cells under control conditions (Fig. 6D) and after tunicamycin pretreatment (not shown). After 6 h of ATP-depletion, Golgi vesicular staining was found to be scattered throughout the cytosol

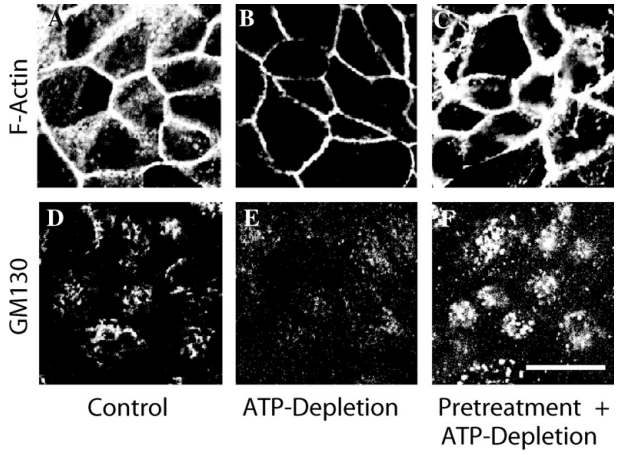


Fig. 6. Cytoskeletal markers show partial preservation of the actin cytoskeleton and the Golgi apparatus in tunicamycin pretreated cells. Confocal analysis of MDCK monolayers stained for the cytoskeletal marker F-actin (A–C), and the Golgi-marker GM130 (D–F) were obtained under control conditions, after 6 h of ATP-depletion, and after tunicamycin pretreatment and 6 h of ATP-depletion as indicated. Bar 20 μm. Note that ATP-depletion resulted in a progressive destruction of the cytoskeletal architecture of the cell, which was prevented by tunicamycin.

(Fig. 6E) consistent with earlier reports documenting disruption of Golgi structure [29]. Tunicamycin pretreatment preserved the native appearance of the Golgi apparatus despite ATP-depletion (Fig. 6F). GM130 staining after tunicamycin treatment and ATP-depletion was not identical to control (the staining appeared more centered around the nucleus), but was much less scattered than under ATP-depletion conditions. Thus, not only are junctions, cell–substratum interactions, and the cytoskeleton protected, but organelle preservation also occurs (consistent with the electron microscopy data).

Discussion

Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, induces a stress response in the endoplasmic reticulum (ER) leading to the upregulation of a number of ER resident molecular chaperones [8,30,31]. Recent computational modeling of ischemic cells suggests that ischemia induces chaotic behavior of chaperone-mediated protein folding in the cell; preinduction of chaperones is predicted to lead to more rapid restoration of protein folding dynamics [32]. It has recently been shown that tunicamycin pretreatment confers a significant survival benefit to cultured renal epithelial cells undergoing ATP-depletion [15]. The data presented here suggest that tunicamycin-mediated cytoprotection in the face of ATP-depletion involves the maintenance of cell–cell and cell–substratum interactions, as well as intracellular architecture characteristic of polarized epithelial cells.

In a recent study, we demonstrated that ATP-depletion of MDCK cells activates caspase-mediated pathways that lead to the cleavage of E-cadherin [33]. Caspase activation is central to the process of apoptotic cell death [34]. Caspase-12, which is associated with the cytosolic face of the ER, is activated by ER stress and participates in ER stress-induced apoptosis [35–40]. A recent study found that the expression of GRP78/BiP, the luminal ER homolog of the cytoplasmic molecular chaperone HSP-70, inhibits caspase activation and caspase-mediated cell death [39]. GRP78, the expression of which is upregulated by treatment with tunicamycin, was found to form an inhibitory complex with caspase-12 controlling its activation by preventing its release into the cytosol [39]. Such a complex is thought to form due to the redistribution of a subpopulation of GRP78 from the ER lumen to other cellular sites including the cytosol and ER membrane following episodes of ER stress [39]. Caspase-12 have been shown to directly cleave procaspase-9 leading to caspase-9-dependent activation of caspase-3 [39,41,42]. Interestingly, the ATP-depletion-dependent cleavage of E-cadherin is thought to be a caspase-3-mediated event [33]. This raises the possibility that in this study, a potential mechanism of cytoprotection following pretreatment with tunicamycin is due to the formation of a GRP78/caspase-12 inhibitory complex which would block the cleavage of E-cadherin by caspase-3. This in turn would lead to protection of the polarized epithelial cell phenotype due to the preservation of the adherens junction.

Another possible explanation for the observed cytoprotection following pretreatment with tunicamycin has to do with the link between E-cadherin and Bcl-2. Bcl-2 is a member of the apoptosis-regulating family of proteins which acts to increase cell survival by inhibiting apoptosis [43,44]. In recent studies the expression of Bcl-2 has been found to have an inverse relationship with the functional expression of E-cadherin [45,46]. For example, decreased expression and membrane association of E-cadherin has been found to lead to increased expression of Bcl-2 and resistance of cells to etoposide-induced apoptosis [45]. In our study, tunicamycin pretreatment was found to reduce the appearance of E-cadherin at the plasma membrane and induce the accumulation of unglycosylated E-cadherin in the ER due to its inability to move through the secretory pathway (Fig. 2). Based on the data described above, this reduction in membrane associated E-cadherin would be expected to lead to increases in the expression (or level) of Bcl-2, thus providing a resistance to ATP-depletion-mediated apoptosis.

Other tunicamycin-mediated effects that could influence ischemic epithelial cells have also been described. For example, tunicamycin has been shown to block the initiation of DNA synthesis in 3T3 cells, thereby decreasing the rate of cell entry into the S-1 growth phase and inhibiting cell proliferation [47]. Arresting cells in the G1 phase, which is characterized by limited metabolic demand, may enable cells to tolerate ischemia more favorably. ATP-depletion also induces Fas- and caspase-mediated apoptosis in MDCK cells [48]. Tunicamycin is reported to inhibit Fas-mediated apoptosis in thyroid follicular cells [49] and TNF induced apoptosis in hepatocytes [50]. Thus, inhibition or prevention of apoptosis by tunicamycin could represent another cytoprotective pathway. Also, the importance of N-linked glycosylation for normal targeting of proteins to the apical compartment of the cell has been shown under physiologic conditions [51,52]. It is possible that proteins involved in the generation of the ischemic epithelial cell phenotype also depend on N-linked glycosylation. Tunicamycin, which directly inhibits N-linked glycosylation, could therefore interfere with the functionality, targeting the half-life of such "toxic" proteins. Whether any or all of these mechanisms actually play a role in the observed cytoprotection is unclear. However, it is clear that pretreatment with tunicamycin confers a cytoprotective effect on epithelial cells subjected to ischemic insult as modeled in vitro by ATP-depletion. This effect protects epithelial cells at a fundamental level, preserving cell junctions and the cellular architecture necessary for the proper functioning of epithelial cells and constitutes a potential approach to cytoprotection.

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