Nucleotide depletion increases trafficking of gentamicin to the Golgi complex in LLC-PK₁ cells

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Sandoval, Ruben M., Robert L. Bacallao, Kenneth W. Dunn, Jeffrey D. Leiser, and Bruce A. Molitoris. Nucleotide depletion increases trafficking of gentamicin to the Golgi complex in LLC-PK₁ cells. Am J Physiol Renal Physiol 283: F1422–F1429, 2002.—Having shown rapid trafficking of aminoglycosides to the Golgi complex in cell culture, we focused on the injurious interaction that occurs when gentamicin administration is preceded by renal ischemia. Using Texas red-labeled gentamicin as a tracer, we determined that 15 min of cellular nucleotide depletion did not significantly increase subsequent uptake. However, cells previously depleted of nucleotides accumulated significantly more Texas red-labeled gentamicin within a dispersed Golgi complex. Using Ricinus communis and Lens culinaris lectins, which label specific compartments of the Golgi complex (trans-Golgi network/medialcis compartments, respectively), we determined that the medialcis compartment dispersed after 15 min of nucleotide depletion but the trans-Golgi network/trans compartment remained unaffected. An increase in the number of cells exhibiting disrupted medialcis-Golgi morphology after repletion in physiological media containing gentamicin was also seen. In summary, the increase in nephrotoxicity seen when ischemia precedes aminoglycoside uptake may be part of a complex mechanism initially involving increased Golgi accumulation and prolonged Golgi dispersion. The Golgi complex must then endure the effects of gentamicin accumulated in larger quantities in an aberrant physiological state.

ATP depletion; ischemia; nephrotoxicity; acute renal failure; proximal tubule cells; cell toxicity; aminoglycosides

Certain patient subgroups are more susceptible to the nephrotoxic effects of aminoglycoside antibiotics. Increases in the incidence and a more rapid onset of nephrotoxicity have been documented in the elderly and patients suffering from hypotension or an episode of renal ischemia, decreased cardiac output, and liver disease (4, 16, 21, 30). Animal studies that mimic these clinical factors for these higher risk subgroups, particularly renal ischemia, have demonstrated a significant increase in proximal tubule cell aminoglycoside binding and uptake and a more rapid rate of development of acute renal failure (15, 31, 35, 36).

The mechanism responsible for these observations remains to be determined. In particular, models of aminoglycoside-induced nephrotoxicity in renal proximal tubule cells have shown that the amount of aminoglycosides accumulated within the lysosomal pool, followed by subsequent lysis and release, do not correlate with observed secondary effects, such as the rapid inhibition of protein synthesis (2, 3, 23). Using a purified form of a 1:1 fluorescent conjugate of gentamicin and Texas red-labeled gentamicin (TRG) characterized in a previous study (23), we have recently demonstrated the existence of a pathway that traffics ~10–20% of internalized aminoglycosides directly and rapidly to the Golgi complex (24). This observation was made possible by utilizing techniques that quench the fluorescence of TRG emanating from lysosomes to reveal Golgi-associated fluorescence. Recent studies conducted in our laboratory that utilized transmission electron microscopy techniques have corroborated these findings in vivo with a Sprague-Dawley model (32). Localization of aminoglycoside antibiotics within the Golgi complex may represent an intermediary juncture in the trafficking of aminoglycosides via this newly described pathway. From here, it is possible that the Golgi complex may act as a hub and direct gentamicin to other organelles, validating data describing accumulation alterations induced by gentamicin therein (1, 13, 16, 32, 33). Concomitantly, gentamicin may follow retrograde transport, described for other toxins such as Shiga and ricin (12, 25, 26), with eventual accumulation within the endoplasmic reticulum (ER).

Therefore, the present study was conducted to determine whether conditions in cell culture that mimic renal ischemia (nucleotide depletion) would lead to an increase in the amount of aminoglycoside entering the Golgi complex. A more recent study has determined that antimycin A, a compound commonly used to deplete ATP levels in cells, depletes GTP levels as well; hence, the distinction in terminology used in Ref. 6.

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Employing techniques previously established in our laboratory, we found that depleting cells of cellular nucleotide levels for 15 min caused dispersion of the medial/cis compartments of the Golgi complex. However, this was insufficient to cause dispersal of the trans-Golgi network (TGN)/trans compartments (7). Subsequent aminoglycoside administration resulted in increased cellular accumulation within the Golgi complex.

Taken together, these data suggest that the synergistic nephrotoxic interactions seen when aminoglycoside administration is preceded by a brief episode of renal ischemia may involve two mechanisms. The first is the initial dispersal of specific elements within the Golgi complex, whereas the second involves an increase in accumulation of aminoglycosides within the Golgi complex.

MATERIALS AND METHODS

Experimental model. Porcine kidney proximal tubule cells (LLC-PK1, American Type Culture Collection, Rockville, MD) were grown on 18-mm-diameter coverslips (Fisher Scientific, Itasca, IL). Cells were maintained in K-P media, a 1:1 mixture of DMEM and Ham’s nutrient mixture (F-12), supplemented with 10% fetal bovine serum and 1 mg/ml penicillin-streptomycin (Sigma, St. Louis, MO). Cells were allowed to reach a state of confluence before being used in experimental protocols.

Lysosomal fluorescence quenching to reveal Golgi-associated fluorescence. TRG fluorescence emanating from lysosomes was quenched by using a previously established protocol (9, 18, 22, 24) involving horseradish peroxidase (HRP) uptake and incubation in a solution containing diaminobenzidine and H2O2 (Vector Laboratories, Burlingame, CA). Our laboratory has previously shown lysosomal quenching without alterations in cellular or subcellular morphology, enabling demonstration of Golgi-associated fluorescence (24).

Nucleotide depletion. Immediately after incubation in physiological media or media containing HRP for the lysosomal fluorescence quenching protocol, cells were thoroughly washed in PBS and incubated in substrate-depleted medium (5) containing 0.1 μM antimycin A for 15 min at 37°C. Untreated cells were instead placed in DMEM/F-12 media for 15 min under physiological growth conditions.

Golgi complex morphology after nucleotide depletion. After nucleotide depletion or maintenance under physiological conditions, LLC-PK1 cells were washed briefly in PBS and then fixed in paraformaldehyde. The cells were then permeabilized in PBS containing 0.1% Triton X-100 (Sigma) for 5 min and blocked with PBS containing 2% defatted BSA (Sigma) for 30 min. Cells were stained sequentially with an FITC-conjugated lectin from R. communis (RCA-I, Vector Laboratories) and rhodamine-conjugated lectin from Lens culinaris [L. culinaris agglutinin (LCA), Vector Laboratories] diluted to a concentration of 5–10 μg/ml in blocking buffer and a rhodamine-conjugated lectin from L. culinaris [L. culinaris agglutinin (LCA), Vector Laboratories] diluted to a concentration of 40 μg/ml in blocking buffer, resulting in labeling of the TGN/trans and medial/cis compartments of the Golgi complex, respectively. Pavelka and Ellinger (19, 20) have extensively characterized the staining pattern of the two lectins by means of electron microscopy in a variety of tissue types, including polarized intestinal epithelia.

Golgi morphology after recovery from nucleotide depletion in gentamicin-containing media. After identifying that the medial/cis compartments of the Golgi complex were susceptible to effects by nucleotide depletion (7), LLC-PK1 cells were left in physiological conditions or placed under nucleotide-depletion conditions for 15 min and allowed to recover in physiological media or physiological media containing 1 mg/ml unlabeled gentamicin (Fluka, Ronkonkoma, NY) for 30 min or 1, 2, or 4 h. They were then fixed and stained with rhodamine-conjugated lectin from L. culinaris to label the medial and cis portions of the Golgi complex. The morphology of the medial/cis compartments was scored in a blinded fashion as having a normal, moderately dispersed, or severely dispersed staining pattern. A total of 20 random fields were collected for each category, and the percentage of cells displaying the established Golgi profile for each category was tallied.

Cell viability studies after recovery from nucleotide depletion in gentamicin-containing media. Cell viability was examined with the nuclear dyes Hoechst 33342 (Molecular Probes, Eugene, OR) to label the nuclei of all cells and propidium iodide (Molecular Probes), a membrane impermeant dye that labels necrotic cells. Briefly, LLC-PK1 cells were first nucleotide depleted for 15 min and then incubated in the presence or absence of gentamicin for 4 h. Additional propidium iodide labeled untreated cells and cells placed in physiological gentamicin-containing media for 4 h. After the requisite time had elapsed, cells were placed in physiological media containing 2.5 μg/ml Hoechst and 1 mM propidium iodide for 5 min. The cells were then imaged live, and cell viability and mitotic indices were generated.

Fluorescent gentamicin uptake in quantitative/colocalization studies after nucleotide depletion. Immediately after 30–60 min of HRP uptake, cells were placed in either K-P media or nucleotide depletion media for 15 min and then placed in K-P media containing 2.0 mg/ml of a fluorescently conjugated TRG (Molecular Probes) for 60 min at 37°C, followed by cell fixation and image acquisition for quantitative studies. Other cells were processed and stained with FITC-LCA (Vector Laboratories) to label the medial/cis compartment as mentioned above. For quantitative studies, a total of 10 fields were taken for each of the four categories (total TRG fluorescence and TRG fluorescence remaining after quenching to reveal Golgi-associated fluorescence in untreated and in nucleotide-depleted cells).

Cell fixation and slide mounting. LLC-PK1 cells were briefly washed with PBS, pH 7.4, at 37°C, and then fixed in fresh 4% paraformaldehyde in PBS, pH 7.4, for 1 h at room temperature or at 4°C overnight. Permanent slides of cover-slip-grown cells were made by mounting coverslips onto a glass slide in a 1:1 solution of PBS and glycerol (Sigma), containing 1% 1,4-diazabicyclo[2.2.2]octane (Sigma) to retard photobleaching, and sealing the edges with clear nail polish.

Image acquisition. Images were acquired on a Bio-Rad (Hercules, CA) MRC-1024 combination two-photon, trans-illumination capability, Kr/Ar laser scanning confocal microscope on a Nikon Diaphot inverted microscope platform with either a ×100 oil immersion objective with a numerical aperture of 1.4 or a ×60 water immersion objective with a numerical aperture of 1.2. To avoid the possibility of spectral overlap in the colocalization studies, the signals from the TRG/rhodamine and FITC/emissions were excited and acquired sequentially with the Kr/Ar laser. For the cell viability studies, images were acquired by using the dual-photon mode at an excitation of 800 nm to localize the Hoechst 33342 and propidium iodide dyes.

Image analysis and processing. The images from the colocalization studies were processed and overlaid by using Metamorph v4.0 image-processing software (Universal Imaging, West Chester, PA). Acquired images for the quantitative studies were first processed by applying a 3 × 3 low-pass...
filter. Using the region toolbars, a 128 × 128 region of interest was selected over confluent areas and a total of four regions were selected for each of the 10 images. Values for average fluorescence intensity from each of the regions were then averaged. Background readings were taken from non-confluent areas, averaged, and subtracted.

To determine the severity of Golgi complex dispersal in nucleotide depletion studies, the Golgi complexes from cells in each field were individually scored blind as being normal, moderately dispersed, or severely dispersed. The percentage included in each category for each of the fields was calculated, and the percentages for each category from all 20 fields were averaged.

Statistical analysis. Average TRG fluorescence intensity results were analyzed using Student’s t-test. Data acquired from the Golgi morphology studies were analyzed using ANOVA comparisons utilizing the Newman-Keuls method for planned comparisons among groups. Differences having a $P$ value of $<0.05$ were considered statistically significant. All data are expressed as means ± SD.

RESULTS

The main focus of this study was to determine whether cellular nucleotide depletion altered subsequent uptake and trafficking of TRG. On the basis of total fluorescence, there was no significant difference in the initial uptake of TRG between the nucleotide-depleted (Fig. 1A) and untreated cells (Fig. 1B). However, in cells processed to reveal Golgi-associated fluorescence (see MATERIALS AND METHODS), the nucleotide-depleted cells (Fig. 1D) retained more TRG than the untreated cells (Fig. 1C). Also, the intracellular distribution of the fluorescence was different between the control and nucleotide-depleted groups. In nucleotide-depleted cells (Fig. 1, B and D), the staining was more evenly distributed throughout the entire cytosol and amorphous perinuclear structures (arrows) were not as numerous. Using Student’s $t$-test, Golgi-associated fluorescence in the nucleotide-depleted cells was found to be significantly higher than that in untreated cells (Fig. 2).

When cells under physiological and nucleotide-depleted conditions were processed to display Golgi-associated fluorescence when labeled with FITC-LCA, the remaining TRG and Golgi complex marker colocalized (Fig. 3). The nucleotide-depleted cells demonstrated more of the TRG staining (Fig. 3D) and, therefore, showed greater colocalization (yellow, Fig. 3F) within the Golgi complex (Fig. 3E). This striking observation was made more pronounced in the previously nucleotide-depleted cells by the fact that the remaining diffuse TRG signal (Fig. 3D) closely matched some of the components of the dispersed and Golgi complex (Fig. 3E). The colocalization pattern present in untreated cells (yellow, Fig. 3C) exhibited between the TRG (Fig. 3A) and the FITC-LCA (Fig. 3B) was confined around the perinuclear area in the centralized, nondispersed Golgi complex.

To investigate the effect of cellular nucleotide depletion on Golgi morphology, the Golgi complex was labeled with compartment-specific markers to determine whether the dispersion seen in the Golgi complex was restricted to certain regions of the Golgi or occurred...
globally. Nucleotide depletion induced dispersion of the medial/cis compartments (as labeled with LCA) into punctate vesicular structures that distributed diffusely throughout the cytosol (Fig. 4E, red in 4F). This differed from the staining seen in untreated cells (Fig. 4B, red in 4C), which kept their classical perinuclear, quarter-moon pattern. Curiously, the TGN/trans compartment (as labeled with RCA) in nucleotide-depleted cells was unaffected by 15 min of nucleotide depletion (Fig. 4D, green in 4F) and exhibited the same staining pattern as seen in untreated cells (Fig. 4A, green in 4C). When overlayed (Fig. 4, C and F), the two probes produce distinct staining patterns, made even more pronounced by nucleotide depletion. In both groups, a series of finger-like projections radiating from the perinuclear area were discernible.

We next investigated whether the phenomena of Golgi dispersal might play a role in the synergistic mechanisms nucleotide depletion and aminoglycosides have on nephrotoxicity. To accomplish this goal, we examined how long Golgi dispersal would persist when, after 15 min of nucleotide depletion, cells were repleted in either physiological media or physiological...
media containing gentamicin. We classified the different dispersal patterns exhibited by the medial/cis compartments of the Golgi complex as being normal (Fig. 5A), moderately dispersed (Fig. 5B), or severely dispersed (Fig. 5C). The cells from all 20 fields for each time course and the presence or absence of gentamicin in the repletion media were scored and graphed, respectively (Fig. 5, D and E). Under physiological conditions, ~75% of all the cells exhibited normal Golgi morphology. This proportion dropped to <50% (P < 0.05 vs. control, ANOVA) immediately after 15 min of nucleotide depletion and subsequently to <25% (P < 0.05 vs. untreated and 15-min nucleotide depleted, ANOVA) during the early phases of nucleotide depletion (30 min–2 h). Finally, at 4 h of recovery, cells repleted in gentamicin-free media exhibited a significant increase in cells displaying normal Golgi morphology (P < 0.05 vs. 30 min and 1 and 2 h) but not to control levels (P < 0.05). Cells repleted in gentamicin-containing media displayed no significant improvement in morphology. To assure the differences observed at 4 h were the result of the prolonged exposure to gentamicin and not due to the possible effects of necrosis or apoptosis, cell viability studies were conducted. In all groups, cell viability was at 100% as determined by exclusion of propidium iodide. Of close to 3,200 cells counted in all the groups, only 1 cell in the nucleotide depleted/recovery in the normal media group was positive for necrosis. Similarly, nuclear morphology as delineated by Hoechst staining failed to localize small, condensed nuclear aggregates associated with apoptosis. Changes in mitotic indices were found. Untreated cells had an index of ~4%, while depleted cells recovered in normal media, depleted

Fig. 4. Effects of nucleotide depletion on the medial/cis and trans-Golgi network (TGN/trans compartments. LLC-PK₁ cells were placed in either physiological media or substrate-depleted media with 0.1 μM antimycin A for 15 min and then fixed and sequentially stained with FITC-RCA-I and rhodamine-LCA. Although the same perinuclear area with each cell was stained with both lectins (A and B, D and E), distinct staining patterns were produced that were apparent in the color overlays (C and F). In general, the RCA-I (TGN/trans marker) produced a much more compact, cisternal staining pattern, with finger-like projections (A and green in C). The LCA (medial/cis marker) produced a denser staining pattern, with thinner cisternae and numerous vesicles in close proximity (B and red in C), which given the specific nature of the stain, may be part of the ER-Golgi intermediate compartment. Nucleotide depletion (D–F) had no apparent effect on the TGN/trans compartment (D and green in F) but caused dispersion of the medial/cis compartment (E and red in F) throughout the cytosol, with some retention of centralized accumulation of vesicles around the perinuclear area. An additional profile of the severity of dispersion in this compartment can be seen in Fig. 5, A–C. Bar = 10 μm.
cells recovered in gentamicin, and gentamicin-only treated cells had indices of 0.5, 2, and 2%, respectively.

**DISCUSSION**

Previous studies have described a synergistic interaction that expedites the onset and increases the severity of aminoglycoside-induced nephrotoxicity when mild renal ischemia precedes antibiotic administration (14, 15, 31, 35, 36). Although the most consistent feature among these investigations is the enhanced surface binding and internalization of aminoglycosides, the complete mechanism behind this observation remains unknown. To best characterize the mechanism, we chose to continue our studies in culture cells. Specifically, we wanted to determine the potential role of the direct Golgi pathway. This newly described pathway demonstrating rapid and direct trafficking of aminoglycosides to the Golgi complex in both cultured cells and animal models (24, 32) was suspect, because alterations induced in either aminoglycoside trafficking to the Golgi complex or to the Golgi complex itself could be of functional significance.
In this study, we demonstrate a statistically significant increase in trafficking of TRG to the Golgi complex in cells previously depleted of nucleotides. When nucleotide-depleted cells were allowed to internalize TRG and were then processed to reveal Golgi-associated fluorescence, the resulting colocalization pattern is distinct. The localized signal from the TRG remained in vesicular structures that colocalized with some of the vesicles from the now fragmented Golgi complex. This pattern was more striking than those seen in cells under physiological conditions that retained the normal Golgi complex morphology. This finding suggests an aberration in the mechanism that shuttles TRG to the now dispersed Golgi complex.

The positive results obtained from our colocalization studies with the medial/cis compartment marker prompted us to more closely examine one of the most striking finds, the dispersal of the Golgi complex after nucleotide depletion. The specificity of lectins as selective Golgi compartment markers has been extensively characterized by Pavelka and Ellinger (19, 20) using electron microscopy techniques. The differential staining pattern obtained in our studies confirms these observations. Moreover, the work done by del Valle et al. (7) supports our observation of preferential dispersion of the medial/cis compartment from short episodes of nucleotide depletion. This group reported that much longer time points, in excess of 45 min, were still insufficient to cause dispersion of the TGN/trans compartment. What role selective dispersal of the medial/cis, but not the TGN/trans, compartment at this level of injury plays on this synergistic nephrotoxic mechanism remains to be determined (7). Curiously, the medial/cis compartment, the one susceptible to dispersion by nucleotide depletion, showed almost perfect colocalization with the accumulated TRG. Moreover, our studies demonstrated that nucleotide repletion only exacerbated the insult, as more cells exhibited disrupted Golgi morphology than was observed immediately after 15 min of nucleotide depletion. This observation persisted over a period of several hours and was made worse when recovery after depletion occurred in media containing gentamicin. This setting has clinical relevance when one considers that at-risk patients would have a longer clearance time for the drug because of reduced renal function and would have a higher circulating concentration, perhaps enough to mimic conditions in this experimental model. This study and our laboratory’s other work (24) have focused on the aspect of rapid trafficking of gentamicin to the Golgi complex. Adhering to this criteria, we chose to terminate our repletion study at 4 h, concerned that at longer time points we may be inadvertently observing alterations due to secondary effects of gentamicin toxicity, such as a decrease in protein synthesis or rupture of lysosomes and release of gentamicin into the cytosol.

These two observations may be at the core of this pathology. Studies utilizing the fungal toxin brefeldin A to disperse the Golgi complex, by causing it to coalesce with the ER, have shown perturbation of normal Golgi function (11). Additionally, in vitro studies have shown vesicle fusion to be severely inhibited in the presence of gentamicin (10). This observation is directly applicable because unlike brefeldin A, which causes the Golgi and ER to coalesce, nucleotide depletion disperses the Golgi throughout the cytosol in discrete vesicles, which may subsequently have some difficulty fusing to reform a normal Golgi complex in the presence of gentamicin. In any event, it is clearly the trafficking of gentamicin to the Golgi complex, under physiological or after ischemic conditions, that constitutes a likely candidate for a mechanism of aminoglycoside toxicity (24). Having found no evidence of necrosis or apoptosis at the 4-h time point, the continued dispersal of the Golgi complex is likely to be a direct result of the presence of gentamicin.

Previous studies using animal models have described increased accumulation when mild renal ischemia preceded gentamicin administration (15), a finding we could not support here. This difference may be due in part to the decreased number and complexity of the apical microvilli characteristic of cultured LLC-PK1 cells. This finding fortuitously serves only to underscore and validate the role the Golgi complex plays in this nephrotoxic mechanism, because uptake of gentamicin between nucleotide-depleted and untreated cells was not significantly increased. Increased accumulation of gentamicin within this organelle directly facilitates its ability to rapidly induce alterations in protein synthesis previously described (2, 3).

In conclusion, depleting cellular nucleotides for 15 min caused dispersion of the medial/cis compartment of the Golgi complex. An increase in accumulation of gentamicin within the Golgi complex subsequently occurred. We believe that the increased Golgi trafficking of gentamicin in conjunction with the persistence of Golgi complex dispersal in the presence of gentamicin are important factors in the synergistic increase in cellular toxicity these two events generate.

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