Gentamicin traffics rapidly and directly to the Golgi complex in LLC-PK₁ cells

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Sandoval, Ruben M., Kenneth W. Dunn, and Bruce A. Molitoris. Gentamicin traffics rapidly and directly to the Golgi complex in LLC-PK₁ cells. Am J Physiol Renal Physiol 279: F884–F890, 2000.—To study the intracellular mechanisms of aminoglycoside toxicity, we used a 1:1 fluorescent conjugate of Texas Red and gentamicin (TRG) to quantify early uptake dynamics in renal epithelial (LLC-PK₁) cells. Utilizing a protocol that quenches TRG fluorescence from lysosomes, the bulk of intracellular accumulation, we determined a portion rapidly trafficked directly to the Golgi complex when identified by a FITC-conjugated lectin from Lens culinaris agglutinin (LCA). A kinetic study over 120 min on cells showing total and quenched TRG fluorescence was then carried out, and the fluorescence intensity from the images was quantified. Trafficking of TRG to the Golgi complex occurred within 15 min and accounted for ~20% of total cellular accumulation in the kinetic study. Colocalization studies using compartment-specific markers, 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl sphingosine (C6-NBD ceramide) and LCA, for the TGN trans-Golgi network, and the cis medial-Golgi compartments, respectively, determined colocalization occurred with both Golgi compartments. These data support the existence of a pathway that directly and rapidly shuttles a portion of internalized gentamicin to the Golgi complex. We believe this pathway may be responsible for the early negative effects seen on protein synthesis in renal proximal epithelia after aminoglycoside administration.

aminoglycosides; lysosomal-fluorescence quenching; nephrotoxicity

Despite recent advances in the understanding of aminoglycoside nephrotoxicity, a complete picture of the complex cellular mechanisms involved remains elusive. One area of much speculation involves the relatively short time interval between aminoglycoside administration and protein-synthesis inhibition (2, 3). The time required for cellular uptake and sequestering of aminoglycosides to lysosomes, followed by subsequent rupture, release, and association with the protein-synthetic machinery, as previously suspected, exceeds the earliest time for which protein-synthesis inhibition has been observed.

We have previously demonstrated a novel pathway for the uptake and trafficking of aminoglycoside anti-

biotics to the Golgi complex by employing a fluorescent tracer of gentamicin (15). By using protocols involving both transmission electron microscopy (TEM) and confocal microscopy, we reported a fraction of the internalized aminoglycoside pool traffics to the Golgi complex. However, due to the low fluorophore-to-conjugate ratio of the probe used in this study, we were unable to determine whether Golgi complex accumulation of gentamicin resulted from direct trafficking to the Golgi complex after endocytosis, and if so, the kinetics of accumulation.

Therefore, the present study was conducted to determine the kinetics involved in the trafficking of aminoglycosides to the Golgi complex. To investigate this phenomenon, we employed confocal microscopy to follow a highly enriched form of the tracer Texas Red and gentamicin (TRG), used and characterized extensively in our previous study (15). We also utilized a technique that quenched fluorescence emission from the lysosomal compartment, thereby revealing only the fluorescence from nonlysosomal structures (7, 11, 14). This was crucial because the intense fluorescence originating from the lysosomes overwhelms fluorescence from less intense structures, making them undetectable.

Results from the kinetic studies revealed accumulation of gentamicin within the Golgi complex occurred within 15 min of incubation. Finally, cells grown on grid coverslips were used to visualize the different populations of TRG within the same field. These different populations were colocalized to both the trans-Golgi network (TGN)/trans-compartment and cis medial-Golgi-compartment probes. The data gathered suggest the fluorescent nephrotoxin sequesters equally among the compartments of the Golgi complex. We postulate accumulation of TRG within the Golgi complex may represent an intermediary locale before eventual retrograde transport into the endoplasmic reticulum (ER), or trafficking throughout the cell from the Golgi complex.

MATERIALS AND METHODS

Experimental model. Porcine kidney proximal tubule cells (LLC-PK₁, ATCC, Rockville, MD) were grown on 18-mm-
diameter coverslips (Fisher Scientific, Itasca, IL) for quantitative uptake experiments, or 35-mm-diameter cell culture dishes with a grid coverslip attached to the bottom (MaTek, Ashland, MA) for colocalization experiments. Cells were maintained in a 1:1 mixture of DMEM and Ham’s nutrient mixture (F-12) (K-P media), supplemented with 10% fetal bovine serum and 1 mg/ml penicillin streptomycin (Sigma, St. Louis, MO). Cells were allowed to reach a state of ~40–70% confluence before being used in experimental protocols.

**Lysosomal fluorescence quenching.** Before aminoglycoside uptake, the LLC-PK1 cells in all experiments were preincubated in media containing 2 mg/ml horseradish peroxidase (HRP; Sigma) for 30–60 min under normal growth conditions. After termination of the TRG uptake protocol and fixation, but before permeabilization, some cells were incubated for 2–5 min in a solution of diaminobenzidine (DAB), without the Nickel solution as described in the peroxidase substrate kit DAB (SK-4100, Vector Laboratories, Burlingame, CA) to form an electron-dense product inside the lysosomes. This is known to prevent fluorescence from these structures (7, 11, 14).

**Quantitative TRG uptake studies.** After preincubation in HRP, cells were placed in K-P Media containing 2 mg/ml TRG (Molecular Probes, Eugene, OR) for 0, 15, 30, 60, and 120 min. Cells were then washed twice briefly in normal media and incubated in normal media for 30 min under normal growth conditions. After termination of the TRG uptake protocol and fixation, but before permeabilization, some cells were incubated for 2–5 min in a solution of diaminobenzidine (DAB), without the Nickel solution as described in the peroxidase substrate kit DAB (SK-4100, Vector Laboratories, Burlingame, CA) to form an electron-dense product inside the lysosomes. This is known to prevent fluorescence from these structures (7, 11, 14).

**Image analysis and processing.** All of the experimental protocols were repeated a total of three times to confirm initial findings. Moreover, time course studies for these experiments exhibit identical staining patterns between groups, with differences occurring only in the intensity of staining. The images from the colocalization studies were processed and overlaid by using the Kr/Ar laser in a single photon mode. In these studies, the acquisition channel not used for collecting fluorescence data was set to acquire a transillumination brightfield image of the alpha-numerical markers from the grid-etched coverslips. About four-hundred microliters of PBS were placed over the grid to prevent depression of the 35-mm dish, and an 18-mm diameter coverslip was placed over it to facilitate the acquisition of the transillumination brightfield image.

**RESULTS**

Quenching of fluorescence originating from lysosomes was a crucial step in detecting TRG emission from organelles with smaller, less intense accumulations. In Fig. 1, the dramatic decrease in TRG fluorescence can be seen between unquenched (A) and quenched (B) images. Acquisition parameters changed due to the decrease in fluorescence intensity of the quenched samples; usually, detection of these samples required a threefold increase in laser emissions. Permeabilizing the cells to allow penetration of the FITC-conjugated lectin to label the Golgi complex also caused...
a decrease in the TRG fluorescence intensity in all cell groups. Costaining of both unquenched and quenched samples produced slight differences in the staining patterns. As expected, the vast majority of the TRG (red) in the unquenched cells (Fig. 1A) was localized to vesicular structures previously shown to be lysosomes (arrowheads) and could be seen as bright-red punctate spots that did not colocalize with the Golgi complex marker. A small portion of the TRG did colocalize with the Golgi complex (arrows), appearing yellow, around the perinuclear area. In contrast, the fluorescent TRG in the lysosomal quenched cells (Fig. 1B) colocalized with the FITC-lectin Golgi complex marker (arrows), appearing yellow. Quenching of TRG lysosomal emissions also allowed us to determine how quickly amino-glycosides accumulated in the Golgi complex via colocalization with the fluorescent lectin. In Fig. 1 (C-F), both the untreated time point 0 (C) and 15 min of TRG exposure (D) showed no detectable levels of TRG accumulation. However, for the 30 (E)- and 60-min (F) time points, there was enough accumulation of TRG in the Golgi complex to be detected. For both of these time points, the remaining TRG colocalized with the Golgi complex marker.

Because permeabilizing cells to localize the Golgi complex with the FITC-conjugated lectin decreased the overall staining intensity of TRG, a study was undertaken to quantify TRG fluorescence at different points of uptake without permeabilizing the cells. The images from this study (Fig. 2, A-D, Total) exhibited a typical TRG staining pattern sequestered within punctate vesicular structures for cells showing total fluorescence.
at all time points [15 (A), 30 (B), 60 (C), and 120 min (D)] except for the 0-min time point that accumulated no TRG (data not shown). In cells processed for lysosomal fluorescence quenching (Fig. 2, A-D, quenched), the majority of the retained signal did not appear as bright punctate spots, as is typical of lysosomal accumulation. The signal was confined mainly around the perinuclear area and its staining pattern was comparable to Golgi complex morphology (Fig. 2 A-D, quenched, *). The quantitative data from this study (Fig. 3A) showed that a pool of nonlysosomal-associated TRG was detected within 15 min of exposure (A). Laser emissions needed to detect TRG accumulations for this pool were ~3 times higher than these needed to detect this same pool in lysosomal fluorescence-quenched cells with longer incubation periods. For all time points, images showing total fluorescence exhibited the typical staining pattern for TRG localized within punctate, vesicular structures. In the images showing the staining pattern in lysosomal fluorescence-quenched cells (A-D), the residual fluorescence appeared perinuclear in localization, and reminiscent of Golgi complex staining in cells (*). Bar = 20 μm.

Having determined that a portion of the gentamicin internalized rapidly, trafficked to, and sequestered within the Golgi complex, we next set out to determine within which compartment of the Golgi complex accumulation of TRG occurred. The timeline diagram in Fig. 4 graphically demonstrates the protocol used to meticulously document different distributions of TRG, accumulated after 60 min of uptake (lysosomal vs. nonlysosomal associated) within the exact same cell, as well as colocalization with the two different Golgi complex probes. A group of cells showing total TRG fluorescence in Fig. 5A (inset) was processed for lysosomal fluorescence quenching and imaged (B) along with its NBD-ceramide counterpart (D). Next, the cells were imaged after membrane permeabilization (C) and stained with FITC-LCA (E). The dramatic decrease in the fluorescence within the same cells among Fig. 5, A-C demonstrated how effectively the lysosomal fluorescence quenching protocol worked. Certain portions of the Golgi complex-accumulated TRG was associated with a detergent-insoluble component (Fig. 5, B and C, cells 1 and 4, arrows), whereas others (Fig. 5, B and C, cells 2 and 3, arrowheads) lost a majority of their accumulation on detergent extraction. In general, both detergent-soluble and -insoluble accumulations of TRG showed a high degree of colocalization with both Golgi complex probes, suggesting accumulation occurred within both the TGN/trans-Golgi portions and cis/medial portions of the Golgi complex. Only cell 1 in Fig. 5B appeared to have localized all of the Golgi complex-associated TRG exclusively with the cis/medial portion.
as can be demonstrated by the lack of association with the NBD-ceramide marker in Fig. 5D (cell 1, arrow).

**DISCUSSION**

Rapid uptake and accumulation of aminoglycosides within proximal tubule cells is responsible for the cascade of events leading to acute nephrotoxicity (1, 6, 21). Studies from multiple laboratories have shown cellular uptake occurs primarily through endocytosis after binding to acidic phospholipids and the multiligand receptor megalin (1, 9, 18, 23). Once internalized, the bulk of this pool is sequestered within the lysosomal compartment. In a previous study, we demonstrated accumulation of the aminoglycoside gentamicin in the Golgi complex within 8 h (15). Our present results indicate this accumulation occurs via direct trafficking of TRG to the Golgi complex from the surface membrane. This newly emerging pathway from the surface membrane to the Golgi complex is exciting because cellular effects secondary to aminoglycoside toxicity, such as a decrease in protein synthesis, occur quickly (8, 16, 17). These rapid intracellular effects are not likely attributable to sequestering of aminoglycosides within the lysosomal pool proceeded by subsequent rupture, release, and association with the protein-synthetic machinery because previous animal studies have demonstrated a lack of proximal tubular morphologic injury by light microscopy even after 2 days of gentamicin administration (2).

This study confirms and expands on data previously describing this new pathway of gentamicin trafficking (15). Here, we show trafficking occurs rapidly and directly to the Golgi complex and, through the use of lysosomal fluorescence quenching and image analysis, determines the sequestration kinetics of this pool. We hypothesized that gentamicin was directly targeted to the Golgi complex after endocytosis. To test this hypothesis, our first challenge was to delineate the earliest time point when gentamicin could be detected within the Golgi complex. Using a highly purified TRG fluorescent probe, with a 1:1 ratio of TRG, we were able to detect trafficking to the Golgi complex within as little as 30 min via direct colocalization with a specific Golgi complex marker. Even earlier detection was possible at 15 min in unpermeabilized cells by comparison of the staining pattern to established Golgi complex morphology. These data strongly suggest a pathway exists that directly shuttles aminoglycosides, after cellular uptake, to the Golgi complex where it can affect mechanisms such as protein sorting and protein synthesis. In our previous study, we were probably unable to detect a TRG-Golgi association at early time points because of the previous TRG probe's low fluorescence stoichiometry and the overwhelming intensity emanating from lysosomal accumulations of TRG. Detecting and quantifying dimly fluorescent objects in the presence of more intense structures are extremely problematic. Setting acquisition parameters to detect the dimmer objects generally causes oversaturation of detectors (20). The resulting images have no discernible details.
just a sea of bright white signal. Therefore, utilization of a protocol for quenching TRG fluorescence emanating from the lysosomal pool was crucial for our studies (1, 11, 14).

We next set out to determine where within the Golgi complex gentamicin trafficked. The two markers used, NBD-ceramide and FITC-LCA, have been extensively characterized and binding has been established within the TGN/Trans compartments and cis/medial compartments of the Golgi complex, respectively (12, 13). As expected, the two markers exhibited some degree of overlap in certain cytosolic regions around the nucleus; however, they also revealed distinct staining areas. Without three dimensional rendering it was difficult to determine which of the Golgi-complex markers occupied the greatest region. Accumulations of detergent-soluble and -insoluble pools of TRG seemed to accumulate equally among the two Golgi complex probes, suggesting a steady retrograde transport of the aminoglycoside through the Golgi complex occurred after 60 min of uptake. It is possible that this steady transport of TRG continues not only through the Golgi complex but may reach the ER. However, in the present study, we were unable to detect any appreciable accumulations of TRG within the ER, as fluorescence from this pool is most likely undetectable via confocal fluorescence microscopy at these short time points.

A mechanism for retrograde transport of toxins after endocytosis from the Golgi complex to the ER has been recently reported for other toxins. Both Shiga and Ricin toxins exploit this pathway, and their toxicity is dependent on accumulation within the ER and subsequent release into the cytosol via the same mechanisms that allow small glycopeptides to translocate the ER (8). The unique structure of gentamicin, an aminoglycoside composed of three O-linked aminated carbohydrate moieties, may be best suited to utilize all of the mechanisms mentioned above. Its carbohydrate component may be responsible for the retrograde transport to components throughout the Golgi complex. Whether

Fig. 5. Colocalization experiments of total and lysosomal quenched TRG fluorescence demonstrate strong colocalization between residual TRG after lysosomal fluorescence quenching and both Golgi complex probes. LLC-PK1 cells imaged during the initial portion of the colocalization protocol exhibited typical lysosomal TRG accumulation after 60 min of exposure (A). After a group of cells showing total TRG fluorescence was processed for lysosomal fluorescence quenching (inset in A), lysosomal quenching dramatically decreased fluorescence of the same field, and residual staining was primarily localized to perinuclear structures in certain cells (B, cells 1, 2, 3, and 4, arrows and arrowheads). These residual structures localized with both the TGN/trans-compartment probe NBD-ceramide (D), and the cis/medial-compartment probe FITC-LCA (E, cells 1, 2, 3, and 4, arrows and arrowheads). On membrane extraction (C), portions of the residual TRG fluorescence seen in B were washed away (cells 2 and 3, arrowheads), yet other portions remained (cells 1 and 4, arrows). As with the membrane-bound accumulations, the detergent-insoluble pool of TRG colocalized with both Golgi complex probes, but to a greater extent with the FITC-LCA (D and E, cells 1 and 4, arrows). Bar = 20 μm.
or not further retrograde movement to the ER occurs, the aminoglycoside is in a position to directly affect protein synthesis or may conceivably become expelled into the cytosol via the mechanisms that translocate small glycopeptides into the cytosol (8). Detectability of TRG, either ER associated or free within the cytosol, through fluorescence microscopy or biochemical techniques involving cell fractionation, would be extremely problematic because of its low concentrations.

In conclusion, through the use of a fluorescent probe with a markedly improved fluorescence, and a protocol that eliminates fluorescence from the lysosomal pool, we demonstrated and quantified rapid association of gentamicin with the Golgi complex consistent with direct trafficking from the surface membrane. Moreover, the use of compartment-specific markers to the Golgi complex indicated that accumulation of TRG over, the use of compartment-specific markers to the Golgi complex consistent with rapid association of gentamicin with the Golgi complex, would be extremely problematic because of its low concentrations.

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REFERENCES


