Gentamicin Inhibits Renal Protein and Phospholipid Metabolism in Rats: Implications Involving Intracellular Trafficking

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Abstract. Studies were undertaken to characterize the mechanism of aminoglycoside-induced nephrotoxicity. Early time points in gentamicin treatment (1 to 3 d) were used to investigate the development of toxic events without the complication of gross morphologic cellular alterations. Enzyme activities of cortical homogenates and brush border membrane (BBM) preparations documented little effect on specific activities or the ability to isolate representative membrane fractions. In vivo protein synthesis experiments demonstrated that gentamicin reduced cellular protein synthesis after 2 d of treatment. This inhibition increased to 50% on the third day. Total cellular protein synthesis was inhibited to the same extent as BBM protein synthesis. However, gentamicin had different effects on homogenate versus BBM phospholipids. The total phospholipid contents in cortical homogenates and BBM from treated animals were increased, compared with control animals. A significant decrease in phospholipid synthesis was observed only in homogenates from treated animals. When effects on individual phospholipids were investigated, only an increase in phosphatidylinositol levels was observed in cortical homogenates from treated rats. However, gentamicin treatment was demonstrated to increase the levels of phosphatidylinositol and phosphatidylcholine, while decreasing the level of sphingomyelin (SPH), in BBM. Incorporation of $^{32}$P into SPH, phosphatidylserine, and phosphatidylethanolamine was inhibited in cortical homogenates from gentamicin-treated animals; among BBM phospholipids, however, a significant decrease was observed only for SPH synthesis. It was concluded that inhibition of phospholipid degradation was quantitatively the major contributor to the effects of gentamicin on phospholipid metabolism. Confocal microscopic studies, using tracer amounts of fluorescently labeled gentamicin, revealed gentamicin in large, mostly basal structures. Correlative electron microscopic studies, using photo-oxidation techniques, demonstrated that these structures consisted of lysosomal, Golgi complex, and mitochondrial structures. These observations suggest retrograde trafficking of gentamicin and indicate a general mechanism of gentamicin-induced nephrotoxicity.

Aminoglycoside antibiotics are widely used in the treatment and prevention of Gram-negative bacterial infections. Their bactericidal activity is derived from their ability to bind prokaryotic ribosomes. This action blocks the ribosomal initiation complex and/or causes mistranslation (1,2). As a result, protein synthesis is inhibited or deranged, causing bacterial death.

Aminoglycoside use can be complicated by well described nephrotoxic and ototoxic effects (3–5). Complications attributable to aminoglycoside toxicity rank as one of the most common reasons for prolonging hospital stays in the developed world (6). Nephrotoxic effects are most common and develop after relatively short periods of treatment (7,8). In many cases, these effects are reversible if administration is discontinued.

Although many hypotheses have been proposed and tested (3,9,10), the precise mechanisms of aminoglycoside-induced nephrotoxicity remain unclear. Alterations in phospholipid metabolism after aminoglycoside treatment have been described by several laboratories, including our own (11–15). These changes occur rapidly and persist for extended periods, even after recovery, in the rat model (11,14–17). Inhibition of lysosomal phospholipases, subsequent accumulation of phospholipids, and formation of lysosomal myeloid bodies have been implicated as direct mechanisms of nephrotoxicity (11–13,16,18).

Accumulating evidence has also suggested aminoglycoside-induced inhibition of protein synthesis as a mechanism of nephrotoxicity. It has been demonstrated that elevated concentrations of aminoglycosides can cause mistranslation or block incorporation of amino acids by eukaryotic ribosomes in vitro (19–21). In addition, concentrations of gentamicin attained in the rat renal cortex during in vivo treatment were demonstrated to inhibit subsequent in vitro microsomal protein synthesis (19,20,22). This effect was observed before a decline in renal function or significant alterations in cellular enzyme activities and morphologic features were observed.

In large part, aminoglycoside effects on protein and phospholipid synthesis and metabolism have not been determined in completely in vivo experiments. Therefore, the purpose of the
studies described here was to determine the effect of aminoglycosides on these factors in completely in vivo experiments. Studies were performed at time points (1 to 3 d) when little or no alteration of cellular enzyme activities, morphologic features, or function was observed. Correlative confocal microscopic and electron microscopic (EM) studies were also performed and suggested a role for intracellular trafficking in aminoglycoside-induced nephrotoxicity.

Materials and Methods

Conjugation of Texas Red and Gentamicin

Conjugation of Texas Red to gentamicin was performed as we described previously (23). All steps were performed at 4°C. Texas Red (sulfonfyl chloride form; Molecular Probes, Eugene, OR), at 20 mg/ml in dimethylformamide (Sigma Chemical Co., St. Louis, MO), was added to a rapidly stirred solution of 0.1 M K$_2$CO$_3$, pH 8.5, and 5 to 10 mg/ml gentamicin (Fluka Biochemika, Ronkonkoma, NY) and was incubated for 4 to 5 h at 4°C. A gentamicin/Texas Red molar ratio of 10:1 was used, to minimize formation of multiply substituted Texas Red–gentamicin (TRG) conjugates. After conjugation, the reaction mixture was centrifuged (20 min at 2000 g at 4°C), and the supernatant was removed, placed in a 1000-D molecular mass cut-off dialysis membrane, and dialyzed extensively against 0.9% NaCl/10% glycerol/50 mmol/L Tris, pH 8.7. The final conjugate was obtained and either photo-oxidized (see below) or mounted in Gelvex and TCA precipitation, as described for filters above (homogenates were used for all experiments). They were fed standard rat chow and allowed free access to food and water during the experiments. Rats were given intraperitoneal injections of either gentamicin (gentamicin sulfate, 40 mg/ml; Elkins-Sinn, Cherry Hill, NJ), at a dose of 100 mg/kg [short-term gentamicin (STG)], or vehicle (vehicle control) for 1 to 3 d. In all cases, rats were given injections for the indicated times and euthanized the next day. The dosage was used previously demonstrated, by our laboratory and others, to induce reproducible and reversible nephrotoxicity (12,15,16,24). The times chosen ensured the occurrence of only mild toxicity (22,25). Serum creatinine levels were measured enzymatically using the BBM marker leucine aminopeptidase and the basolateral membrane marker Na$^+$/K$^+$-ATPase, as described previously (15,27,29). Marker enzymes used to determine contamination by other intracellular organelles were succinate dehydrogenase (mitochondria) and KCN-resistant NADH dehydrogenase [endoplasmic reticulum (ER)], as described previously (15,27,28).

Animal Protocol

Male Sprague-Dawley rats (200 to 250 g; Harlan, Indianapolis, IN) were used for all experiments. They were fed standard rat chow and were allowed free access to food and water during the experiments. Rats were given intraperitoneal injections of either gentamicin (gentamicin sulfate, 40 mg/ml; Elkins-Sinn, Cherry Hill, NJ), at a dose of 100 mg/kg [short-term gentamicin (STG)], or vehicle (vehicle control) for 1 to 3 d. In all cases, rats were given injections for the indicated times and euthanized the next day. The dosage used was previously demonstrated, by our laboratory and others, to induce reproducible and reversible nephrotoxicity (12,15,16,24). The times chosen ensured the occurrence of only mild toxicity (22,25). Serum creatinine levels were measured enzymatically using the BBM marker leucine aminopeptidase and the basolateral membrane marker NADH dehydrogenase [endoplasmic reticulum (ER)], as described previously (15,27,28).

TRG Studies

In these studies, tracer amounts of Texas Red–labeled gentamicin (i.e., 354 ng TRG/mg regular gentamicin, 0.04% TRG) were included in the normal gentamicin solution (see above). Rats that received this solution were treated exactly like those given gentamicin only. Only the 3-d time point was used in these studies. Rats were anesthetized with pentobarbital, the abdominal cavity was opened, the portal vein was catheterized, and phosphate-buffered saline (PBS) infusion was begun. The vena cava was then cut, and the vascular tree was flushed by continued perfusion with PBS (approximately 200 ml). Fixation of the tissue was accomplished by perfusion with 180 to 200 ml of freshly prepared 4% paraformaldehyde in PBS. Kidneys were then decapsulated and cut into approximately 5-mm$^3$ pieces, and fixation was continued overnight at 4°C in the same fixative. The next day, kidney pieces were washed three times with PBS and placed in 0.25% paraformaldehyde/PBS until sectioning. Sections of 50 $\mu$m were obtained and either photo-oxidized (see below) or mounted in Gelvex overlay mounting medium (Aldrich Chemical Co., Inc., Milwaukee, WI); coverslips were sealed with clear acrylic nail polish. TRG staining was examined by confocal microscopy (see below).

Brush Border Membrane (BBM) Preparation and Characterization

Control and gentamicin-treated rats were lightly anesthetized with metofane, decapitated, and exsanguinated, and their kidney cortices were dissected and homogenized. Homogenates for all rats were prepared using exactly the same volume of buffer and as close to the same amount of tissue as possible. The beginning and ending weights did not vary by >5% between control and STG-treated rats in any experiment. BBM were isolated from cortical homogenates using Mg$^{2+}$ precipitation and differential centrifugation techniques, as described in detail elsewhere (15,26–29). Relative enrichments of BBM preparations were documented enzymatically using the BBM marker leucine aminopeptidase and the basolateral membrane marker Na$^+$/K$^+$-ATPase, as described previously (15,27–29). Marker enzymes used to determine contamination by other intracellular organelles were succinate dehydrogenase (mitochondria) and KCN-resistant NADH dehydrogenase [endoplasmic reticulum (ER)], as described previously (15,27,28).

Biosynthetic Labeling of Proteins and Phospholipids

Control and gentamicin-treated (1 to 3 d) rats were given intraperitoneal injections, on the day of euthanization, of either 0.1 mCi of $[^3]$H]leucine or 0.5 mCi of $^{32}$P (Amersham, Arlington Heights, IL), both in 0.5 ml of normal saline solution. $[^3]$H]Leucine was injected 1 h before euthanasia and $^{32}$P was injected 2 h before euthanasia. After euthanasia, cortical homogenates were made and BBM were prepared from them as described above. In both cases, aliquots were added to scintillation fluid (Beckman, Fullerton, CA) and counted in a Beckman LS1801 β-counter. Incorporation of radioactivity into protein or lipid was then determined as described below. We previously used similar procedures to quantify phospholipid synthesis (27).

Protein Determinations

Protein concentrations were determined using the method of Lowry et al. (30) or the Bradford dye binding assay (31), and synthesis was expressed as counts incorporated per milligram of protein. Incorporation of $[^3]$H]leucine into protein was determined by TCA precipitation. Samples (homogenates or BBM) were incubated on ice in 10% TCA for >1 h and were filtered using Millipore filtration techniques we described previously (29,32). Each filter was rinsed, suspended in scintillation cocktail, and counted. Alternatively, TCA-precipitated samples were centrifuged at 16,000 × g for 10 min, and the TCA pellet was solubilized with 1 N NaOH, mixed with scintillation cocktail, and counted.

Lipid Determinations

These determinations were performed as we described in detail previously (15,27,28). Briefly, lipids from 0.5 to 1.0 mg of cortical homogenate or BBM were extracted with chloroform/methanol (1:2, vol/vol) and isolated according to the method of Bligh and Dyer (33). Total phospholipid levels were determined using the method of Ames and Dubin (34), and individual phospholipid species were separated by two-dimensional thin layer chromatography, using a modification (27) of the method of Esko and Raetz (35). Incorporation of $^{32}$P into total cellular membranes, BBM, and individual lipid species was determined by counting aliquots after chloroform/methanol extraction and TCA precipitation, as described for filters above (homogenates...
and BBM, or counting resuspended scrapings of individual lipid species after two-dimensional thin layer chromatography (individual phospholipid species; see above).

Confocal Microscopy
TRG conjugate fluorescence was examined using a Bio-Rad MRC-1024 scanning laser confocal microscope (Bio-Rad, Richmond, CA), with a Nikon ×100 1.4-NA PLAN APO objective (Nikon, Natick, MA). Samples were illuminated using an argon-krypton laser. We successfully used this system in previous studies (15,23).

Photo-Oxidation of TRG and EM

The protocol for and mechanism of photo-oxidation of ringed structures in the presence of diaminobenzidine (DAB) have been described by others (36). We previously described our modifications of the photo-oxidation reaction (23). An excitation filter (filter 560DF40; Optical Omega, Brattleboro, VT) was positioned directly over sections (see above) immersed in DAB substrate solution (Vector Laboratories, Burlingame, CA) at 4°C. A mercury lamp suspended 30 cm above the filter cube was used to excite the sample with 560-nm light for 2.5 min three times. After each excitation, fresh 4°C DAB solution was added to the sample well. Reactive species produced by photo-oxidation of the Texas Red fluorophore react with DAB to form a dense EM-observable reaction product wherever TRG is present. Structures in the presence of diaminobenzidine (DAB) have been described by others (36). We previously described our modifications of the photo-oxidation reaction (23). An excitation filter (filter 560DF40; Optical Omega, Brattleboro, VT) was positioned directly over sections (see above) immersed in DAB substrate solution (Vector Laboratories, Burlingame, CA) at 4°C. A mercury lamp suspended 30 cm above the filter cube was used to excite the sample with 560-nm light for 2.5 min three times. After each excitation, fresh 4°C DAB solution was added to the sample well. Reactive species produced by photo-oxidation of the Texas Red fluorophore react with DAB to form a dense EM-observable reaction product wherever TRG is present. Additional processing of the photo-oxidized tissue for EM was performed using procedures similar to those we have used before (15,29,32). Samples were fixed with 1% glutaraldehyde for 1 h, washed four times with PBS, postfixed with 0.14% osmium tetroxide/0.04% tannic acid/0.50% K3Fe(CN)6 in PBS, dehydrated with graded ethanol, infiltrated with graded solutions of propylene oxide/Spurr’s resin to 100% Spurr’s resin (incubated for 2 h at 100%), and then polymerized overnight at 65°C. Ultrathin sections were cut using a MT6000-XL ultramicrotome (RMC Inc., Tucson, AZ) and observed with a Philips CM-12 electron microscope (Wilmington, DE), using 80- to 100-kV accelerating voltage.

### Table 1. Effects of gentamicin treatment on homogenate and BBM protein concentrations

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Homogenates</th>
<th>BBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.07 ± 0.89 (15)</td>
<td>2.34 ± 0.33 (15)</td>
</tr>
<tr>
<td>1-d STG</td>
<td>8.27 ± 0.61 (4)</td>
<td>2.34 ± 0.18 (4)</td>
</tr>
<tr>
<td>2-d STG</td>
<td>8.86 ± 0.77 (8)b</td>
<td>1.81 ± 0.28 (8)b</td>
</tr>
<tr>
<td>3-d STG</td>
<td>8.19 ± 0.98 (14)</td>
<td>2.22 ± 0.38 (14)</td>
</tr>
</tbody>
</table>

*a Rats were treated with vehicle or gentamicin for 3 d. The day after the last injection, cortical homogenates and brush border membrane (BBM) preparations were prepared and protein concentrations were determined as described in Materials and Methods. The n values are indicated by values in parentheses.

*b Statistical significance of P < 0.03 and P < 0.0001 for 2-d short-term gentamicin (STG)-treated homogenates and BBM, respectively.

### Table 2. Enzyme activities for animals treated with gentamicin for 3 d

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (μmol/h per mg protein)</th>
<th>Enrichment (BBM specific activity/homogenate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>3-d STG</td>
</tr>
<tr>
<td>LAP</td>
<td>3.27 ± 0.49 (7)</td>
<td>2.94 ± 0.37 (10)</td>
</tr>
<tr>
<td>BBM</td>
<td>29.88 ± 4.93 (7)</td>
<td>29.76 ± 5.45 (10)</td>
</tr>
<tr>
<td>Na+K+ATPase homogenates</td>
<td>14.59 ± 1.82 (7)</td>
<td>10.95 ± 1.66 (10)b</td>
</tr>
<tr>
<td>BBM</td>
<td>29.67 ± 3.20 (7)</td>
<td>31.52 ± 4.09 (10)</td>
</tr>
<tr>
<td>SDH</td>
<td>19.75 (avg.) (2)</td>
<td>20.72 ± 3.88 (5)</td>
</tr>
<tr>
<td>NADH dehydrogenase homogenates</td>
<td>25.23 (avg.) (2)</td>
<td>28.70 ± 4.37 (5)</td>
</tr>
<tr>
<td>BBM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Rats were treated with gentamicin for 3 d. The day after the last injection, cortical homogenates and BBM preparations were prepared and protein concentrations and enzyme activities were determined as described in Materials and Methods. The n values for each enzyme are indicated by the values in parentheses. LAP, leucine aminopeptidase; SDH, succinate dehydrogenase; avg., average.

*b Statistical significance of P < 0.01.
BBM preparations, on days 1 and 3 (Table 1). A significant difference in protein concentrations was observed on day 2 for treated rats.

Similarly to previous studies (16), we observed little effect of STG treatment on several cellular homogenate enzyme activities and their enrichment in BBM preparations. The BBM marker leucine aminopeptidase exhibited no significant change in either specific activity or BBM enrichment (Table 2). Likewise, markers of two intracellular organelles, i.e., succinate dehydrogenase (mitochondria) and KCN-resistant NADH dehydrogenase (ER), did not exhibit any significant change in enrichment (Table 2). However, statistically significant alterations were observed for the basolateral membrane marker Na\(^+\)/K\(^+\)-ATPase. The specific activity in homogenates from STG-treated animals was reduced, whereas the BBM enrichment was increased. No alterations in enzyme activities or BBM enrichment were observed at time points earlier than 3 d (data not shown).

The next experiments directly assessed the effects of gentamicin on in vivo protein synthesis. Rats were treated with gentamicin for 1 to 3 d and then injected with \([\text H]\)leucine 1 h before euthanasia, for determination of newly synthesized protein in homogenates and BBM preparations. As presented in Figure 1A, a significant decrease in \([\text H]\)leucine incorporation into total cellular protein was observed by the second day of treatment. Protein synthesis decreased further on the third day of treatment. When total cellular and BBM protein syntheses were compared at 3 d (Figure 1B), both were decreased by approximately 50%. Effects on protein degradation were not directly measured.

It has been demonstrated that short-term (11,12,16,37) and long-term (15) aminoglycoside exposure produces rapid and persistent alterations in renal phospholipid contents. These changes are thought to result from inhibition of phospholipid degradation (13,37–39). It is also possible that synthesis and/or incorporation of phospholipids into their appropriate membranes may be affected. To explore these possibilities, experiments were performed to better characterize the effects of gentamicin on phospholipid synthesis and incorporation into membranes. Results are presented in Table 3. After treatment of rats with gentamicin for 3 d, the total phospholipid content of homogenates and BBM preparations was significantly increased (both 21%), compared with control homogenates and BBM. Although incorporation of \(^{32}\text P\) into total cellular phospholipids (i.e., synthesis) was moderately decreased (11%) and increased (7%) in gentamicin-treated homogenates and BBM, respectively, neither change was statistically significant.

Our next experiments characterized the effects of 3 d of gentamicin treatment on individual phospholipid contents. No significant effect on the percentage composition of any phospholipid analyzed in STG cellular homogenates was observed, except for phosphatidylserine (PS), which exhibited an increase of 34% (Figure 2A). However, when individual phospholipids of BBM preparations from STG-treated and control rats were compared, significant increases in phosphatidylethanolamine (PE) and PI levels (37 and 64%, respectively) were observed, whereas a significant decrease (23%) in sphingomyelin (SPH) content was observed (Figure 2B).

To evaluate whether synthesis of individual phospholipids was being altered, rats were treated with gentamicin exactly as described above, except they were injected with \(^{32}\text P\) 2 h before euthanasia. Significant decreases in the rate of incorporation of \(^{32}\text P\) into SPH (\(P < 0.05\)) and phosphatidylserine (PS) (\(P < 0.05\)) were observed, whereas a significant increase was noted for phosphatidylethanolamine (PE) (\(P < 0.02\)) (Table 4). The same analysis performed on lipids extracted from BBM preparations from treated animals revealed only a significant decrease in \(^{32}\text P\) incorporation into SPH (\(P < 0.001\)) (Table 4). No consistent significant change in total or individual phospho-
lipid composition or in total or individual phospholipid synthesis was observed at times earlier than 3 d (data not shown). Both membrane protein and phospholipid biosynthetic pathways use cytosolic enzymes. However, they are also both dependent on and intimately associated with the membranes of intracellular organelles, specifically the ER and Golgi complex. The last series of experiments was performed to morphologically characterize where gentamicin was trafficked and concentrated during STG treatment. These experiments were performed exactly as described above, except that tracer amounts of TRG were added to the native gentamicin used for injection (see Materials and Methods).

Confocal microscopic results from these experiments are presented in Figure 3; large bright fluorescent structures are clearly evident. Similar to native gentamicin, the TRG was taken up, retained, and concentrated in numerous, relatively large structures (probably lysosomes) located predominantly in the basal region of proximal tubule cells (PTC). Only minimal plasma membrane staining was observed 24 h after the final injection. No fluorescence staining was observed in proximal tubules of control animals (data not shown). Little, if any, intracellular staining was observed in glomeruli or distal tubules.

When the fluorescent structures were examined in greater detail using photo-oxidation of TRG and EM, it became evident that TRG was associated with a variety of structures. The classic effects of gentamicin on lysosomes can be seen in Figure 4. Electron micrographs of photo-oxidized tissue sections from animals given injections of vehicle (Figure 4A), native gentamicin (Figure 4B), and TRG-spiked gentamicin (Figure 4C) are shown. It was evident that the photo-oxidation reaction itself did not induce significant reaction product in either control tissue (Figure 4A) or native gentamicin-treated tissue (Figure 4B), which contained myeloid material. However, in the tissue treated with gentamicin and tracer amounts of TRG, much of the myeloid material was darkly stained (Figure 4C). Not all lysosomes or myeloid material in the same section or cell was labeled. Much of the TRG-stained myeloid material appeared as heavily stained, collapsed/aggregated membranes that lacked the classic concentric circular, lamellar appearance (compare Figure 4, B and C) (25,40). Whether TRG was associated with lipid, protein, or both within the membrane was not determined.

Table 3. Effects of gentamicin on total phospholipid contents and $^{32}$P incorporation into total phospholipids

<table>
<thead>
<tr>
<th></th>
<th>Total Phospholipids (nmol/mg protein)</th>
<th>$^{32}$P Incorporation (cpm/mg protein per 2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>Homogenates</td>
<td>180 ± 7 (5)</td>
<td>218 ± 21 (5)$^b$</td>
</tr>
<tr>
<td>BBM</td>
<td>648 ± 42 (5)</td>
<td>784 ± 53 (5)$^b$</td>
</tr>
</tbody>
</table>

$^a$ Rats were treated with gentamicin for 3 d. On the fourth day, cortical homogenates and BBM preparations were prepared and phospholipids were isolated and quantified or rats were given injections of $^{32}$P, 2 h later the same procedures were performed and radioactivity was then determined. Gentamicin-treated homogenates and BBM were compared with control samples. The n values are indicated by the values in parentheses.

$^b$ Statistical significance of $P < 0.02$ for homogenates and $P < 0.01$ for BBM (total phospholipid content only).

Figure 2. Effects of gentamicin on homogenate and BBM individual phospholipid contents. Rats were treated with gentamicin for 3 d. The day after the last injection, cortical homogenates and BBM were prepared, total phospholipids were extracted and isolated, and individual phospholipids were separated and then quantified as described in Materials and Methods. (A) Individual phospholipids as percentages of total phospholipids in cortical homogenates. (B) Individual phospholipids as percentages of total phospholipids in BBM preparations. The statistical significance of the difference between individual phospholipids for homogenates (A) and BBM (B) is indicated above the specific phospholipid ($n \geq 3$ for all groups). SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylserine; PS, phosphatidylethanolamine.
More detailed examination revealed evidence of other intracellular organelle staining (Figure 5). Darkly stained structures could be observed in the basal region of cells from adjacent proximal tubules of gentamicin/TRG-treated animals (Figure 5B). This staining was very well correlated with the confocal microscopic data presented in Figure 3. Dark staining, as seen in Figure 5B, was not observed in vehicle-treated control samples photo-oxidized exactly like the gentamicin/TRG-treated samples (Figure 5A). At higher magnification, it was clear that much of this staining was lysosomal (Figure 5C).

However, some of the darkly stained structures did not appear to be lysosomal (Figure 5B). In additional experiments, which were designed to specifically enhance this staining with the injection of TRG into STG-treated rats 30 min before perfusion-fixation, we observed unequivocal staining of classic Golgi stacks (Figure 5D) and mitochondria, according to morphologic criteria (Figure 5E). Although the results were not rigorously quantified, we estimate that $\leq10\%$ of the Golgi complexes and mitochondria observed were stained in this manner.

Unequivocal evidence of mitochondrial staining was observed in the TRG accumulation experiments (Figure 5, B and C) and the uptake experiments (Figure 5D). At high magnification (Figure 5E), it was clear that the outer membrane and intermembrane space were heavily stained. However, it was not clear whether the inner membrane was labeled. If labeled, it did not appear to extend deeply into the cristae of the mitochondria. Staining of the matrix was not observed in any of the heavily stained mitochondria observed.

**Discussion**

It has long been known that gentamicin treatment can induce toxicity in PTC (4,18,25,40); however, the mechanisms involved in the toxic injury remain unresolved. Although *in vitro* and partially *in vivo* studies have demonstrated that protein synthesis is inhibited with gentamicin (19–22), it had not been determined whether this inhibition occurs in entirely *in vivo* experiments. Phospholipase inhibition, which is thought to induce myeloid formation and phospholipidosis, has been directly demonstrated *in vitro* and implicated *in vivo* (13,37–39) but, to our knowledge, alteration of *in vivo* phospholipid biosynthesis had not been demonstrated. The purpose of the studies presented here was to determine the effects of aminoglycoside treatment on protein and phospholipid metabolism in entirely *in vivo* experiments.

Using a rat model with little other evidence of PTC toxicity, we demonstrated that protein synthesis was significantly inhibited after 2 d of gentamicin treatment. This inhibition in-

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**Table 4. Effects of gentamicin on homogenate and BBM individual phospholipid synthesis**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>3-d STG</th>
<th>Control</th>
<th>3-d STG</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPH</td>
<td>224 ± 17 (5)</td>
<td>138 ± 38 (3)</td>
<td>1,156 ± 150 (5)</td>
<td>671 ± 104 (4)</td>
</tr>
<tr>
<td>PC</td>
<td>17,573 ± 2,118 (5)</td>
<td>16,231 ± 2,416 (3)</td>
<td>28,515 ± 1,434 (5)</td>
<td>27,954 ± 9,005 (4)</td>
</tr>
<tr>
<td>PI</td>
<td>1,493 ± 505 (5)</td>
<td>1,845 ± 389 (3)</td>
<td>1,879 ± 709 (5)</td>
<td>2,853 ± 1,052 (4)</td>
</tr>
<tr>
<td>PS</td>
<td>110 ± 26 (5)</td>
<td>68 ± 18 (3)</td>
<td>703 ± 260 (5)</td>
<td>464 ± 156 (4)</td>
</tr>
<tr>
<td>PE</td>
<td>2,156 ± 238 (5)</td>
<td>2,897 ± 240 (3)</td>
<td>9,809 ± 1,476 (5)</td>
<td>9,507 ± 2,989 (4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Homogenate</th>
<th>BBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPH</td>
<td>224 ± 17 (5)</td>
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<td>9,809 ± 1,476 (5)</td>
</tr>
</tbody>
</table>

*Note:* Rats were treated with gentamicin for 3 d. The day after the last dose, rats were given intraperitoneal injections of $^{32}$P. After 2 h, cortical homogenates and BBM preparations were prepared, phospholipids were isolated and separated, and the radioactivity of the separated individual phospholipids was determined. Incorporation of radioactivity into individual phospholipids of cortical homogenates and BBM preparations is shown. SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine. All $n$ values are indicated by the values in parentheses.

*Statistical significance of $P<0.05$ for homogenate SPH and PS, $P<0.02$ for homogenate PE, and $P<0.001$ for BBM SPH.*
Figure 4. Photo-oxidation and electron microscopic (EM) analysis of TRG accumulation in lysosomal myeloid material. Animals were treated as described for Figure 3. However, after sectioning, samples were subjected to photo-oxidation procedures and were then embedded and processed for EM. (A) Photo-oxidized cortical section from a vehicle-treated animal. Magnification, ×23,380. (B) Photo-oxidized section from a gentamicin-treated animal. Magnification, ×23,380. (C) Photo-oxidized section from a gentamicin/TRG solution-treated animal. Magnification, ×25,200.

Figure 5. Photo-oxidation/EM analysis of intracellular organelles (lysosomes, Golgi complexes, and mitochondria). Animals and sections were treated as described for Figures 3 and 4 (A to C and E) or were treated with TRG 30 min before perfusion-fixation (D), as described in the text. (A) Control. Magnification, ×5800. (B) Gentamicin/TRG-treated. Magnification, ×5800. (C) Gentamicin/TRG-treated, lysosomal staining. Magnification, ×35,000. (D) Gentamicin/TRG-treated, 30-min TRG uptake, Golgi complex staining. Magnification, ×35,000. (E) Gentamicin/TRG-treated, mitochondrial staining. Magnification, ×45,000.
creased to 50% by the third day. Furthermore, this inhibition appeared to affect BBM protein synthesis in a similar manner and to the same degree as total cellular protein synthesis. Effects on protein degradation were not directly determined. These studies agree with and expand earlier, partly in vivo studies performed somewhat differently than ours (19–22).

With the same model, we also observed disruption of individual phospholipid synthesis and accompanying inhibition of phospholipid degradation. In contrast to our studies of protein synthesis, total cellular and BBM phospholipid syntheses were not significantly affected by gentamicin treatment. However, there were significant alterations in the incorporation of $^{32}\text{P}$ into individual phospholipids. Incorporation of $^{32}\text{P}$ into PE was significantly increased in STG-treated homogenates, whereas incorporation into SPH and PS was reduced. Only a reduction in the incorporation of $^{32}\text{P}$ into SPH in BBM preparations was observed with STG treatment. Previous in vivo studies, which were performed differently than ours, demonstrated either no change in $^{32}\text{P}$ incorporation into any phospholipid in a rat model (16) or an increase in the synthesis of at least two phospholipids using a rabbit primary culture PTC model (14). In our studies, we observed no change (PC and PI in STG-treated homogenates and PC, PE, PI, and PS in STG-treated BBM), an increase (PE in STG-treated homogenates), and decreases (PS and SPH in STG-treated homogenates and SPH in STG-treated BBM) in phospholipid levels.

Similar to findings reported by others (11–13,16,37–39), our studies showed that phospholipid degradation was inhibited at early time points after gentamicin treatment (in these studies, at 3 d). We demonstrated that total phospholipid contents in homogenates and BBM preparations were increased 21%, compared with control samples. We also showed that the individual phospholipid contents of BBM were affected to a greater extent by STG treatment than were total cellular membranes, which is, to our knowledge, a new finding. The only significant effect on the percentage composition of any phospholipid in STG-treated homogenates was an increase in PI content (34%). However, in STG-treated BBM preparations, significant increases in PC and PI levels (37 and 64%, respectively) were observed, whereas a significant decrease (23%) in the SPH content was observed, compared with control samples. It was clear from these studies that any increase in phospholipid content observed as a result of gentamicin treatment was predominantly the result of the inhibition of degradation, rather than any effect on synthesis. Finally, the phospholipid degradation and synthesis effects were somewhat delayed, compared with the protein synthesis effects, and did not become statistically significant until the third day of treatment. This is relevant because the previously mentioned animal studies that examined lipid synthesis were performed at 2 d (16).

Our last, and perhaps most interesting, results were from the morphologic studies. Confocal microscopic studies revealed accumulation of fluorescently labeled gentamicin in large basal structures. It was clear that most of these structures were lysosomes. However, more sensitive EM techniques revealed transport of gentamicin not only to lysosomes but also to Golgi complexes and mitochondria, again in entirely in vivo experiments. Photo-oxidized TRG darkly labeled not only membranes of myeloid bodies within lysosomes but also membranes of Golgi cisternae and the outer membrane and intermembrane space of mitochondria.

Although disruptions of protein and phospholipid synthesis could occur at many sites, a common site for both would be at the level of the Golgi complex, ER, or cell cytoplasm, particularly in the early stages. We are aware of only one study that suggests that gentamicin may be released into the cytosol at early time points after uptake (41). In the morphologic studies we have performed, we have never observed evidence of gentamicin being released into the cytosol in living cells. Therefore, for alteration of protein and phospholipid synthesis, we think that retrograde trafficking of gentamicin to the Golgi complex and perhaps the ER must occur. Our recent cell culture studies (23) and the in vivo morphologic data presented here, as well as implications from studies performed by other investigators (12,14,24,42), support this possibility. We do not now have a good explanation for the mitochondrial staining. However, if gentamicin reaches the ER, it might be able to traffic to the mitochondria from there.

Retrograde trafficking of a number of bacterial and plant protein toxins after endocytosis is now well established (43). These protein toxins kill eukaryotic cells after translocation of an active part of the molecule from various sites into the cytoplasm, where they inactivate the 60S subunit of the ribosome or elongation factor 2 (see reference 43 and references cited therein). Gentamicin inhibits bacterial protein synthesis by binding to the 30S subunit of the bacterial ribosome and inhibiting elongation or by causing mistranslation (1,2). In general, it is thought that gentamicin is not released into the cytoplasm of kidney PTC in the early stages of toxicity. As a result, the gentamicin-induced disruption of protein and phospholipid synthesis observed in these studies seems to be produced via a different, more indirect mechanism than that of the protein toxins. We suggest that gentamicin may interact with components of the protein and/or phospholipid biosynthetic machinery from the lumen of the Golgi complex or ER to induce the alterations characterized in these studies. The possibility that aminoglycosides might directly interact with the Golgi complex or ER was suggested previously (12,24,37).

It is also possible that normal membrane traffic and fusion become increasingly perturbed during gentamicin treatment, resulting in disruption of many cellular activities and ultimately cell death. The evidence of apparent retrograde movement of gentamicin observed in this study is consistent with this possibility. Additionally, the subtle but significant alteration in Na$^{+}$/K$^{+}$-ATPase enrichment in BBM preparations supports the possibility of altered trafficking resulting from gentamicin treatment.

Other investigators, using similar STG treatment models, also proposed alterations in membrane traffic (12,14,16,24,42). Relevant to this concept is evidence that gentamicin treatment inhibits vesicle/lysosome fusion in vivo (42) and homotypic endosome fusion in vitro (44), as well as the suggestion that longer-term gentamicin treatment causes swelling of the ER (25,45). It has also been speculated that myeloid bodies may be
formed as a result of decreased degradation of smooth ER (46). Furthermore, in a long-term model of gentamicin treatment in which rats recover from gentamicin-induced toxicity, we observed an apparent reduction of the endosomal compartment (15). This hypothesis suggests that disruption of normal trafficking and fusion could lead to aberrant trafficking and fusion. Because gentamicin is normally delivered to lysosomes and contains no known targeting sequence, altered trafficking and fusion could provide a mechanism for inducing the putative aberrant retrograde movement.

Although we do not present direct evidence to confirm this hypothesis, disruption of membrane trafficking provides a unique but consistent explanation of the results observed in this study. Significantly, it also provides an encompassing mechanism of gentamicin-induced toxicity that could explain the different effects described in detail by many investigators over several decades.

In summary, the results of our studies provide, for the first time, direct and completely in vivo evidence that gentamicin treatment inhibits protein synthesis in an animal model. They also provide new evidence that individual phospholipid synthesis and degradation are disrupted in vivo. Lastly, they provide unique evidence that gentamicin treatment causes trafficking of gentamicin to the Golgi complex and mitochondria. Together, these observations provide a mechanistic basis for gentamicin-induced nephrotoxicity. As a result of treatment, gentamicin is delivered to multiple sites, perhaps by altered trafficking and fusion. These sites then correspond with multiple sites of potential toxicity. The implication may be that the observed toxicity is the result of multiple minor toxic effects, which may act synergistically, as suggested previously (47).

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References


Gentamicin Inhibits Renal Metabolism