Inhibition of kidney lysosomal phospholipases A and C by aminoglycoside antibiotics: Possible mechanism of aminoglycoside toxicity

(streptomycin/amikacin/dibekacin/gentamicin/tobramycin)

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ABSTRACT Nephrotoxicity is an important side effect of aminoglycoside antibiotics, which are used to control infections caused by Gram-negative bacteria. Accumulation of aminoglycosides and phospholipids in the lysosomes is a prominent and early feature of aminoglycoside nephrotoxicity and is characterized histologically by the presence of numerous multilamellar bodies in kidney proximal tubule cells. Previous studies have shown that the drug-induced phospholipid fatty liver in man and animals is due to concentration of certain cationic amphiphilic drugs in lysosomes with inhibition of lysosomal phospholipases. It seemed possible that this mechanism might also explain the elevated levels of phospholipid and increased numbers of multilamellar bodies reported in the kidney cortex in aminoglycoside nephrotoxicity. In this study, subcellular localization of acid phospholipases A and C has been shown to be lysosomal in rat kidney cortex. A soluble lysosomal protein fraction was isolated and found to contain both phospholipase A and phospholipase C activity. Streptomycin did not inhibit the release of fatty acids from [3H]diethanolamphosphatidylcholine. However, amikacin, dibekacin, gentamicin, and tobramycin inhibited both phospholipase A and phospholipase C. Our results suggest that the accumulation of phospholipids in lysosomes of kidney cortex, an early and pervasive feature of acute aminoglycoside nephrotoxicity, is due to inhibition of lysosomal phospholipases.

In 1971, Yamamoto and co-workers in Osaka noted that patients who had been taking the coronary vasodilator, 4,4'-bis(diyethylaminoethoxy)-α,β-diyethylidiphenylethane (DH; 4,4'-diethanolamphophathidylcholine) developed an acquired lipid storage disease that resembled Niemann–Pick disease (1, 2). A major ultrastructural feature of this disorder is the presence of many multilamellar bodies (myelin figures) in the liver and other tissues (3–5). All classes of phospholipids were substantially increased in liver and other tissues but sphingomyelin was not increased out of proportion as in Niemann–Pick disease (2). Since this report, a large number of drugs with widely different actions have been shown to cause phospholipid storage in a variety of tissues in vivo and in cultured cells. Although their pharmacological actions are different, these agents share cationic amphiphilic structural features. (For review of these disorders, see refs. 6–8).

Studies of the lipidosis induced in rats by the cationic amphiphilic agents chloroquine and DH have shown that phospholipid storage in liver is limited to the lysosomal compartment (9). Other intracellular organelles, such as mitochondria and microsomes, retain a normal phospholipid/protein ratio and the phospholipid and protein pool sizes represented by these organelles are not statistically different from those of controls (10). However, lysosomal protein and phospholipid pools are greatly increased in rats treated with DH or chloroquine (10). In addition, these two drugs concentrate in lysosomes to levels that are 10–13 times greater than that of the liver homogenate (9). These findings suggested that cationic amphiphilic drugs might cause acquired lipidosis by blocking the action of lysosomal phospholipases. We subsequently found that both chloroquine and DH are potent inhibitors of lysosomal phospholipase A and phospholipase C activities in vitro (11). These results were then extended to include seven additional cationic amphiphilic agents (12). Our current hypothesis for the mechanism of cationic amphiphilic drug-induced phospholipidosis is that these agents concentrate in lysosomes and inhibit lysosomal phospholipases, resulting in phospholipid accumulation and multilamellar body formation in affected tissues (11, 12).

Aminoglycoside antibiotics are widely used in clinical medicine because of their efficacy in treatment of infections caused by Gram-negative bacteria. However, their use is sometimes complicated by acute nephrotoxicity involving the proximal tubular cells and may result in serious damage to the kidney. Multilamellar bodies (also called myeloid bodies or myelin figures) are a prominent early histologic feature of aminoglycoside nephrotoxicity (13–15). Aminoglycoside antibiotics have been shown to concentrate in the proximal tubular cells and in kidney lysosomes (16). One report indicates that the phospholipid content of kidney cortex is increased (17). Gentamicin also concentrates in lysosomes and induces phospholipid storage in cultured rat fibroblasts (18, 19). These findings suggested that aminoglycosides might be inhibitors of lysosomal phospholipases. This paper presents evidence showing that acid phospholipases A and C are lysosomal in rat kidney cortex and that they are inhibited by four aminoglycoside antibiotics in vitro.

MATERIALS AND METHODS

Isolation of Subcellular Fractions and Lysosomal Soluble Delipidated Protein from Rat Kidney Cortex. Male Sprague–Dawley rats were injected intraperitoneally with Triton WR-1339 (850 mg/kg of body weight; Supelco, Bellafonte, PA) in normal saline. After 54 hr, the rats were fasted overnight. The kidneys were removed and the cortex was excised, minced with scissors, and rinsed with 0.25 M sucrose/5 mM Tris-HCl, pH 7.4/1 mM EDTA (SET buffer). A 10% homogenate was prepared in SET buffer using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 800 x g for 5 min. The

Abbreviation: DH, 4,4'-bis(diethylaminoethoxy)-α,β-diethyldiphenylethane (4,4'-diethanolamphophathidylcholine).
nuclear pellet was removed and the postnuclear supernatant was centrifuged at 20,000 × g for 30 min in a Sorvall SS34 rotor. The resulting pellet, consisting of heavy and light mitochondria and lysosomes, was suspended in 45% sucrose and applied beneath a three-step gradient consisting of 11 ml of 45% sucrose, 15 ml of 34% sucrose, and 7.5 ml of 14.7% sucrose as described by Trouet (20). The gradient tubes were centrifuged at 27,000 × g in a Beckman SW 27 rotor for 3 hr. A dark brown pellet, representing mitochondria and heavy lysosomes, was obtained, as well as a light brown floating band at the interface between the 34% and 11% sucrose layers representing a low-density population of lysosomes. The postmitochondrial supernatant was centrifuged at 120,000 × g for 1 hr to obtain the microsomal pellet and the supernatant fraction. The pellets were suspended in SET buffer; protein was measured by the method of Lowry (21) using bovine serum albumin as a standard.

Soluble lysosomal protein was obtained by 10 cycles of freezing and thawing of the low-density-lysosomal fraction in half-isotonic SET buffer followed by centrifugation at 120,000 × g for 1 hr to remove the membranous protein. After determination of the protein concentration (21), small aliquots of the kidney lysosomal soluble protein fraction were stored at -60°C until use.

Marker Enzyme Assays. The following marker enzymes were measured in the various subcellular fractions from kidney cortex: Succinate INT reductase (mitochondria) was measured by the method of Pennington (22); NADPH cytochrome c reductase (microsomes) was assayed in the presence of rotenone as described by Sottaca et al. (23); and N-acetyl-β-D-glucosaminidase (lysosomes) was determined by the method of Kolovsky and Palmieri (24).

Preparation of [9,10-3H]Dioleoylphosphatidylcholine. [9,10-3H]Oleic acid was obtained from New England Nuclear and glycerol-3-phosphocholine (tetrabutylammonium salt) was purchased from Calbiochem. [9,10-3H]Dioleoylphosphatidylcholine was synthesized chemically by the method of Warner and Benson and purified by TLC (25). Purity of the final product was estimated by TLC to be >98%.

Assay of [9,10-3H]Dioleoylphosphatidylcholine Hydrolysis. Incubation mixtures were 50 mM NaOAc, pH 4.0 unless otherwise noted/45 μM [9,10-3H]dioleoylphosphatidylcholine (specific activity, 7.5 mCi/mmol; 1 Ci = 3.7 × 1010 becquerels), which had been dispersed in water by brief sonication as described (11), and protein in a volume of 0.200 ml. In the subcellular fractionation experiments, 100–200 μg of protein was used; in studies with the soluble lysosomal fraction, 15–20 μg of protein was used. The samples were incubated for 20 min in a shaking water bath at 37°C; the reactions were stopped by addition of 4 ml of chloroform/methanol, 2:1 (vol/vol). Total lipid extracts were prepared by the method of Folch et al. (26) and analyzed by TLC on 0.25-mm-thick layers of silica gel H (20 × 20 cm) (EM Laboratories, Elmsford, NY). Areas representing fatty acid, diacyl- and monacylglycerol, phosphatidylcholine, and lysophosphatidylcholine were located by using iodine vapor and analyzed for radioactivity as described (11).

Rates were calculated after correction for blank values from an incubated sample that did not contain protein. In making calculations, a specific activity of 7.5 mCi/mmol was used for diglyceride while 3.75 mCi/mmol was used for fatty acid, lysophosphatidylcholine, and monoglyceride.

Aminoglycoside Antibiotics. Amikacin, dibekacin, and streptomycin were generously provided by H. Lüllmann (Kiel, Federal Republic of Germany); their potencies were 0.92, 0.70, and 0.74 mg/mg of powder, respectively. Gentamicin and tobramycin were obtained from Sigma; their potencies were 0.55 and 0.99 mg/mg of powder, respectively.

RESULTS

Subcellular Localization in Kidney Cortex of Acid Phospholipases. Acid phospholipase A and C activities have been demonstrated previously in homogenates of rat kidney (27). However, to our knowledge, the subcellular localization of these phospholipases has not previously been examined in the kidney. We isolated the following subcellular fractions from rat kidney cortex homogenates: nuclear and debris, heavy and light mitochondrial, microsomal, and supernatant. The crude mitochondrial fraction was further separated into low-density and high-density subfractions. The results (Fig. 1) indicate that succinate INT reductase (A) and NADPH cytochrome c reductase (B) are highly enriched in the mitochondrial and microsomal fractions (relative specific activities, 2.8 and 4.8, respectively). The lysosomal marker enzyme, N-acetylglucosaminidase, is most highly enriched in the low-density-lysosomal fraction (relative specific activity, 4.6). However, substantial amounts of total enzyme activity are also present in the nuclear/debris and high density mitochondrial fractions, as is often the case in other tissues. Acid phospholipase A activity (D) is greatly enriched in the low-density-lysosomal fraction. Acid phospholipase C activity (not shown) had a similar distribution but was difficult to measure accurately owing to its low activity. However, it was enriched over the homogenate only in the low-density-lysoso-
were also present in the mitochondrial and supernatant fractions. Both phospholipase A and phospholipase C activities were also present in the mitochondrial fraction, in which they represented 27% and 64%, respectively, of the total activity. This was also true for the lysosomal marker enzyme, N-acetylglucosaminidase (32% of total activity).

The nuclear and debris fraction contained significant amounts of all the marker enzymes, suggesting some nonspecific absorption of membranous material to nuclei. In the case of lysosomal enzymes, this could also represent sedimentation of heavier lysosomes in the nuclear fraction. Nevertheless, taken together, these results indicate that the subcellular distribution of acid phospholipases A and C is essentially the same as that of lysosomes, based on N-acetylglucosaminidase, indicating that these phospholipases are lysosomal.

**Solubilization and Properties of Kidney Lysosomal Phospholipases.** A soluble protein fraction was obtained by subjecting the low-density-lysosomal fraction to 10 cycles of freezing and thawing in dilute buffer. Ninety-eight percent of the phospholipase A activity and 88% of the phospholipase C activity were recovered in the resulting soluble fraction, while only 20% of the protein was solubilized by freezing and thawing.

The pH dependence of [3H]dilysophosphatidylcholine hydrolysis by the kidney lysosomal soluble protein fraction is shown in Fig. 2. Maximal phospholipase A and C activities were obtained at pH 3.7–4.0. The apparent contribution of phospholipase A to [3H]dilysophosphatidylcholine degradation is 7- to 8-fold greater than that of phospholipase C at pH 3.7–4.0. The activity of lysophospholipase and monoglyceride lipase in these soluble lysosomal preparations appears to be much less than that of the two phospholipases since the generation of fatty acid is only slightly greater than the formation of lysophosphatidylcholine and monoacylglycerol. No phospholipase activity was observed in incubations at pH 6.4–9.2 (data not shown). At pH 4.0, the activity of phospholipases A and C was linear with time to 20 min and with protein to 100 μg/ml (not shown).

The effects of four aminoglycoside antibiotics on the formation of [3H]labeled lysophosphatidylcholine (phospholipase A) and monoaclglycerol/diaclyglycerol (phospholipase C) from [9,10-3H]dioleoylphosphatidylcholine by a lysosomal soluble protein fraction isolated from kidney cortex are shown in Fig. 3. [3H]-Labeled fatty acid release was also determined (data not shown). The IC50 values of the various aminoglycosides are given in Table 1. Amikacin, dibekacin, gentamicin, and tobramycin inhibited lysosomal phospholipase A and C activities, as well as the overall release of fatty acids from [3H]dioleoylphosphatidylcholine. Phospholipase A was substantially inhibited, especially by tobramycin (IC50, 0.4 mM). For phospholipase A, IC50 values of the other agents were 3.3–6.2 mM. The inhibition of phospholipase C by these agents was similar to that found with phospholipase A except with gentamicin, which inhibited to 60% of control at 1 mM but did not cause further reduction of activity at 5 and 10 mM. The degree of inhibition of fatty acid release by all hydrolytic mechanisms was intermediate between that of phospholipases A and C for amikacin, dibekacin, and tobramycin (IC50, 0.24–4.1 mM). However, the overall release of fatty acid from [3H]dioleoylphosphatidylcholine was inhibited by gentamicin to a greater degree than was the activity of phospholipase A, sug-

**Fig. 2.** Dependence of kidney lysosomal phospholipases on pH. Buffers used: pH 3.7–5.2, NaOAc; pH 5.6–9.2, Tris maleate. O, Fatty acid; •, lysophosphatidylcholine (phospholipase A); ○, monoaclglycerol/diaclyglycerol (phospholipase C). Results represent mean ± SD of three experiments.

**Fig. 3.** Effect of amikacin, dibekacin, gentamicin, and tobramycin on hydrolysis of [9,10-3H]dioleoylphosphatidylcholine by a soluble delipidated protein fraction obtained from rat kidney cortex lysosomes. O, Amikacin; ●, gentamicin; •, dibekacin; ○, tobramycin. (A) Phospholipase A. (B) Phospholipase C. Lysosomal protein (16 μg) was incubated in 50 mM NaOAc, pH 4.0/45 μM [9,10-3H]dioleoylphosphatidylcholine (specific activity, 7.5 mCi/mmol) at 37°C for 20 min. Final volume, 0.200 ml. Reaction was stopped by addition of 20 μl of chloroform/methanol, 2:1 (vol/vol), and the lipids were extracted and analyzed by TLC as described (11). Reactions were linear with time and amount of protein. Control values (mean ± SD; n = 3): lysophosphatidylcholine, 248 ± 42 nmol·mg⁻¹·hr⁻¹; monoaclglycerol/diaclyglycerol, 33 ± 5 nmol·mg⁻¹·hr⁻¹; fatty acid, 364 ± 20 nmol·mg⁻¹·hr⁻¹; monoaclglycerol/diglyceride produced (mol/mol), 2.0.
These studies show that weight (16, 17).

We also need to consider that the lysosomal phospholipases and degree of phospholipid storage produced, at least in well-defined situations in cell culture. However, it is more difficult to draw conclusions about the order of toxicity in vivo because these agents show wide variations in uptake by kidney cortex (17). Thus, there are several factors that may affect the potential of an aminoglycoside to cause nephrotoxicity including (i) ability to concentrate in tubular cells, (ii) ability to concentrate in lysosomes, and (iii) ability to block phospholipid catabolism by inhibiting phospholipase A2. Our studies pinpoint these last as a potentially important feature not previously demonstrated.

The molecular mechanism of aminoglycoside and cationic amphiphilic drug inhibition of lysosomal phospholipase activity is unknown, but a major theory, proposed by Lüllmann and coworkers (6–8), is that the drugs form complexes with phospholipids and that the complexes are less susceptible to degradation by phospholipases. The formation of these complexes has been convincingly demonstrated by several different approaches using various cationic amphiphilic drugs such as chlorpromazine, chloroquine, propranolol, and imipramine (29–31). The cationic amphiphilic agents generally bind most strongly to acidic phospholipids such as phosphatidylinerine and phosphatidylinositol; phosphatidylethanolamine is intermediate, while phosphatidylcholine, the substrate used in our experiments, has the lowest tendency to form complexes with cationic amphiphilic drugs (30). Recently, aminoglycosides have been shown to form complexes with phosphatidylinerine (32). However, definitive proof that these two classes of agents do not inhibit by direct interaction with the enzymes awaits studies of purified lysosomal phospholipases.

In conclusion, our studies show that four aminoglycoside antibiotics inhibit the activity of lysosomal phospholipases A and C from rat kidney in vivo, suggesting that inhibition of lysosomal phospholipases may play important roles in aminoglycoside nephrotoxicity and in gentamicin-induced phospholipidosis in cultured fibroblasts. Interestingly, streptomycin, an aminoglycoside that does not cause nephrotoxicity, did not inhibit kidney phospholipases. There may be a rough parallel between the ability of an aminoglycoside to cause cellular phospholipid storage and its ability to inhibit lysosomal phospholipase. To better assess the physiological significance of these findings, further studies to estimate the intralysosomal concentration of these agents are needed to see whether aminoglycoside levels in lysosomes are high enough to account for the apparent blockage of phospholipid catabolism that is reflected by increased amounts of phospholipid and increased numbers of multilamellar bodies in the proximal tubule cells of the kidney.

DISCUSSION

These studies show that aminoglycosides, with the exception of streptomycin, inhibit the activity of kidney lysosomal phospholipases A and C. Although the aminoglycosides are not as effective as inhibitors as cationic amphiphilic drugs (11, 12), they would be expected to impair lysosomal phospholipid breakdown because they become highly concentrated in the lysosomes of kidney proximal tubule cells (16) and of fibroblasts (18). This general type of mechanism has been postulated to be an important factor in aminoglycoside nephrotoxicity, having been termed the “lysosomal dysfunction hypothesis” by Kaloyanides and Pastoriza-Munoz (17). Our studies, using a soluble lysosomal phospholipase preparation isolated from rat kidney cortex, provide direct support for this hypothesis. In addition, these findings may also explain the lysosomal phospholipidosis reported in gentamicin-treated rat fibroblasts (19). This proposed mechanism — i.e., inhibition of phospholipid catabolism in kidney lysosomes by aminoglycosides — is essentially identical to that which we have previously put forward to explain the phospholipid fatty liver caused by certain cationic amphiphilic drugs (11, 12). Inhibition of sphingomyelinase by gentamicin has been reported in cultured rat fibroblasts (19).

In view of the fact that concentrations of gentamicin in the millimolar range are required to substantially inhibit kidney lysosomal phospholipases, one might question the physiological significance of our findings. Of critical importance is the intralysosomal concentration of the aminoglycosides in the proximal tubule cell. Although no direct measurements have been carried out, estimates can be made by using data from the literature. In gentamicin-toxic rats, cortical gentamicin concentrations have been shown to range from 900 to 2230 μg/kg wet weight (16, 17). If one assumes that the lysosomal volume represents 5–10% of the cell volume and that most of the gentamicin is lysosomal, as shown by Morin et al. (16), it can be calculated that intralysosomal gentamicin concentrations may be 18–80 mM. These values should be considered to be minimal estimates because the kidney cortex gentamicin concentration will certainly underestimate the concentration in the proximal tubule cells, which accumulate the drug more avidly in vivo than other cell types (16). Intralysosomal levels of 18–80 mM gentamicin would certainly be of physiological importance in view of our in vitro findings (Fig. 3 and Table 1).

It is interesting to note that streptomycin, which is not nephrotoxic, does not inhibit kidney lysosomal phospholipases. Furthermore, Tulkens and van Hoof (28) have shown that the phospholipid content of cultured fibroblasts is increased by aminoglycosides in the order amikacin (9%) < gentamicin (36%) < tobramycin (42%). This is roughly the order we found for inhibition of kidney lysosomal phospholipases by these agents in vitro. Thus, there may be a rough parallel between ability to inhibit phospholipases and degree of phospholipid storage produced, at least in well-defined situations in cell culture.

Table 1. Inhibition of kidney cortex lysosomal phospholipases by aminoglycosides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50, mM</th>
<th>Phospholipase A</th>
<th>Phospholipase C</th>
<th>Total fatty acid release</th>
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</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>6.2</td>
<td>4.0</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Dibekacin</td>
<td>3.3</td>
<td>2.0</td>
<td>2.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Gentamicin</td>
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<td>2.0</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.40</td>
<td>0.20</td>
<td>0.24</td>
<td></td>
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</tbody>
</table>

Results are calculated from the data in Fig. 1. Ind, indeterminate.

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**References**