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IN VITRO RESPONSE OF RAT AND HUMAN KIDNEY LYSOSOMES TO AMINOGLYCOSIDES

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Abstract—To study aminoglycoside nephrotoxicity, renal cortical lysosomes were prepared from rat kidneys and from healthy portions of five human kidneys removed for tumor. The renal cortex was homogenized in 1 mM EDTA with 0.3 M sucrose, and the lysosomes were separated by differential centrifugation. Lysosomes were incubated in isotonic sucrose solution with various drug concentrations for 1 hr at 37°. They were resedimented and the *N*-acetyl-β-glucosaminidase (NAG) activity was measured in the supernatant fraction and in the disrupted pellet. Incubation with four aminoglycosides at therapeutic plasma concentrations lowered the percentage of NAG released into the supernatant fraction in a dose-related fashion. Incubation with the polyamines spermine and spermidine also produced this effect, with spermine and gentamicin being additive. This apparent lysosomal stabilization at clinically achieved plasma concentrations was also observed after substituting isotonic glycine for sucrose in the incubation mixture. High concentrations of aminoglycoside consistent with those accumulated in the renal cortex of patients and rats produced a dose-dependent release of lysosomal NAG with a rank order of potency paralleling their clinically observed potential for producing nephrotoxicity. Rats were treated with 20 mg/kg gentamicin twice a day for 28 days producing kidneys resistant to aminoglycoside nephrotoxicity. Lysosomes prepared from these animals compared to saline-treated controls showed decreased reponse to gentamicin at 2 and 4 μg/ml and to spermine. Human renal cortical lysosomes also exhibited aminoglycoside- and polyamine-induced changes in NAG release. We conclude that the lysosome is a site of action for aminoglycoside nephrotoxicity. We propose that aminoglycoside stabilization of this lysosomal membrane may lead to eventual disruption of the proximal tubular lysosomal system and cell injury.

The acute renal tubular necrosis that is produced by aminoglycoside antibiotics contributes to the morbidity associated with the clinical use of these drugs [1]. Though the mechanism of the nephrotoxic action is obscure, the aminoglycosides are known to accumulate in the proximal tubule lysosomes of laboratory animals [2, 3] and in the renal cortex of man [4–6]. This accumulation may be related to the initiation of nephrotoxicity [6, 7].

The aminoglycosides are highly water-soluble cationic compounds which appear to concentrate in the lysosome through a pinocytotic mechanism [2, 3]. This leads to lysosomal swelling visualized by electron microscopy [7]. There is also accumulation of undigested lipid membranous material or myeloid bodies in the proximal tubular lysosome [8] and in lysosomes of cultured rat fibroblast [9] possibly as a result of inhibition of lysosomal phospholipid metabolism by the aminoglycoside.

The purpose of our study was to determine whether the renal tubular cell lysosome is a site of action for aminoglycosides and, if so, what effect these agents have on both rat and human renal cortical lysosomes.

The order of clinically observed potential for producing nephrotoxicity for four aminoglycosides is generally accepted to be neomycin > gentamicin > tobramycin > streptomycin [1]. This nearly parallels in decreasing order the number of cationic groups on these molecules of 6, 5, 5 and 3 respectively. To determine whether amino groups are important in causing the nephrotoxic effect, we examined the polyamines spermine and spermidine, in addition to the aminoglycosides, for their effects on the lysosome. These simple aliphatic amines are ubiquitous in mammalian systems and are excreted by the kidney [10]. Spermine with four amine groups is nephrotoxic to animals while spermidine with three amine groups probably is not [11, 12].

MATERIALS AND METHODS

Gentamicin sulfate and the Garamycin Injectable brand of gentamicin sulfate were supplied by the Schering Co., Kenilworth, NJ. Powders of neomycin sulfate and tobramycin sulfate were supplied by Eli Lilly & Co., Indianapolis, IN. Cimetidine was supplied by Smith, Kline & French Laboratories, Philadelphia, PA. Streptomycin sulfate, methicillin, vancomycin, penicillin G, spermine and spermidine bases were purchased from the Sigma Chemical Co., St. Louis, MO.

The methods used for isolation of the renal cortical lysosomal fraction were adapted from the technique of Maunsbach [13].

Specimen preparation. Male Fisher 344 rats were anesthetized with sodium pentobarbital. After transcardial perfusion with cooled 1 mM EDTA solution in 0.3 M sucrose at pH 7.0 (hypertonic sucrose), both kidneys were removed. The renal cortical tissue was quickly separated, weighed and minced with sharp scissors. The subsequent entire homogenization and

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isolation procedure was carried out at 0–5°. The tissue was first homogenized in 8:1 hypertonic sucrose with a manual Dounce glass homogenizer for five full strokes. Homogenization was completed by five full strokes with a motorized Potter–Elvehjem homogenizer with a loosely fitting Teflon pestle rotating at 1000 rpm. The homogenate was then centrifuged twice in a Sorvall RC-2B centrifuge with an SS-34 fixed angle rotor at a low speed of 1250 rpm for 10 min, and the pellets containing unbroken cells and nuclei were discarded. The remaining supernatant fractions were recentrifuged at 8750 rpm for 3 min to sediment a lysosomal-mitochondrial fraction distinguished by its black and brown color. The fluffy yellow brush border overlay and the supernatant fraction were discarded. The lysosomal-mitochondrial pellet (lysosomal pellet) was resuspended in 3 ml of hypertonic sucrose as a wash procedure and resedimented, and the supernatant fraction was discarded.

Incubation. A pH 7.0 solution of 0.256 M sucrose (isotonic sucrose) was prepared, and the agents to be tested were dissolved at ten times final concentration in isotonic sucrose. Two-tenths milliliter of drug/sucrose solution was added to 1.8 ml of the lysosomal suspension (incubation mixture) in separate tubes, gently vortexed, and incubated for 1 hr in a gently shaking water bath at 37°. An incubation mixture containing 0.2 ml of isotonic sucrose and 1.8 ml of the lysosomal suspension was used as a control. At the end of the hour incubation, the incubation mixtures were immediately cooled and resedimented, and the supernatant fractions were removed. The remaining pellets were hypotonically disrupted in 2 ml of distilled water or 10 mM KCl. In experiments with glycine, a 0.25 M solution of this amino acid was used in place of isotonic sucrose. Pellets were disrupted in 2 ml of 10 mM KCl or 0.1% Triton X-100. The remainder of the procedure was the same.

Measurements. Both supernatant fractions and ruptured pellets were diluted appropriately and assayed for the activity of the lysosomal enzyme *N*-acetyl-β-glucosaminidase (EC 3.2.1.30, NAG) by our previously published modification [14] of the method of Leaback and Walker [15]. The units of enzyme activity are expressed as nmoles of the product, methylumbelliferone (MU), liberated per hr per ml of assay mixture. NAG enzyme was assayed on the same day in experiments with rats to avoid problems of enzyme instability. The percentage of NAG released was calculated as the ratio of the activity in the supernatant fraction to the activity in the supernatant fraction plus pellet, times 100.

Human tissue experiments. Human renal cortical lysosomes were isolated from healthy appearing portions of kidneys from five patients who underwent radical nephrectomy for tumor. The patients, four male and one female, had normal BUN and creatinine prior to surgery and no patient had received an aminoglycoside prior to surgery or intravenous radiographic contrast material within 48 hr prior to nephrectomy. Renal cortical tissue was removed from the surgical specimen and placed in cool hypertonic sucrose. The tissue was finely minced with sharp scissors and filtered through 420 μm Teflon mesh. This suspension was homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle rotating at approximately 1500 rpm for ten full strokes. The homogenate was then treated in the same manner as that of the rat renal cortical tissue for the remainder of the procedure.

Chronic rat study. To obtain a group of rats resistant to the nephrotoxic effects of gentamicin, we adapted the technique of Gilbert *et al.* [16]. Male Fisher 344 rats were divided into two groups having equal mean weights of 145 ± 4 (S.D.) g. They were housed in standard metal cages and allowed water and standard lab chow *ad lib*. One group was given sterile injectable gentamicin, 20 mg/kg subcutaneously, at 8:00 a.m. and 4:00 p.m. daily. The second group received an equal volume of saline as concurrent controls. Serum was taken from four animals in both groups on days 10 and 14 and two animals from both groups on day 22, and creatinine was measured. All animals survived until time of sacrifice. The gentamicin-treated animals demonstrated a period of acute renal failure from days 10–17. This was manifested by a decline in activity, a slowed rate of growth [at day 28, the gentamicin-treated rats weighed an average of 214 ± 9 (S.D.) g vs 236 ± 10 g for the controls, $P < 0.001$], and an elevation of serum creatinine. Animals from both groups were killed after day 28, renal cortical lysosomes were isolated, and their gentamicin dose–response curves were compared.

All statistics were done using the Mann-Whitney U-test.

RESULTS

Incubation of rat renal cortical lysosomes in isotonic sucrose caused a release of NAG from the lysosome into the medium. Adding gentamicin to the incubation mixture caused a dose-related inhibition of release of NAG into the supernatant fraction (Fig. 1). A near zero percent release was obtained at a concentration of 15 μg/ml of gentamicin, compared to a mean release of about 49%

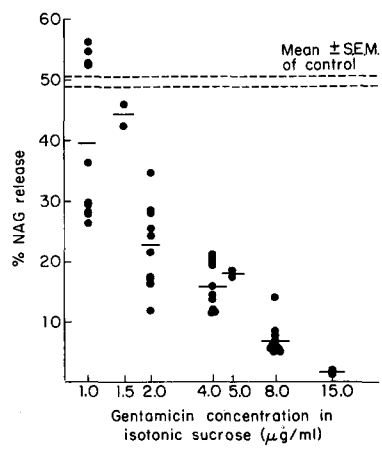

Fig. 1. Gentamicin effect on rat renal cortical lysosomes. Lysosomes from rat renal cortex were incubated for 1 hr at 37° in isotonic sucrose containing various gentamicin concentrations. The release of the lysosomal enzyme, *N*-acetyl-β-glucosaminidase (NAG) is shown as a function of the gentamicin concentration.

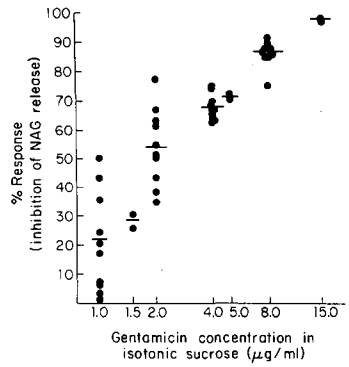

Fig. 2. Gentamicin dose–response curve. The gentamicin inhibition of release of rat renal cortical lysosomal NAG (data from Fig. 1) was converted to a dose–response curve. A 100% response corresponds to no NAG activity in the supernatant fraction. A 0% response corresponds to the mean percent NAG activity released into the supernatant fraction in the absence of drug.

for the sucrose control. The sum of the NAG activities present in the supernatant fraction and the pellet was the same in each incubation tube, indicating no inhibition of the NAG enzyme by the drug.

To convert this drug-related inhibition of NAG release to a dose–response curve, the zero percent response was set equal to the percentage of NAG released in the incubation mixture without drug, and the 100% response was that of an incubation with no NAG activity in the supernatant fraction. The response was then defined by the following equation:

$$\% \text{ Response} = \frac{\text{mean \% release by control} - \text{\% release by drug}}{\text{mean \% release by control}} \times 100$$

The transformed gentamicin dose–response relationship (Fig. 2) indicates a clear gentamicin effect at the concentrations observed in plasma of patients receiving the drug. Furthermore, the aminoglycosides streptomycin, tobramycin and neomycin and the polyamines spermine and spermidine were tested and shown to have these effects (Fig. 3). The same effect of inhibition of lysosomal NAG release caused by gentamicin and streptomycin was also observed on incubating renal cortical lysosomes in isotonic glycine (Fig. 4).

To see if gentamicin and spermine produced additive effects, a mixing experiment was done. A solution containing 0.45 μg/ml spermine (EC_{40}) with 1.25 μg/ml gentamicin (EC_{25}) produced a 65 ± 2% (S.E.) response. Thus, an additive effect of gentamicin and spermine occurred.

Aminoglycosides are known to reach high concentrations in renal cortex compared to elsewhere in the body of both humans [4] and rats [17] after administration of therapeutic amounts. We observed the pattern of renal cortical lysosomal NAG release with a wide range of concentrations of three aminoglycosides in isotonic glycine (Fig. 5). From these data it appears that the curve of percent NAG release as a function of aminoglycoside concentration follows a biphasic pattern of inhibition of release at lower concentrations followed by enhanced release at higher concentrations.

Other renally excreted compounds, some of which

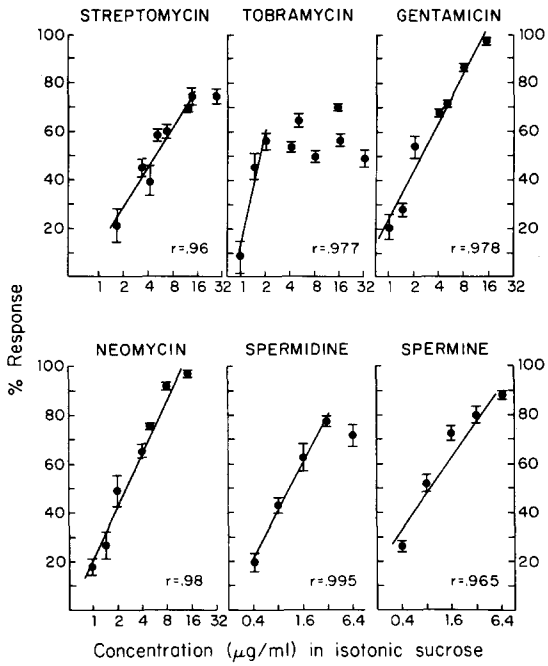

Fig. 3. Aminoglycoside and polyamine dose–response. The responses of rat renal cortical lysosomes to four aminoglycosides and two polyamines are shown. Concentrations of streptomycin greater than 16 μg/ml, tobramycin greater than 2 μg/ml and spermidine greater than 3.2 μg/ml were excluded from the calculation of the r values due to an apparent ceiling effect.

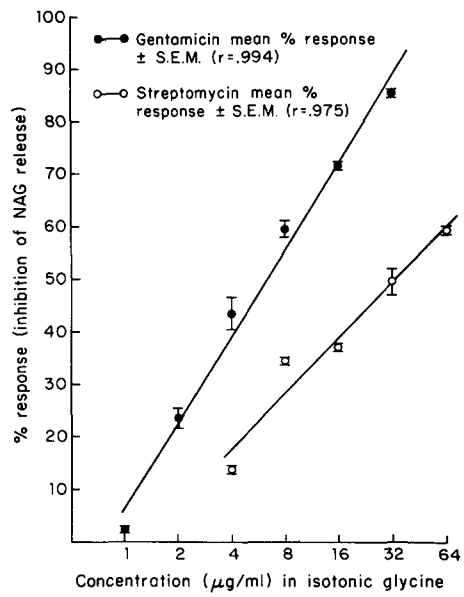

Fig. 4. Aminoglycoside dose–response in isotonic glycine medium. The mean percent release of NAG enzyme after incubation of rat renal cortical lysosomes for 1 hr at 37° in isotonic glycine was 83.6 ± 0.3% (S.E.M.). The NAG release as a function of streptomycin and gentamicin concentrations is shown here as a dose–response curve.

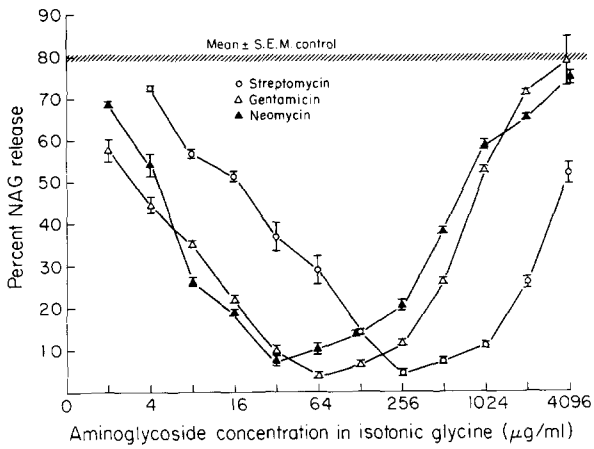

Fig. 5. Percent NAG release in the presence of aminoglycosides. Rat renal cortical lysosomes were incubated in isotonic glycine containing a wide range of aminoglycoside concentrations. Lysosomal NAG release followed a biphasic pattern in relation to aminoglycoside concentration.

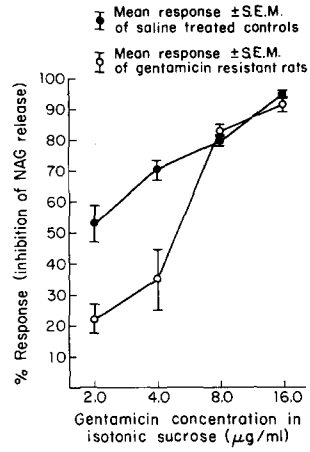

Fig. 6. Gentamicin dose–response in gentamicin-resistant and control rats. The dose–response curves are for renal cortical lysosomes from saline-treated control rats and for those isolated from rats in an induced state of *in vivo* gentamicin resistance. All points represent the mean of eight experiments with the exception of seven experiments at the 4 μg/ml concentration in the gentamicin-resistant animals. The level of significance is $P < 0.002$ and $P < 0.004$ for the 2 and 4 μg/ml concentrations respectively.

Table 1. Response of rat renal cortical lysosomes to therapeutic levels of some renally excreted drugs

Drug	Concn (μg/ml)	% Response*
Penicillin G	10.0	4,3
	20.0	2,7
Cimetidine	1.0	9,9
	5.0	0,9
Methicillin	10.0	−11,+6
	20.0	− 5,−5
Vancomycin	2.0	−24,+8
	10.0	−23,−19

* Percent response is equivalent to the percent inhibition of NAG release into the supernatant fraction on incubation.

are nephrotoxic and others not, were tested at concentrations achieved in plasma when the drugs are given therapeutically. None showed effects similar to the aminoglycosides (Table 1).

Renal cortical lysosomes were isolated both from rats given gentamicin chronically (more than 28 days) that had kidneys in an induced state of resistance to gentamicin nephrotoxicity and from saline-treated control rats. The gentamicin-treated rats showed an *in vitro* lysosomal resistance to the inhibition of NAG release caused by gentamicin at the 2 and 4 μg/ml concentrations ($P < 0.002$ and $P < 0.004$ respec-

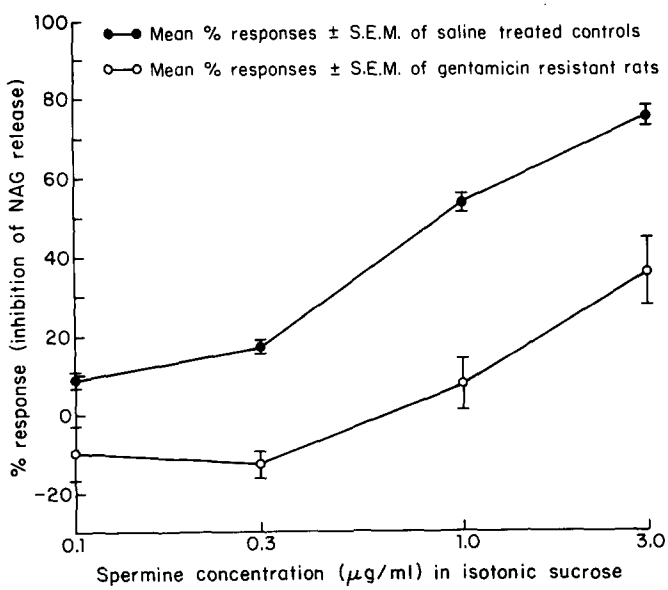

Fig. 7. Spermine dose–response curves of renal cortical lysosomes from saline-treated control rats and gentamicin-resistant rats. All points represent the mean of four experiments. The level of significance is $P < 0.01$ at each concentration.

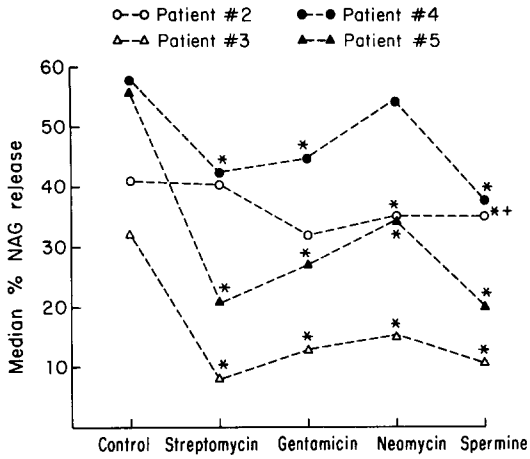

Fig. 8. Response of human renal cortical lysosomes to aminoglycosides. Using renal cortical lysosomes isolated from four patients, the median percent NAG released into the supernatant fraction by the sucrose control is compared with the median percent NAG released on incubation with three aminoglycosides and spermine. The concentrations of aminoglycoside used were 2, 4, 8 and 16 μg/ml. The concentrations of spermine used were 0.4, 0.8, 1.6 and 3.2 μg/ml for all patients except no. 2. Key: (+) the concentrations used were 0.03, 0.1, 0.3 and 1.0 μg/ml; and (*) the level of significance is $P < 0.05$.

tively) compared to control (Fig. 6). Spermine dose–response curves were also compared using renal cortical lysosomes from chronic gentamicin-treated and saline-treated control rats. An *in vitro* resistance to the spermine effect was observed at all four concentrations tested ($P < 0.01$ at each concentration) (Fig. 7).

The aminoglycosides and spermine also showed an *in vitro* effect on human renal cortical lysosomes. Four of the five patients tested had renal cortical lysosomes which showed significant inhibition of NAG release into the supernatant fraction on incubation with aminoglycosides (Fig. 8). In these patients, the baseline control values ranged between 30 and 60% NAG release. The one patient (not shown in figure), who began with a lower baseline control value of about 15% release, demonstrated an enhanced release of NAG into the supernatant fraction with 4 μg/ml concentrations of aminoglycoside ($P < 0.04$). This was the highest concentration tested at that time. All four patients whose renal cortical lysosomes were tested for spermine response demonstrated an inhibition of NAG release. Although we were able to demonstrate responses by lysosomes from all five patients at concentrations of aminoglycosides clinically achieved in plasma, we were not able to demonstrate a dose–response relationship over this concentration range in human renal cortical lysosomes.

DISCUSSION

We have shown that neomycin, gentamicin, tobramycin and streptomycin decreased, in a concentration-related fashion at low concentrations, the amount of lysosomal NAG enzyme released into the supernatant fraction from rat renal cortical lysosomes incubated in isotonic sucrose. This suggests that these drugs stabilize the lysosomal membrane. The amino acid glycine is able to permeate the membrane more readily than sucrose and is unable to protect the lysosome from osmotic disruption. This can be demonstrated by observing the rate of release of lysosomal NAG from renal cortical lysosomes during incubation in isotonic glycine [18]. Our data indicate that the release of NAG caused by incubation of renal cortical lysosomes in isotonic glycine followed a biphasic pattern as a function of the concentration of aminoglycoside in the medium. The three aminoglycosides tested exhibited an inhibition of NAG release at low concentrations. This was followed by enhancement of NAG release at high drug concentrations.

The ability of renal cortical lysosomes to resist disruption in the presence of aminoglycosides was observed in our study at concentrations routinely achieved in plasma of patients receiving these drugs [19, 20]. The rank order of potency for producing this effect on these lysosomes was neomycin ≈ gentamicin > tobramycin > streptomycin. At high concentrations of three aminoglycosides in glycine, the lysosomes demonstrated an enhanced release of NAG. The rank order of potency for producing this effect was neomycin > gentamicin > streptomycin, paralleling their clinically observed potential for producing nephrotoxicity. These findings suggest that the observed response of an apparent lysosomal membrane stabilization followed by rupture may be involved in the production of nephrotoxicity since the renal cortex of rats [17] and humans [4] is able to accumulate the aminoglycosides. The concentrations required to achieve this disruptive response *in vitro* appear to be well within the range of concentrations found within human [4] and rat [17] renal cortex after administration of therapeutic amounts of these drugs. Furthermore, the data from rat studies indicate that the aminoglycosides accumulated in the renal cortex reside almost entirely within the small fraction of the proximal tubule cell volume that corresponds to the lysosomes [2, 3].

Several investigators have shown that rats treated for a prolonged period of time with gentamicin will undergo a period of acute tubular necrosis followed by tubular regeneration and resistance to the nephrotoxic effects of gentamicin [16, 21]. This occurs with continued drug administration. Gilbert *et al.* [16] also showed that this resistance to gentamicin occurs even though the animals continue to concentrate the drug in the renal cortex. Therefore, if the stabilization of the renal cortical lysosomal membrane is involved in the production of nephrotoxicity, lysosomes from chronically treated animals should show less drug-induced stabilization. We observed a decreased response of lysosomes from chronically treated animals when incubated in medium containing 2 or 4 μg/ml of gentamicin in isotonic sucrose. Drug concentrations higher than 16 μg/ml were not tested in this study.

The human renal cortical lysosomes also exhibited a response to aminoglycosides *in vitro*. Lysosomes from four patients were apparently stabilized similar to the stabilization observed in lysosomes from the

experimental animals. Renal cortical lysosomes from one patient had an opposite response of enhanced release of NAG after incubation in 4 μg/ml of aminoglycoside. This patient had the lowest initial control values for enzyme release. Although data from only five patients are presented, it appears that responses to aminoglycosides may differ, depending on the baseline state of the lysosomal population.

To determine the structure–activity relationship with respect to amine groups and the effect on the lysosomal membrane, the polyamines spermine and spermidine were tested in this system. Gentamicin and spermine were additive in their effects of inhibiting the release of NAG from renal cortical lysosomes. Furthermore, rats in an induced state of renal gentamicin resistance have renal cortical lysosomes which *in vitro* are resistant to the stabilizing effect of spermine. Renal cortical lysosomes from four patients were also tested for an *in vitro* response to spermine. All four sets of lysosomes exhibited responses similar to those produced by aminoglycosides. These data appear to indicate that spermine and gentamicin act at the same site on the lysosomal membrane. The polyamines are known to produce stabilization of biological membranes [22, 23]. The cationic amine groups appear to be the part of the structure that produces this effect.

The stabilization of the renal cortical lysosomal membrane by aminoglycosides appears to be consistent with the data from Weglicki *et al.* [24] suggesting that compounds which decrease the activity of lysosomal phospholipases are usually lysosomal membrane stabilizers. The polyamines are thought to stabilize membranes by binding to the negatively charged pole of the phospholipids, producing a charge reversal and, thereby, inhibiting the interaction of the phospholipase enzyme with the substrate [25, 26]. The inhibition of phospholipid catabolism and the resulting phospholipidosis, which occur with aminoglycosides, are also thought to be due to binding of cationic groups to phospholipids, which prevent lysosomal phospholipase enzyme–substrate interaction [9, 27]. This may also be a means by which the aminoglycosides produce stabilization of the lysosomal membrane since the activity of this enzyme in the membrane may be inversely related to its stability [24]. An additional means of stabilizing the membranes may include crosslinking of phospholipids by these polycationic compounds.

The observation of apparent stabilization of lysosomes at low drug concentrations and disruption of them at high concentrations may be related to the experimental conditions. The lysosomes are isolated in 0.3 M sucrose and resuspended in 0.25 M glycine at 0°. This can cause osmotic swelling of the lysosomes. The aminoglycoside is then added and the temperature raised to 37°. The increase in temperature causes expansion of the fluid volume inside the stabilized lysosomal membrane. A lysosomal membrane very highly stabilized by the inhibition of its phospholipases would have a decreased amount of lysolecithin [28] to form micellar pores that permit diffusion of water-soluble particles out of the lysosome [29]. Marked stabilization of the membrane with few pores could limit the outward diffusion of these particles and the establishment of a new osmotic equilibrium. Thus, pressure equalization across the membrane would not occur. These processes that lead to lysosomal swelling and rupture would occur because of a stabilized membrane. Thus, our apparent dose-dependent difference in the nature of the drug effect, i.e. stabilization versus disruption, might be related to the *in vitro* experimental conditions rather than to two different mechanisms of drug action.

Proximal tubule lysosomal enzymuria consistently occurs during aminoglycoside therapy [1, 7, 30] and with various forms of renal disease [31, 32]. Therefore, the presence of an increased amount of these enzymes in the urine appears to be a non-specific indicator of renal injury [14, 31, 32]. While the initial effect of aminoglycosides on the proximal tubular lysosomes seems to be membrane stabilization, the subsequent rupture of the lysosome would cause the lysosomal enzymes to be free in the cell, injuring the cell and thereby causing leakage of these enzymes into the urine.

Our data are consistent with an inverse relationship between lysosomal phospholipid catabolism and membrane stability, as previously shown in hepatic tritosomes [24]. Since binding to the negatively charged pole of phospholipids with prevention of enzyme–substrate interaction is a well-known mechanism of decreasing phospholipase activity [26, 27], other phospholipase-containing membranes may be responsive to this aminoglycoside effect, *in vitro*, as well. The work of Lehninger [33] established the connection between lipolytic activity in the mitochondrial membrane and the structure-linked function of respiratory control. Therefore, an alteration of mitochondrial respiratory function, *in vitro*, in response to aminoglycosides as described by Fabre *et al.* [34] would be anticipated. Lipsky and Lietman [35] described an *in vitro* inhibition of sodium-potassium ATPase activity as a response to a decrease in the fluidity of the phospholipid annulus of this enzyme induced by neomycin. This is also consistent with a decrease in phospholipid catabolism caused by neomycin. However, it is uncertain if aminoglycosides reach these intracellular sites at significant concentrations *in vivo* [2, 3].

We therefore conclude that the lysosome is a site of action for aminoglycoside nephrotoxicity. We propose that aminoglycoside-induced stabilization of the proximal tubular lysosomal membrane may lead to the disruption of the lysosomal system and cell injury.

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