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RENAL MITOCHONDRIAL INTEGRITY DURING CONTINUOUS GENTAMICIN TREATMENT

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Abstract—Rats were given gentamicin over a period of 21 days. At 5, 10, 14 and 21 days renal cortical mitochondria were isolated, and respiratory and Ca^{2+} transport functions and cytochrome concentrations were determined. The mitochondrial data were correlated with indicators of deteriorating renal function and tissue gentamicin accumulation. During the first 10 days of chronic gentamicin treatment, mitochondrial cytochrome oxidase and cytochrome *c* concentrations declined significantly. This decline was followed by a partial spontaneous recovery by days 14 and 21. Cytochrome *b* concentration was not significantly different from normal. Parallel with the cytochrome concentration changes, State 3 respiratory activities with all substrates studied and the rates of Ca^{2+} accumulation declined during the first 10 days and recovered spontaneously thereafter. It is concluded that chronic gentamicin treatment leading to renal failure inhibits mitochondrial energy-linked functions, which inhibition is induced by rate-limiting synthesis of those mitochondrial respiratory chain enzymes coded outside the mitochondrion.

Aminoglycoside antibiotics are widely used to treat serious infections with aerobic gram negative organisms. Despite rigorous monitoring of serum drug concentrations, nephrotoxicity complicates therapy in 10–20% of cases [1]. The precise cellular mechanisms of toxic damage to the renal tubular cell is unclear with inhibition of plasma membrane and lysosomal phospholipases [2, 3] or basolateral membrane Na^+-K^+-ATPase [4] being proposed as major factors in toxicity. Previous workers have reported *in vitro* and *in vivo* abnormalities of mitochondrial respiration and H^+-ATPase activity after exposure to aminoglycosides [5–7]. Although decreases in State 3 and uncoupled respiration occur prior to overt renal failure or tubular necrosis in gentamicin-treated rats [6], redistribution of drug during homogenization of the renal cortex for mitochondrial preparation has been proposed as an explanation for the abnormalities observed [8].

Gilbert *et al.* [9] and Elliott *et al.* [10] have shown that rats continuously treated with gentamicin for 28–42 days develop spontaneous reversal of renal dysfunction as well as regeneration of renal tubular necrosis despite the continuous therapy. The early renal dysfunction and the following "acquired insensitivity" provide an ideal opportunity to study the role of altered mitochondrial function in the pathogenesis of nephrotoxicity. Since concentrations of aminoglycosides in the renal cortex rise during regeneration, the possibility that mitochondrial respiratory dysfunction is due to an artifact of preparation can be directly tested. In addition, the kinetics of renal mitochondrial uptake of calcium and concentrations of mitochondrial respiratory enzymes have not been measured previously during *in vivo* aminoglycoside treatment of experimental animals.

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MATERIALS AND METHODS

Male Fisher 344 rats (Simonsen; Gilroy, CA) were used in all studies. Animals weighed between 200 and 250 g at the beginning of each experiment. They were housed in groups of five to six and were fed standard rat chow replete in all known dietary requirements including those for sodium, potassium, magnesium and calcium. Animals were given free access to water. Gentamicin (Schering Corp., Kenilworth, NJ) was administered subcutaneously, 20 mg/kg twice a day diluted to a total volume of 0.5 ml. Groups of six to ten animals were treated for 3, 5, 7, 10, 14 and 21 days. Concomitantly, twenty animals of the same age were treated with equivalent volumes of sterile saline, the vehicle for gentamicin.

Twenty-four hours prior to being killed, animals were placed in metabolic cages for collection of accurate 24-hr urine volumes. Inulin clearance was determined within 12 hr of the last dose of gentamicin by the method of Stitzer and Martinez-Maldonado [11] using ^{14}C inulin. Clearances reported are the mean values of four 20-min collection periods.

Tissue from one kidney was prepared for cortical slice studies as previously described in detail [12]. In brief, after weighing, freshly cut 4-mm thick slices were incubated for 90 min in Cross–Taggart medium [13] containing [^3H]-tetraethylammonium (TEA) and *para*-aminohippurate (PAH). The slice to medium ratios of the radioactivity and PAH concentrations reflect the activities of tubular transport systems for organic bases and acids respectively.

Gentamicin concentrations were measured in the

renal cortex by radioimmunoassay (Diagnostic Products, Los Angeles, CA). Tissue was taken for histological examination, embedded in paraffin, and stained with hematoxylin and eosin. The extent of tubular necrosis and regeneration was graded in a blind manner without knowledge of the treatment group of the animal.

Isolation of renal cortical mitochondria. Gentamicin-treated and sham control animals were killed by decapitation after 5, 10, 14 and 21 days of treatment. Renal cortical tissue from both kidneys was collected immediately, minced, and cooled in ice-cold isolation medium and used for mitochondrial isolation.

Mitochondrial isolation from renal cortex was performed without delay according to previously established methods [14]. All procedures were performed at 0–4%. The isolation medium consisted of 120 mM KCl and 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), pH 7.4. The minced tissue was suspended in 30 ml of isolation medium and homogenized in a Potter–Elvehjem tissue homogenizer using a Teflon pestle. The homogenate was centrifuged at 700 *g* for 5 min to remove cell debris and nuclei. The supernatant fraction was then centrifuged at 7700 *g* for 10 min to collect the mitochondrial pellet. The pellet was homogenized gently by hand and suspended in isolation medium to wash the mitochondria. After centrifugation at 7700 *g* for 5 min, the mitochondrial pellet was again gently rehomogenized by hand and the washing procedure was repeated three times in 120 mM KCl (no EGTA) to remove EGTA and contaminating subcellular organelles from the preparation. The final pellet was suspended in 120 mM KCl at 15–25 mg protein/ml and stored on ice.

Mitochondrial respiratory activity. Respiratory activities of the mitochondrial suspensions were determined with a Clark oxygen electrode at 23°. The reaction medium contained 120 mM KCl, 10 mM Tris-Cl, 10 mM Tris-P_i pH 7.4. State 4 (excess substrate, no ADP) and State 3 (substrate plus ADP) respiratory activities were measured with the following substrates as electron donors: 5 mM glutamate and 2 mM malate; 5 mM pyruvate and 2 mM malate; 5 mM β-hydroxybutyrate and 2 mM malate; 5 mM α-ketoisocaproate and 2 mM malate. To initiate State 3 respiratory activity, 500 μM ADP was added to the cuvette. The respiratory rates were calculated as nmoles O_2 utilized per minute per milligram of mitochondrial protein. State 3 rates were also calculated as moles of O_2 utilized per mole of cytochrome oxidase per minute. The data are expressed as means ± standard errors for groups of five to nine animals for each time point.

Mitochondrial calcium accumulation rates. The rates of calcium accumulation by the mitochondria were measured with a Ca^{2+} indicator dye, murexide, according to the method of Mela and Chance [15]. The reaction medium contained 120 mM KCl, 20 mM Tris-Cl, 1 mM Tris-P_i, 40 μM murexide, 4 mM succinate, and 4 μM rotenone, pH 7.4. The absorbance change induced by the complexation of murexide with Ca^{2+} was monitored in a differential dual wavelength spectrophotometer at 540–507 nm. Murexide does not cross the mitochondrial membrane and, thus, detects the extramitochondrial Ca^{2+} concentration. The rate of disappearance of added Ca^{2+} from the extramitochondrial medium into the mitochondria was determined from the initial slope of the absorbance change after the addition of Ca^{2+}. Using this slope, the mitochondrial Ca^{2+} accumulation rate was calculated as nmoles of Ca^{2+} accumulated per minute per milligram of mitochondrial protein or as moles of Ca^{2+} per minute per mole of cytochrome oxidase. The data are expressed as means ± standard errors for groups of five to nine animals each.

Mitochondrial cytochrome concentrations. The reduced minus oxidized difference spectra of the mitochondrial suspensions were obtained in a Hitachi 557 differential dual wavelength spectrophotometer suitable for optical measurements of turbid suspensions. The spectral changes were measured in one cuvette by scanning the optical density within the visible region from 700 to 400 nm against the reference wavelength of 700 nm. The optical density change induced by the transition from a fully oxidized state (+ rotenone) to a fully reduced state (succinate and ADP added to reach anaerobiosis) was measured, and the concentrations of mitochondrial cytochromes were calculated using the following extinction coefficients: cytochrome oxidase $(aa_3)_{605-630\,nm}$, $E = 26\,mM^{-1}cm^{-1}$; cytochrome $c_{550-540\,nm}$, $E = 20\,mM^{-1}\,cm^{-1}$; cytochrome $b_{560-575\,nm}$, $E = 24\,mM^{-1}\,cm^{-1}$. The concentrations of the cytochromes are expressed as nmoles cytochrome per milligram of mitochrondrial protein, and are presented as means ± standard errors of groups of five to nine animals each.

Protein concentrations. Protein concentrations of the mitochondrial suspensions were determined according to Lowry *et al.* [16].

Data analysis. Data are reported as the mean ± S.E. Differences between treatment and control groups were analyzed by Student's *t*-test, while data at various time points were subjected to analysis of variance.

RESULTS

Renal function studies are displayed in Table 1. Azotemia and elevations in creatinine were significantly above control by day 5, corresponding to a decline in glomerular filtration rate (C_{In}) ($P < 0.01$). Abnormalities of tubular function as measured by slice transport and urine volume did not become evident until day 7 (TEA) ($P < 0.01$) and day 10 (PAH) ($P < 0.01$). The nadir of glomerular function measured by inulin clearance and tubular function measured by slice transport was reached at day 14. Despite continuous gentamicin treatment, spontaneous improvement of BUN, creatinine, inulin clearance, slice-to-medium PAH and TEA ratios occurred by day 21 in surviving animals.

Gentamicin-treated animals developed a continuous weight loss which became significant ($P < 0.01$) by the third treatment day. Kidney weight gradually increased becoming significantly elevated over control values at day 7 ($P < 0.01$).

Gentamicin accumulated in the renal cortex to peak values at day 5. With the onset of frank tubular

Table 1. Renal function data

Group	N	BUN (mg/dl)	Cr (mg/dl)	C_{in} (ml/min/100g body wt)	PAH (slice to medium ratio)	TEA	Urine volume (ml/24hr)	Body wt (g)	Kidney wt (g)	Gentamicin concn (μg/g wet wt tissue)
Control	20	22.9 ± 5.0	0.31 ± 0.13	0.78 ± 0.06	5.5 ± 0.61	13.2 ± 2.1	8.8 ± 3.7	255 ± 21	0.95 ± 0.07	
Day 3	6	24.8 ± 7.6	0.48 ± 0.17	0.52 ± 0.17	5.6 ± 0.54	9.4 ± 2.3	9.5 ± 2.8	221 ± 14*	0.96 ± 0.08	496 ± 124
Day 5	9	32 ± 4.5*	0.58 ± 0.16*	0.42 ± 0.16*	5.9 ± 0.74	11.1 ± 3.6	7.3 ± 3	218 ± 15*	0.98 ± 0.06	644 ± 87
Day 7	6	39.3 ± 13*	1.3 ± 0.67†	0.25 ± 0.12†	5.5 ± 0.44	8.4 ± 0.65*	21 ± 11.2*	247 ± 19*	1.17 ± 0.05*	447 ± 63
Day 10	10	163 ± 72†	7.5 ± 3.7†	0.03 ± 0.05†	2.5 ± 1.1*	2.5 ± 1†	10.5 ± 6.3	208 ± 11†	1.05 ± 0.07	266 ± 38
Day 14	4‡	291 ± 40†	10.4 ± 0.64†	0.04 ± 0.04†	1.2 ± 0.07†	0.28 ± 0.05†	10 ± 2.2	192 ± 14†	1.18 ± 0.07†	270 ± 10
Day 21	7	116 ± 81*§	1.8 ± 0.87†‖	0.21 ± 0.2†‖	2.6 ± 0.46*§	4.7 ± 1.8†§	24.8 ± 14.5*	159 ± 23†	1.65 ± 0.29†	399 ± 110§

* $P < 0.01$ vs control.
† $P < 0.001$ vs control.
‡ Three additional animals died from uremia prior to study.
§ $P < 0.05$ vs day 14.
‖ $P < 0.01$ vs day 14.

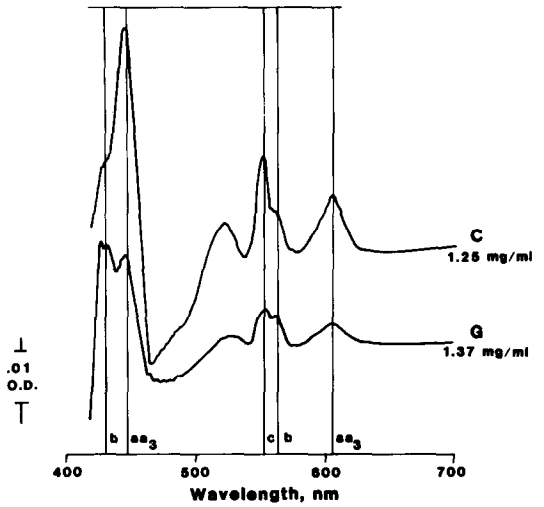

Fig. 1. Reduced–oxidized difference spectra of kidney mitochondrial suspensions from control (C) and gentamicin-treated (G) rats. Mitochondria were suspended at 1.25 mg protein/ml (C) and at 1.37 mg protein/ml (G) in reaction medium containing 120 mM KCl, 10 mM Tris-Cl and 10 mM Tris-phosphate, pH 7.4. The spectra were generated as described in Methods.

necrosis, cortical concentrations decreased to their nadir. With continuous treatment and histological tissue evidence of regeneration, drug concentrations rose by day 21 ($P < 0.05$ vs nadir values).

Control animals developed no histologic abnormalities. In treated animals, proximal tubular necrosis began focally at day 5 and increased in extent until days 10–14. This was followed by epithelial regeneration and tubular restoration during continuous gentamicin administration. The combined extent of regeneration and necrosis was maximal at 14 days. The regenerating epithelium matured

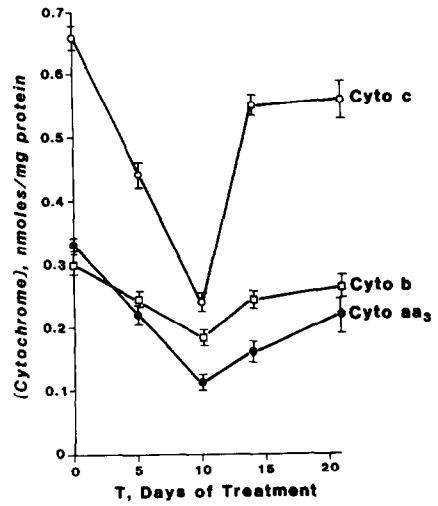

Fig. 2. Concentration changes of cytochromes *c*, *b* and aa_3 of rat kidney mitochondria in control (zero time) and gentamicin-treated rats as a function of the length of treatment. The concentrations of cytochromes were calculated as described in Methods using the α-peaks of the reduced–oxidized difference spectra.

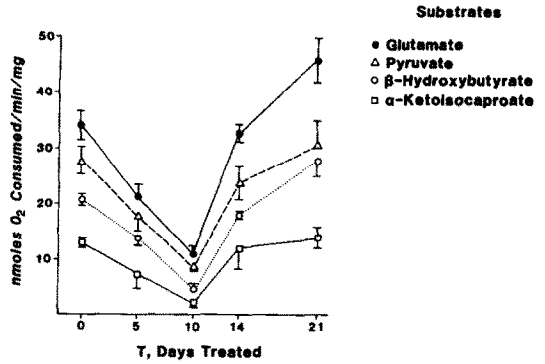

Fig. 3. Kidney mitochondrial State 3 respiratory activities as a function of the length of *in vivo* gentamicin treatment. Glutamate, pyruvate, β-hydroxybutyrate or α-ketoisocaproate in the presence of malate were used as substrates. To induce State 3 respiration 500 μm ADP was added to the cuvette. O_2 consumption rates were determined and calculated as described in Methods.

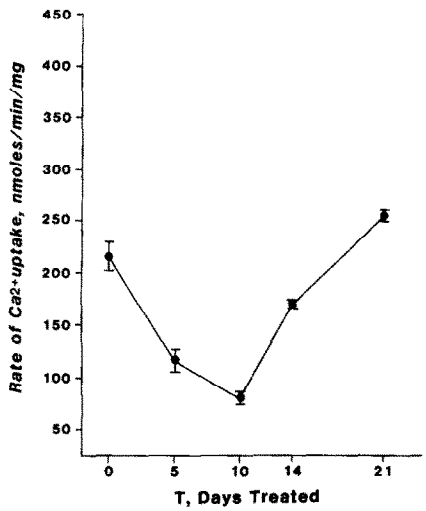

Fig. 4. Initial rates of kidney mitochondrial Ca^{2+} accumulation as a function of the length of gentamicin treatment. As described in Methods, accumulation of Ca^{2+} from the medium into the mitochondria was measured spectrophotometrically with murexide as the Ca^{2+} indicator and the rates calculated as nmoles Ca^{2+} accumulated per min per mg protein.

subsequently so that the histological appearance was nearly normal by day 21.

Concentrations of cytochromes. Characteristic reduced minus oxidized difference spectra of renal cortical mitochondrial cytochromes are shown in Fig. 1. The upper spectrum was obtained in mitochondria isolated from a control animal (C). The mitochondria were suspended at 1.25 mg protein/ml in a single cuvette, and the spectrum was obtained as described in Methods. The α-peaks of cytochrome aa_3 (605 nm), cytochrome *b* (560 nm) and cytochrome *c* (550 nm) can be clearly identified. The corresponding γ-peaks for cytochrome aa_3 at 445 nm and cytochrome *b* at 430 nm can be seen. The lower spectrum (G) was obtained in a similar manner in a suspension of mitochondria at 1.37 mg protein/ml isolated after 10 days of gentamicin treatment. The spectrum demonstrates a reduced level of cytochrome aa_3 measured at 605 nm or 445 nm and a reduced level of cytochrome *c* seen by the small peak at 550 nm. The absorption peaks of cytochrome *b* of gentamicin-treated animals were only slightly below normal.

The calculated concentrations of cytochromes aa_3, *b* and *c* of renal cortical mitochondria are presented in Fig. 2 as a function of the duration of gentamicin treatment. Each point represents the mean value ±S.E. for five animals. At all time points measured, after 5, 10, 14 and 21 days of gentamicin treatment, cytochrome aa_3 was significantly below normal ($P < 0.0005$). Cytochrome *c* was significantly below normal after 5 and 10 days ($P < 0.0005$) as well as after 14 days ($P < 0.005$) and 21 days ($P < 0.05$). Although the concentrations of cytochrome *b* were below normal at all measured time points, the data were not significantly different from control values.

Mitochondrial capacity to consume oxygen and synthesize ATP. Figure 3 illustrates the effect of gentamicin treatment on mitochondrial capacity to respire in State 3 (during ATP synthesis). Respiratory activities of renal cortical mitochondria were determined during utilization of various fuels during the active state of respiration (State 3). Glutamate and pyruvate (glucose derived), and α-ketoisocaproate (branched chain amino acid derived) supported respiratory activities were reduced significantly after 5 and 10 days of gentamicin treatment, reaching a highly significant reduction by 10 days ($P < 0.0005$). A gradual spontaneous recovery of normal respiratory activity occurred between days 10 and 14; this recovery continued throughout the examination period. At 14 and 21 days, the respiratory activities with any of the used substrates were not significantly different from control values. A similar pattern of changes was seen in resting respiratory rates (State 4, data not shown). Thus,

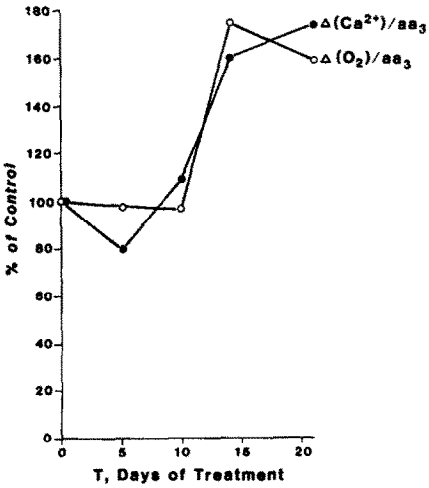

Fig. 5. Kidney mitochondrial State 3 O_2 utilization and Ca^{2+} accumulation rates standardized as moles per min per mole of cytochrome oxidase in control (zero time) and gentamicin-treated animals. Data are derived from experiments presented in Figs. 2–4.

no significant alterations were found in respiratory control ratios (State 3/State 4).

Calcium transport activity. Figure 4 illustrates a kinetic measurement of mitochondrial Ca^{2+} uptake rates using murexide as an extramitochondrial Ca^{2+} indicator. This is an established technique carefully characterized by one of us [15] and subsequently used by many investigators. The experiment illustrated in Fig. 4 shows a Ca^{2+} uptake rate of 244 nmoles Ca^{2+}/min/mg protein after an addition of 100 μM Ca^{2+} in a preparation of control mitochondria suspended at 0.9 mg protein/ml. In Fig. 5 data from control and gentamicin-treated animals are presented. After 5 and 10 days of gentamicin treatment, the Ca^{2+} uptake capacity of renal cortical mitochondria was significantly below control level ($P < 0.0005$). Between days 10 and 14 a spontaneous recovery of the Ca^{2+} uptake activity occurred. After 14 days of treatment the Ca^{2+} uptake activity remained below normal ($P < 0.05$). After 21 days of treatment renal cortical mitochondria exhibited Ca^{2+} uptake activity slightly above normal ($P < 0.05$).

DISCUSSION

The biochemical and toxicologic events leading to tubular necrosis following aminoglycoside treatment are controversial. Since aminoglycosides are rapidly incorporated into lysosomes of proximal tubular cells after glomerular filtration, these organelles are felt to be a primary target for aminoglycoside-induced cell injury [17]. Phospholipases in brush border membranes and within lysosomes are inhibited by concentrations of drug readily achievable within the cell. This inhibition results in an acquired phospholipidosis [3]. The mechanism of cellular necrosis which might follow lysosomal swelling and phospholipid accumulation has never been elucidated.

Plasma membrane enzymes such as renal sodium-potassium activated ATPase are inhibited *in vitro* and *in vivo* by aminoglycosides [8, 18]. The role of membrane ATPase inhibition as the primary mechanism for renal cell injury is unlikely since these abnormalities are evident after a single dose [8], while changes in tubular structure and function become detectable after a 5- to 10-day delay in most experimental models.

Previous workers have demonstrated mitochondrial abnormalities in aminoglycoside-treated animals. Simmons *et al.* [19] showed that kidney ATP content, State 3 respiration, and uncoupled respiration were depressed in rats receiving gentamicin in doses similar to those used in the current study. State 4 respiration was unaffected as were mitochondria isolated from the liver. Since those changes were evident prior to cell necrosis or overt morphologic changes in the mitochondria, it was concluded that they were not terminal, secondary to cell death [19].

Our data indicate that chronic treatment causes several significant alterations of mitochondrial function and respiratory chain enzyme concentrations. After 5 and 10 days of gentamicin treatment, State 3 respiratory activities with all substrates tested were significantly below normal. State 4 respiration (data not shown) was similarly below normal, resulting in no change in the respiratory control ratios. Thus, no indication of uncoupling by gentamicin is seen. The rate of Ca^{2+} accumulation, supported by substrate oxidation as an energy source, was similarly reduced. Since both cytochrome *c* and cytochrome aa_3 were reduced significantly in concentration, it is possible that the reduced concentrations of respiratory chain enzymes are responsible for the reduction of the functions dependent on an intact respiratory chain.

It is particularly interesting that chronic gentamicin treatment influences the synthesis of those mitochondrial components coded outside the mitochondria themselves. Only three major mitochondrial components are coded by the mitochondrial genome: cytochrome *b*, subunits I, II and III of cytochrome oxidase, and subunits VI and IX of the F_1-ATPase [20]. All other mitochondrial components are coded and synthesized elsewhere. It appears from our data that the mitochondria themselves are not primary targets in gentamicin nephrotoxicity. Due to reduced protein synthesis by the extramitochondrial genetic machinery, the mitochondrial concentrations of cytochrome *c* and aa_3 fall significantly. It is also possible that other important mitochondrial respiratory chain components such as the dehydrogenases and the essential enzymes F_1-ATPase and the adenine nucleotide translocase as well as the Ca^{2+} carrier protein are reduced in concentration. We have no direct measurements to confirm this possibility. The decline in activities such as State 3 and 4 respiration and Ca^{2+} uptake, however, could be a result of such decline in enzyme concentrations. This suggestion can be made based on the data presented in Fig. 5. The State 3 respiratory activity and Ca^{2+} transport activity per mole of cytochrome oxidase were calculated. It is clear from these data that, although these activities decline per milligram of total mitochondrial protein, they are not altered per mole of cytochrome oxidase. Thus, no change in the specific *activity* of the respiratory chain occurs. After 10 days of gentamicin treatment, the spontaneous recovery of cytochromes *c* and aa_3, however, occurs at a much slower rate. Thus, it appears that the sudden large increase in mitochondrial respiratory chain capacity is brought about by some other unknown mechanism. This increased activity is followed at a slower rate by recovery of protein synthesis and renal function. The trigger for this sudden recovery is not known.

Our data suggest that mitochondria are indirectly affected by aminoglycosides. Initial mitochondrial abnormalities precede overt cell necrosis, tubular dysfunction, and renal failure whereas improving mitochondrial parameters would herald structural and functional recovery. Falls in cytochrome concentrations suggest interruption of cytochrome enzymes synthesis since both cytochrome *c* and the transmembrane located cytochrome aa_3 were affected. The insignificant fall in cytochrome *b* that is coded by the mitochondrial genome is of interest. Gentamicin is known to kill bacteria by binding irreversibly to the 30S subunit of bacterial ribosomes and causing blockage of protein synthesis through failure of initiation and misreading of messenger RNA [21]. One might speculate that aminoglycosides that gain access to cytosol cause decreases in enzyme

synthesis which ultimately produce the observed mitochondrial functional abnormalities.

Sastrasinh *et al.* [22] have shown inhibition of renal mitochondrial calcium transport when aminoglycosides are incubated with mitochondria *in vitro*. Other *in vitro* effects of aminoglycosides incubated with renal mitochondria include inhibition of State 3 and uncoupled respiration together with stimulation of basal State 4 activity [5]. Because of the differences between the *in vivo* and *in vitro* effects observed, the speculation has arisen that the procedure used to isolate mitochondria allows mitochondria to become exposed to drug artifactually [8]. Our data exclude this possibility since improvement in respiratory activity, cytochrome concentrations and calcium uptake took place at times when cortical gentamicin concentrations were *increasing*. Our *in vitro* data on the effects of gentamicin on renal mitochondrial function do not support the possibility that gentamicin affects renal mitochondria directly during the isolation. We found that gentamicin added *in vitro* at concentrations up to 4 mM had no effect on renal mitochondrial respiratory activity functions (data not shown), whether KCl or mannitol-sucrose was used in the isolation and reaction media.

Our data also speak against the possibility that *in vivo* gentamicin treatment causes generalized damage and leakiness of the mitochondrial membranes. In addition to cytochrome *c*, cytochrome oxidase concentration was reduced, but cytochrome *b* was normal. The respiratory control ratios were not altered by chronic gentamicin treatment. The yields of total mitochondrial protein in each preparation were not significantly different in control and gentamicin-treated animals. These findings speak against decreased purity of the mitochondrial fraction in gentamicin-treated animals.

The present data confirm our earlier observations of spontaneous functional and structural recovery from aminoglycoside-induced renal tubular injury during continuous therapy [9, 10]. Although this phenomenon has been designated as "acquired resistance," the mechanisms are unclear. It is of some interest that radioautographic studies have documented drug entry into tubular cells without apparent detrimental effects during the phase of insensitivity [23]. Powell and Reidenberg [2] have reported that lysosomes are resistant to aminoglycosides during this period of insensitivity. Perhaps this allows the resynthesis of respiratory enzymes and the observed improvement in mitochondrial function that precedes recovery of GFR and histologic cell regeneration.

It is possible and probably likely that the mechanism of cellular necrosis due to aminoglycosides is complex. It is, however, appealing to consider that processes similar to those described for the beneficial effects of aminoglycoside antibiotics also play a role in nephrotoxicity.

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