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Induction of Nephrotoxicity by High Doses of Gentamicin in Diabetic Rats (42618)

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Abstract. Rats with streptozotocin-induced diabetes mellitus (DM) are resistant to aminoglycoside (AG) nephrotoxicity presumably because of defective transport and accumulation of drug by proximal tubular cells. To test this hypothesis we injected DM rats with saline or with gentamicin, 100, 200, and 400 mg/kg per day for 6 days, to determine if the renal cortical concentration of gentamicin could be raised to toxic levels. Nephrotoxicity was assessed by monitoring for evidence of accelerated lipid peroxidation in the renal cortex, for elevation of the serum creatinine concentration, and for evidence of proximal tubular cell injury and necrosis by light and electron microscopy. At 100 mg/kg per day renal cortical gentamicin was 454 ± 85 μg/g. Except for an increase in renal cortical phospholipids these rats manifested no evidence of accelerated lipid peroxidation or elevation of serum creatinine. At 200 mg/kg per day renal cortical gentamicin rose to 636 ± 20 μg/g. These rats manifested mild functional and morphological evidence of toxicity. At 400 mg/kg renal cortical gentamicin rose to 741 ± 43 μg/g. These rats developed severe nephrotoxic injury as manifested by a marked increase of lipid peroxidation evident by an increase of malondialdehyde from a control level of 0.48 ± 0.02 to 1.72 ± 0.12 nmole/mg protein, a shift from unsaturated to saturated fatty acids esterified in renal cortical phospholipids, depression of superoxide dismutase and catalase, and a shift from reduced to oxidized glutathione. The serum creatinine rose from a baseline level of 0.24 ± 0.01 to 0.46 ± 0.05 mg/dl. Light and electron microscopy revealed enlarged lysosomes distended with typical myeloid bodies and extensive proximal tubular cell necrosis. These observations provide compelling evidence in support of the view that the resistance of DM rats to AG nephrotoxicity is causally linked to the low rate of drug uptake by renal proximal tubular cells. When the renal cortical concentration reaches a critical level, it elicits a pattern of toxic injury indistinguishable from that of nondiabetic rats. Thus, there is nothing inherent to the diabetic state that prevents AGs from causing their usual adverse effects on the metabolism of renal proximal tubular cells once they gain access in sufficient quantity into these cells.

Aminoglycoside (AG) antibiotics are widely used in clinical medicine for the therapy of gram-negative bacterial infections (1). The major drawback of these agents is the risk of nephrotoxicity (2) and ototoxicity (3). At the level of the kidney these drugs induce proximal tubular cell injury and necrosis which clinically is typically expressed as nonoliguric acute renal failure (4). In considering why renal proximal tubular cells and cells of the inner ear are more susceptible to toxic injury from AGs than other cells of the body, the most relevant characteristic shared by these cells is that they actively transport and accumulate these drugs to a far greater extent than other cells (5, 6). This fundamental observation has led to the hypothesis that the pathogenesis of AG nephrotoxicity involves a two-step process: the first step involves the uptake of drug by proximal tubular cells; the second step can be characterized as an adverse interaction of these organic polycations with one or more intracellular metabolic processes (7).

The rat with streptozotocin-induced diabetes mellitus (DM) has been shown to be remarkably resistant to the induction of acute renal failure by aminoglycosides (8–11). This resistance has been attributed to the low rate of drug accumulation in the renal cortex of these animals (8, 9, 11) which we have shown in the case of netilmicin is secondary to a depressed rate of transport as well as a defect in the intracellular trapping of drug by proximal tubular cells (12). In the present study we sought to directly test this

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hypothesis by injecting diabetic rats with large doses of gentamicin in an attempt to raise the concentration of drug within renal proximal tubular cells and induce toxicity.

Methods. DM was induced in male Sprague-Dawley rats weighing 200–225 g by injecting streptozotocin, 65 mg/kg body weight, iv. The rats were studied approximately 4 to 5 weeks later at which time they manifested hyperglycemia (>500 mg/dl), 4+ glucosuria, and polyuria. Rats were injected with saline, or gentamicin, 100 mg (base)/kg body wt qd or 100 or 200 mg/kg b.i.d. for 6 days. Twenty-four hours after the last injection the rats were anesthetized with pentobarbitol, 50 mg ip, and sacrificed by exsanguination from the aorta. The kidneys were removed and the renal cortex was dissected and homogenized in ice-cold 0.9% NaCl buffered to pH 7.4 with sodium phosphate. Aliquots of homogenate were assayed for total phospholipid, malondialdehyde (MDA), superoxide dismutase (SOD), catalase, glutathione, protein, and gentamicin. Blood was assayed for creatinine. In four rats from each group the kidneys were perfused *in situ* with glutaraldehyde and processed for subsequent examination by light and electron microscopy as previously reported from this laboratory (13).

Renal cortical phospholipids were extracted in chloroform:methanol (2:1, v:v) containing 10 m*M* tetrabutyl ammonium sulfate and quantitated by measuring inorganic phosphorus (14). Fatty acids were removed from phospholipids by alkaline hydrolysis and simultaneously converted to methyl ester derivatives which were analyzed by GLC (15). MDA was assayed by the thiobarbituric acid procedure (16). SOD was measured as the rate of inhibition of ferricytochrome *c* reduction in a xanthine:xanthine oxidase-generating system (17). Catalase activity was assayed by titrating $KMnO_4$ as described by Cohen *et al.* (18) and expressed as the first-order reaction rate constant, k, which is defined by the equation $k = \log (S_0/S_t) \times 2.3/t$ where t = time, S_0 = initial concentration of $KMnO_4$, and S_t = the concentration of $KMnO_4$ at time t. Total glutathione, defined as the sum of reduced (GSH) and oxidized (GSSG) glutathione, was assayed on the supernatant fraction remaining after TCA precipitation and ether extraction by monitoring spectrophotometrically the reduction of 5,5-dithiobis-2-nitrobenzoic acid by NADPH (19). This reaction is catalyzed by GSH or GSSG and is linearly dependent on the concentration of glutathione. GSSG was measured after removing GSH by trapping with *N*-ethylmaleimide and extracting with ether. Protein was assayed by the method of Lowry *et al.* (20). Gentamicin was measured by an EMIT assay as previously reported (21). Serum creatinine was measured by the method of Mitchell (22).

The data were subjected to analyses of variance and the Duncan multiple-range test to identify statistically significant differences among the groups.

Results. DM rats injected with gentamicin developed significant increases of renal cortical phospholipids ranging from 8% in rats injected with 100 mg/kg per day to 17% in rats injected with 400 mg/kg per day (Table I). With respect to the other variables measured no significant changes from control were detected in rats injected with 100 mg of gentamicin/kg per day. In contrast, rats injected with 200 and 400 mg of drug/kg per day manifested dose-related increases of MDA, an end-product of lipid peroxidation in the renal cortex (Table I) and a shift from polyunsaturated to saturated fatty acids esterified in renal cortical phospholipids (Fig. 1). This shift is reflected by the progressive decline of the average number of double bonds (Δ) per mole of fatty acid (Table I). These rats also manifested a dose-related depression of SOD and catalase (Table I) and an increased ratio of oxidized (GSSG) to total glutathione (Fig. 2). Rats injected with gentamicin at 200 mg/kg per day experienced a slight rise of the serum creatinine concentration whereas in rats injected with 400 mg/kg per day the serum creatinine increased to almost twice that of the control group (Table I).

The concentration of gentamicin in renal cortex increased in a dose-dependent fashion (Table I). In view of the fact that rats injected with 400 mg of gentamicin/kg per day experienced extensive proximal tubular cell necrosis (see below), the measured concentration of drug in renal cortex underestimates the maximal concentration of drug present

TABLE I. RESPONSE OF DM RATS TO INCREASING DOSES OF GENTAMICIN[a]

	Control (N = 16)	Gentamicin 100 mg/kg (N = 6)	Gentamicin 200 mg/kg (N = 12)	Gentamicin 400 mg/kg (N = 11)
Phospholipid (μmol/g dry wt)	183 ± 2	204 ± 2*	199 ± 3*	214 ± 8*
MDA (nmol/mg protein)	0.48 ± 0.02	0.53 ± 0.04	0.66 ± 0.04*,**	1.72 ± 0.12*,***
Δ/mol FA	1.32 ± 0.02	1.33 ± 0.05	1.14 ± 0.02*,***	1.09 ± 0.06*
SOD (units/mg protein)	18.7 ± 0.6	18.9 ± 2.6	15.3 ± 0.5*,**	10.1 ± 1.0*,***
Catalase (k/min)	0.180 ± 0.008	0.177 ± 0.012	0.109 ± 0.003*,***	0.044 ± 0.003*,***
Serum Creatinine (mg/dl)	0.24 ± 0.01	0.25 ± 0.01	0.29 ± 0.02	0.46 ± 0.05*,***
Gentamicin (μg/g cortex)	—	454 ± 85	636 ± 29***	741 ± 43**

[a] MDA, malondialdehyde; Δ/mole FA, number of double bonds per mole fatty acid; SOD, superoxide dismutase.
* Significantly different from control, $P < 0.01$.
** Significantly different from preceding value, $P < 0.05$.
*** Significantly different from preceding value, $P < 0.01$.

in renal cortex prior to the onset of acute tubular necrosis and the resultant sloughing of drug-ladened cells.[1]

Pathology. Paraffin sections stained with hematoxylin and eosin revealed no abnormalities of kidneys from control DM rats. In DM rats injected with gentamicin irregularly stained cytoplasmic inclusions were present in proximal tubular cells; these increased in number and size as a function of drug dose and were accompanied by increased proximal tubular cell necrosis and cell dropout. In rats injected with 100 mg/kg per day rare single cell necrosis or dropout was seen. In rats injected with 200 mg/kg pre day cell necrosis or dropout was evident in 10 to 20% of proximal tubular profiles. In rats injected with 400 mg/kg per day extensive proximal tubular cell necrosis or dropout was observed in more than 60% of proximal tubular profiles. At the ultrastructural level most of the inclusions in proximal tubular cells were irregularly shaped membrane-bound areas with one or more myeloid bodies. The inclusions occupied 8 ± 3% of the cytoplasm of proximal tubular cells of inner cortex in rats injected with 100 mg of gentamicin/kg per day, 14 ± 8% in rats injected with 200 mg/kg per day, and 17 ± 10% in rats injected with 400 mg/kg per day.

Discussion. Rats with streptozotocin-induced DM exhibit decreased transport and accumulation of AG by renal proximal tubular cells (8, 9, 11, 12) and this finding has been advanced as the explanation for the resistance of DM rats to AG nephrotoxicity. To directly test this hypothesis we injected DM rats with two- and fourfold the dose of gentamicin, which we have shown previously causes a reproducible degree of renal injury in non-DM rats (7, 15, 21), in an attempt to raise the renal cortical concentration of gentamicin to toxic levels. Nephrotoxicity was assessed in terms of lipid peroxidation, elevation of the serum creatinine, and morphologic lesions. Recent studies reported from our laboratory demonstrate that accelerated lipid peroxidation is an early and sensitive index of nephrotoxicity. For this purpose we measured MDA, an end-product of lipid peroxidation, as well as the shift from polyunsaturated to saturated fatty acids esterified in renal cortical phospholipids, which pro-

[1] The sloughing of necrotic proximal tubular cells observed in DM rats injected with 400 mg of gentamicin/kg is likely to have resulted also in an underestimation of total renal cortical phospholipids and MDA as well as an underestimation of the magnitude of alterations of the glutathione cascade, of catalase activity and of SOD. This possibility does not change the interpretation of the data.

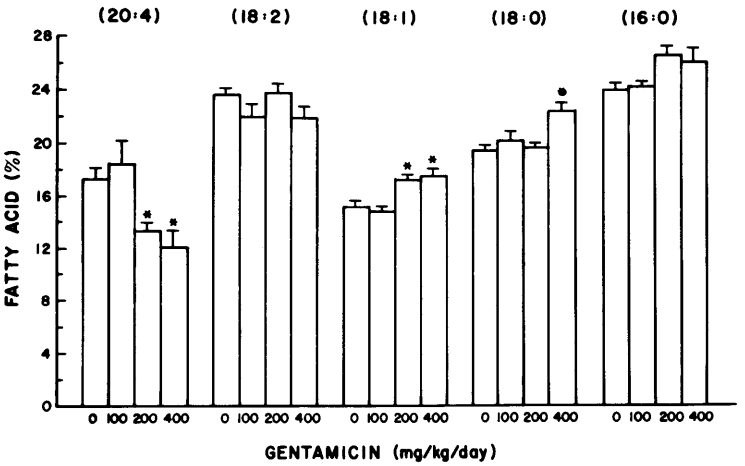

FIG. 1. Effect of gentamicin on the fractional distribution of fatty acids esterified in renal cortical phospholipids. Data represent means ± SE. Numbers in parentheses above bars indicate numbers of carbons and double bonds per fatty acid. 20:4, arachidonic acid; 18:2, linoleic acid; 18:1, oleic acid; 18:0, stearic acid; 16:0, palmitic acid. *Significantly different from control, $P < 0.05$.

vides another measure of lipid peroxidation (23).

We have shown previously that non-DM rats injected with gentamicin at 100 mg/kg for 6 days accumulated 1276 ± 60 μg of drug per gram of renal cortex and manifested frank nephrotoxicity evident by accelerated lipid peroxidation in the renal cortex, elevation of the serum creatinine concentration, and the presence of widespread proximal tubular cell necrosis (24). In contrast, in DM rats injected with gentamicin at 100 mg/kg per day for 6 days the amount of drug accumulated in renal cortex measured only 454 ± 85 μg/g, and these rats did not manifest evidence of renal injury as assessed by the absence of accelerated lipid peroxidation, by the failure of the serum creatinine concentration to rise, and by the virtual absence of proximal tubular cell necrosis. These rats did develop a phospholipidosis, which implies that the drug was sequestered within lysosomes and impaired the degradation of phospholipid as evident by an ultrastructural le-

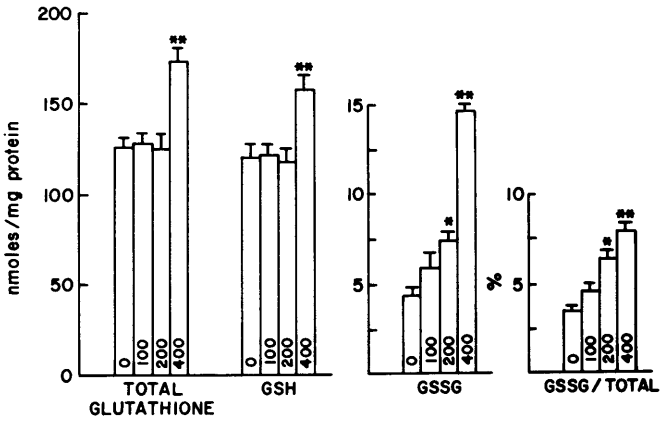

FIG. 2. Effect of gentamicin on total glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG) in renal cortex. Data represent means ± SE. *Significantly different from control, $P < 0.05$; **significantly different from control, $P < 0.01$.

sion, the lysosomal myeloid body (25). Thus, this aspect of our study confirms the observation of other investigators (8–11) that at a dose of gentamicin which causes renal failure in non-DM rats no appreciable renal dysfunction or injury is evident in DM rats.

That a higher dose of gentamicin might overcome the resistance of DM rats to nephrotoxicity was suggested originally by the study of Vaamonde *et al.* (26) who injected rats with 200 mg of drug in two daily doses for 11 days. Whereas all non-DM rats were dead by Day 6, all DM rats survived the experiment although they manifested mild renal impairment. The present study confirms and expands upon these findings.

When the dose of gentamicin was increased to 200 and 400 mg/kg per day for 6 days, the amount of drug present in renal cortex progressively increased and was accompanied by unequivocal evidence of nephrotoxic injury. In rats injected with 200 mg of gentamicin/kg per day MDA exceeded the control level by 37% whereas in rats injected with 400 mg/kg per day it exceeded the control level by 358%. Moreover, there was a dose-related decline in polyunsaturated fatty acids esterified in renal cortical phospholipid. For example, arachidonic acid (20:4) is the most abundant polyunsaturated fatty acid in renal cortical phospholipids of non-DM rats (15, 21) and with four double bonds is highly susceptible to lipid peroxidation (23). In control DM rats we found, in agreement with previous reports (27, 28), that the fraction of phospholipid acyl chains comprised of arachidonate (17 to 18%) was significantly lower than the 26 to 28% typical of non-DM rats (15, 21). Consequently DM rats manifested a shift toward more saturated fatty acids esterified in phospholipids even before the initiation of gentamicin injections. Despite this reduction in baseline arachidonic acid, gentamicin at 200 and 400 mg/kg per day caused significant declines of arachidonate accompanied by a shift to more saturated fatty acids. These changes mimic very closely our previous findings of accelerated lipid peroxidation in non-DM rats injected with nephrotoxic dose of gentamicin (15, 21, 24).

We also examined whether gentamicin affected the three antioxidant systems —SOD, catalase, and glutathione—which are responsible for processing free radicals and protecting the cell against peroxidative injury (29, 30). Rats injected at a dose of 100 mg of gentamicin/kg per day manifested no alterations in these systems, whereas at 200 and 400 mg/kg per day SOD and catalase showed a dose-dependent depression. In previous studies of gentamicin nephrotoxicity (15, 21, 24) we observed reductions of total and reduced glutathione accompanied by an increase of oxidized glutathione and of the ratio of oxidized to total glutathione. The shift from reduced to oxidized glutathione is indicative of oxidative stress such as attends accelerated lipid peroxidation (31). In DM rats injected with 200 mg/kg per day total and reduced glutathione were not different from control whereas there was a modest increase of oxidized glutathione and of the ratio of oxidized to total glutathione (Fig. 2). In contrast, in rats injected with 400 mg of gentamicin/kg per day all components of the glutathione system were significantly increased. The increases of total and reduced glutathione are distinctly different from the typical pattern of reductions of the variables observed in our previous studies in non-DM rats (15, 21, 24). While this pattern may reflect a response that is peculiar to the diabetic state, we cannot exclude the possibility of an artifact. Nevertheless, it is noteworthy that rats injected with the highest dose of gentamicin also had the highest ratio of oxidized to total glutathione which is consistent with evidence summarized above that these rats experienced the greatest degree of lipid peroxidation in the renal cortex.

The changes in lipid peroxidation were accompanied by elevation of the serum creatinine which in the case of rats injected with 400 mg of gentamicin/kg per day increased almost 100% above that of the control rats and signifies approximately a 50% reduction of the glomerular filtration rate. Moreover, these functional changes were accompanied by unequivocal evidence of proximal tubular cell injury and necrosis.

The results of our study provide the most compelling evidence available in support of the concept that the resistance of DM rats to AG nephrotoxicity is causally linked to the low rate of transport and accumulation of

drug by renal proximal tubular cells. When the drug attains a critical concentration within these cells, it elicits a pattern of toxic injury indistinguishable from that of non-DM rats. Thus, there appears to be nothing inherent in the diabetic state which prevents these polycationic drugs from causing their usual toxic effects on the metabolism of renal proximal tubular cells once they gain access into these cells.

Vaamonde and co-workers (32) have reached a similar conclusion based on their observations that even at low concentrations of AG in renal cortex DM rats manifested alterations of phospholipid metabolism, of thymidine incorporation into DNA, and of ultrastructure that were indistinguishable from those of non-DM rats with similar concentrations of drug in renal cortex.

In summary our studies provide strong support for the concept that the uptake of AG by renal proximal tubular cells is a required first step in the pathogenesis of AG nephrotoxicity and that depression of this transport step underlies the resistance of DM rats to AG-induced acute renal failure.

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