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EFFECT OF GENTAMICIN ON LIPID PEROXIDATION IN RAT RENAL CORTEX

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Abstract—We examined the hypothesis that lipid peroxidation participates in the pathogenesis of aminoglycoside-induced nephrotoxicity. Male Sprague–Dawley rats were injected subcutaneously with gentamicin, 100 mg/kg per day, for 1–4 days. Twenty-four or forty-eight hours after the last injection the rats were killed and the renal cortex was processed for total phospholipids, malondialdehyde (MDA), phospholipid fatty acid composition, superoxide dismutase, catalase and glutathione. Gentamicin induced a significant increase in total renal cortical phospholipids which was evident after a single injection and by the third injection reached a plateau 17% above the baseline level. MDA, an end product of lipid peroxidation, increased from 0.674 ± 0.021 nmole/mg protein in the control group to 0.931 ± 0.053 nmole/mg protein ($P < 0.001$) 48 hr after the fourth injection. As another index of lipid peroxidation, we determined the shift from polyunsaturated to saturated fatty acids of renal cortical phospholipids. By the second injection of gentamicin we detected a significant decline of arachidonic acid (20:4) present in phospholipid. By the fourth injection, arachidonic acid had fallen 48% below control and was accompanied by reciprocal increases of more saturated fatty acids including linoleic (18:2), oleic (18:1) and palmitic (16:0) acids. The number of double bonds per mole of fatty acid declined from a baseline value of 1.62 ± 0.01 to 1.20 ± 0.02 ($P < 0.001$) by the fourth injection of drug. Superoxide dismutase showed no consistent alteration, whereas catalase activity (k) fell from the control value of 0.221 ± 0.007 min to 0.155 ± 0.009 min ($P < 0.01$) by the third injection, where k is the first-order rate constant. Total and reduced glutathione declined after the fourth injection of gentamicin accompanied by a shift to oxidized glutathione with an increase in the ratio of oxidized to total glutathione. These data support the conclusion that accelerated lipid peroxidation occurs early in the course of gentamicin administration and raise the possibility that lipid peroxidation is a proximal event in the injury cascade of gentamicin nephrotoxicity.

Aminoglycoside nephrotoxicity has been studied extensively, and the pathophysiology is well described [1–3]; however, the pathogenesis of the proximal tubular cell injury has not been elucidated. Several hypotheses have been proposed to explain the mechanism of cellular injury; these include derangements of lysosomal [4–7], mitochondrial [8–11] and plasma membrane [12, 13] structure and function. Aso *et al.* [14] recently observed an increase in lipid peroxides in the renal cortex of rats injected with gentamicin. Ngaha *et al.* [15] have reported that administration of selenium, an antioxidant that has been shown to decrease lipid peroxidation *in vivo* [16], decreases gentamicin nephrotoxicity in rats. Lipid peroxidation has been implicated in the pathogenesis of cellular damage caused by a number of toxic agents including mercury [17, 18], paraquat [19], carbon tetrachloride [20], cephaloridine [21], paracetamol [22], and adriamycin [23]. It has also been implicated in the cellular injury of renal ischemia [24]. Thus, we were stimulated to investigate whether lipid peroxidation participates in the pathogenesis of gentamicin nephrotoxicity.

One approach to the study of cellular lipid peroxidation is to measure malondialdehyde (MDA†), a major end product [25]. Another sensitive measure of lipid peroxidation is to determine the loss of polyunsaturated fatty acids from phospholipids [26]. Membrane phospholipids abound with unsaturated fatty acids [27], particularly arachidonic acid [28] which is highly susceptible to lipid peroxidation [26]. The cell is endowed with several antioxidant systems which function to limit the extent of lipid peroxidation; these include the enzymes catalase, superoxide dismutase and glutathione peroxidase [29]. A primary increase in the rate of lipid peroxidation will elicit an increase in the activity of one or more of these enzymes [30]. Conversely, inhibition of one or more of these antioxidant systems can lead to an increase in lipid peroxidation [22, 30, 31].

In the present study, we examined the effects of gentamicin administration on lipid peroxidation in the renal cortex of a well characterized rat model of gentamicin nephrotoxicity. Renal cortex of rats

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† Abbreviations: DTNB, 5,5-dithiobis-2-nitrobenzoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; and SOD, superoxide dismutase.

injected with gentamicin was assayed for MDA, fatty acid composition of phospholipids, the activities of catalase and superoxide dismutase, and the content of oxidized and reduced glutathione.

METHODS

Male Sprague–Dawley rats, weighing 200–250 g, were injected subcutaneously with saline or gentamicin (100 mg/kg per day) for up to 4 days and were killed under pentobarbitol anesthesia by exsanguination from the aorta 24 or 48 hr after the last dose. The renal cortex was rapidly dissected free and homogenized in 0.9% NaCl buffered to pH 7.4 with 0.01 M sodium phosphate. Aliquots of homogenate were processed for phospholipids, MDA, glutathione, catalase, superoxide dismutase, and protein.

Renal cortical phospholipids were extracted in chloroform–methanol (2:1, v:v) containing 0.01 M tetrabutylammonium sulfate and were quantitated by inorganic phosphorus determination [32]. Fatty acids were removed from phospholipids by alkaline hydrolysis with simultaneous formation of methyl ester derivatives [33]. Fatty acids were determined by gas chromatography on an HP 5750 GLC equipped with a stainless steel column packed with 10% SP-2330 cyano-silicone powder. The carrier gas was helium. The temperatures of the oven, injection port and flame ionization detector were 160°, 220° and 240° respectively. The chromatograph was equipped with an HP 3390A recorder which automatically calculated the area under the peaks. MDA concentration was determined in duplicate in renal cortical homogenates, after addition of deoxycholate and trichloroacetic acid (TCA) precipitation of protein, using the thiobarbituric acid procedure [34]. Thiobarbituric acid (2 ml of 0.8%) was added to each sample followed by incubation for 60 min at 95° in a shaking water bath. After cooling, the thiobarbituric acid reactive MDA was extracted in 4 ml of butanol–pyridine (15:1, v:v), and the concentration of MDA in the butanol phase was determined spectrophotometrically at 533 nm using an extinction coefficient of 0.154×10^9/pmole. Glutathione in renal cortical homogenates was determined after TCA precipitation of protein followed by five extractions of the supernatant fraction with ether. After adjusting the pH to 6.5, total glutathione, defined as the sum of reduced (GSH) and oxidized (GSSG) glutathione, was assayed by monitoring spectrophotometrically at 412 nm the reduction of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) by NADPH [35]. This reaction is catalyzed by GSH or GSSG and is linearly dependent on the concentration of glutathione [35]. GSSG was measured after first removing GSH from the supernatant fraction by trapping with *N*-ethyl maleimide and extracting with ether [36]. GSH was determined as the difference between total glutathione and GSSG.

Catalase activity was determined by the method of Cohen [37] which involves titration of $KMnO_4$. The activity of catalase was expressed as the first-order reaction rate constant, k, which is defined by the equation:

$$k = \log (S_o/S_t) \times 2.3/t$$

where t = time, S_o = initial concentration of $KMnO_4$ and S_t = the concentration of $KMnO_4$ at time t. Superoxide dismutase (SOD) activity was determined on the 700 g supernatant fraction of renal cortical homogenate containing both cytosolic and mitochondrial enzyme and was measured as the rate of inhibition of ferricytochrome c reduction in a xanthine–xanthine oxidase generating system [38]. One unit of SOD is defined as that quantity required to inhibit the rate of reduction of ferricytochrome c by 50%. Protein was determined by the method of Lowry *et al.* [39].

The data were subjected to statistical analysis using analysis of variance and the Duncan multiple range test. Where appropriate, Student's t-test for non-paired data was also used. The results in the text, table and figures are expressed as the mean ± S.E.M. unless specified otherwise.

Gentamicin sulfate was a gift from the Schering Corp., Bloomfield, NJ. Thiobarbituric acid, glutathione, glutathione reductase, cytochrome c, 5,5-dithiobis-2-nitrobenzoic acid, NADPH, *N*-ethyl-maleimide, xanthine and xanthine oxidase were obtained from the Sigma Chemical Co., St. Louis, MO.

RESULTS

Figure 1 illustrates that gentamicin administration induced a progressive increase in total phospholipid of rat renal cortex during the first 3 days of drug administration after which a plateau was reached 17% above baseline. Gentamicin administration also augmented lipid peroxidation in the renal cortex as assessed by the accumulation of the lipid peroxidation product, MDA (Fig. 2). By 48 hr after the fourth injection of drug, MDA had increased 38%

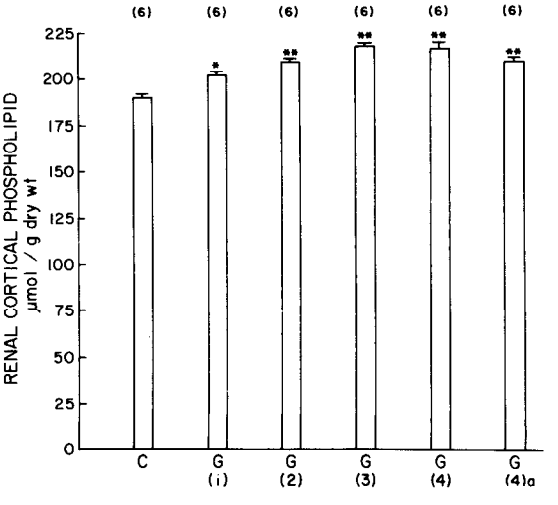

Fig. 1. Renal cortical phospholipid of control (C) rats and of rats injected with gentamicin (G), 100 mg/kg per day, for 1–4 days. The numbers in parentheses at the bottom of the figure indicate the number of injections; "(4)a" signifies that the rats were killed 48 hr after the fourth injection. The numbers in parentheses at the top of the figure indicate the number of rats in each group. The data represent the mean ± S.E. Key: (*) significantly different from C, $P < 0.05$; and (**) significantly different from C. $P < 0.01$.

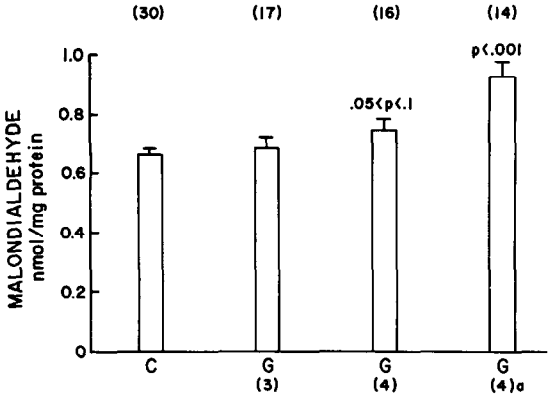

Fig. 2. Renal cortical malondialdehyde levels of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (3) or (4) days. "(4)a" signifies that rats were killed 48 hr after the fourth injection. The numbers in parentheses at the top of the figure indicate the number of rats in each group. The data represent the mean ± S.E.

above the control level ($P < 0.001$). During the same time interval, we did not detect significantly increased urinary excretion of MDA (data not shown).

MDA is a relatively insensitive measure of lipid peroxidation. A more sensitive index is the finding of a shift from polyunsaturated to saturated fatty acids [26]. As shown in Fig. 3, after the second injection of gentamicin we detected a significant decline in the fractional content of arachidonic acid (20:4) present in phospholipid. By the fourth dose of gentamicin, arachidonic acid had declined by 48%. Since total phospholipid had increased only 17% (Fig. 1), it follows that the absolute quantity of phospholipid arachidonic acid had declined sharply and was accompanied by reciprocal increases of more saturated fatty acids including linoleic (18:2), oleic (18:1) and palmitic (16:0) acids (Fig. 3). As another index of the shift from polyunsaturated to saturated fatty acids, we calculated the ratio of double bonds (Δ) per mole of fatty acid. By the fourth dose of

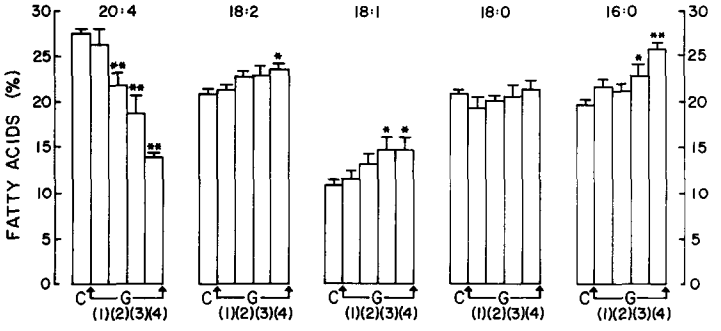

Fig. 3. Fatty acid composition of renal cortical phospholipids of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (1), (2), (3) or (4) days. The numbers at the top of the figure indicate the number of carbon atoms followed by the number of double bonds per fatty acid: arachidonic acid (20:4); linoleic acid (18:2); oleic acid (18:1); stearic acid (18:0); palmitic acid (16:0). The data represent the mean ± S.E. Key: (*) significantly different from C, $P < 0.05$; and (**) significantly different from C, $P < 0.01$.

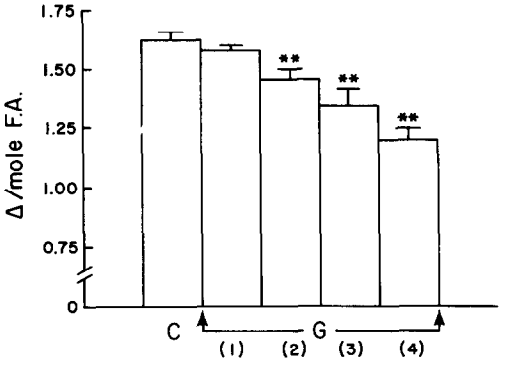

Fig. 4. Mean number of double bonds (Δ) per mole of renal cortical phospholipid fatty acid of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (1), (2), (3) or (4) days. Data represent the mean ± S.E.. A double asterisk (**) indicates significantly different from C, $P < 0.01$.

gentamicin, the number of double bonds per mole of fatty acid had declined 26% (Fig. 4).

We next examined whether gentamicin administration affected the activity of three antioxidant systems, superoxide dismutase, catalase and the glutathione cycle, which are responsible for processing free radicals and, thereby, protecting the cell against lipid peroxidative injury. Superoxide dismutase activity was not altered consistently by gentamicin administration (Fig. 5). Catalase activity, in contrast, was depressed slightly after the second injection of gentamicin and by the third injection had declined 30% below that of the control group (Fig. 6). The activity of the third antioxidant system, glutathione peroxidase, was determined indirectly by measuring total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG) and the ratio of oxidized to total glutathione (Fig. 7). By the fourth injection of gentamicin the suggestion of a decline in total glutathione and reduced glutathione was first

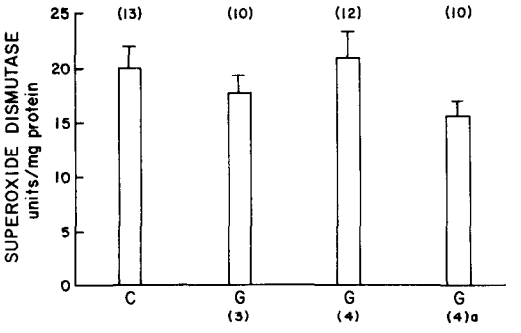

Fig. 5. Superoxide dismutase activity in renal cortex of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (3) or (4) days. "(4)a" signifies that the rats were killed 48 hr after the fourth injection. The numbers in parentheses at the top of the figure indicate the number of rats per group. The mean values (±S.E.) for each time period are: 20.0 ± 2.2, 17.6 ± 1.8, 20.9 ± 2.3 and 15.4 ± 1.4.

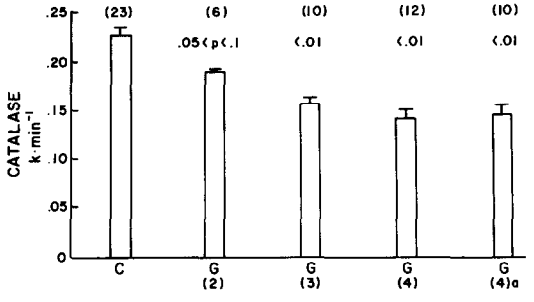

Fig. 6. Catalase activity in renal cortex of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (2), (3), or (4) days. "(4)a" signifies that the rats were killed 48 hr after the fourth injection. The numbers in parentheses at the top of the figure indicate the number of rats in each group. The data represent the mean ± S.E. The k is the first-order rate constant.

detected. Forty-eight hours after the fourth injection, these changes reached statistical significance. The reduction of total and reduced glutathione was accompanied by a shift to oxidized glutathione and an increased ratio of oxidized to total glutathione. The shift from reduced to oxidized glutathione is indicative of oxidative stress such as attends accelerated lipid peroxidation [40].

DISCUSSION

We have shown previously that administration of gentamicin, 100 mg/kg per day for 4 days to Sprague–Dawley rats, initiates an injury cascade which eventuates in proximal tubular cell necrosis and renal failure during the subsequent 4 days despite discontinuation of the drug [41]. During the first 4 days of drug treatment, proximal tubular function and glomerular filtration rate are well preserved; but, thereafter, the glomerular filtration rate begins to fall in association with depression of proximal tubular cell transport of organic acid, organic base, and low molecular weight protein [3, 41, 42]. The urinary excretion of brush-border and lysosomal enzymes rises sharply, coincident with the appearance of diffuse proximal tubular cell necrosis [43].

In an attempt to define the early biochemical alterations that participate in the pathogenesis of aminoglycoside-induced proximal tubular cell injury, we have confined our observations to those events occurring within 48 hr of the fourth dose of gentamicin administration. The results of our studies demonstrate that gentamicin induces increased lipid peroxidation. This conclusion rests in part on the observation that MDA, an end product of lipid peroxidation, increased significantly in response to gentamicin administration. The rise in MDA, however, did not reach statistical significance until after the fourth injection so that it remains uncertain based on the MDA data whether the increase in

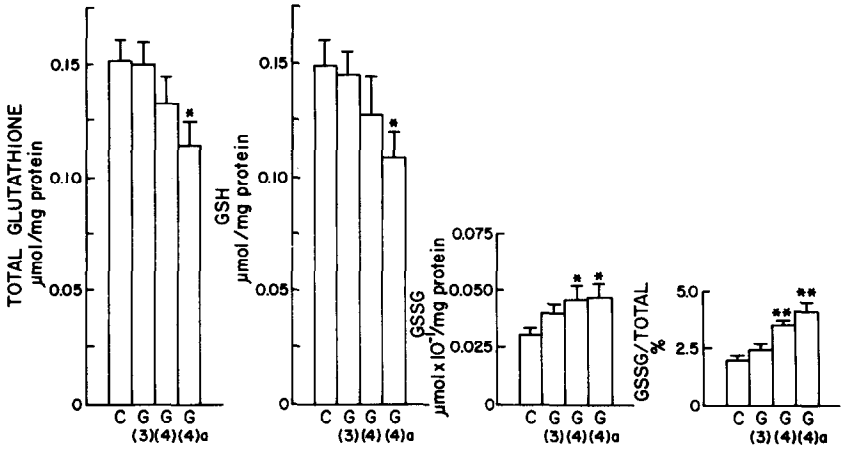

Fig. 7. Total glutathione, reduced (GSH) glutathione and oxidized (GSSG) glutathione levels in renal cortex of control (C) rats and rats injected with gentamicin (G), 100 mg/kg per day, for (3) or (4) days. "(4)a" signifies that the rats were killed 48 hr after the fourth injection. The data represent the mean ± S.E. Key: (*) significantly different from C, $P < 0.05$; and (**) significantly different from C, $P < 0.01$.

lipid peroxidation is a cause or a consequence of nephrotoxicity.

To examine this question further, we monitored the change in polyunsaturated fatty acids of renal cortical phospholipids. Arachidonic acid is the most abundant polyunsaturated fatty acid present in renal cortical phospholipids [28] and with four double bonds is highly susceptible to lipid peroxidation [44]. We observed a sharp decline in arachidonic acid as early as the second dose of gentamicin, and it was accompanied by a shift to more saturated fatty acids. This response pattern is characteristic of lipid peroxidation [26].

Increased prostaglandin synthesis may have contributed to the decline of arachidonic acid. McNeil *et al.* [45] reported that administration of gentamicin to dogs is associated with increased urinary excretion of prostaglandin E_2 (PGE_2). In the rat, Lipsky *et al.* [46] observed an initial rise of PGE_2 excretion followed by a decline. Negri *et al.* [47] reported that prostacyclin, but not thromboxane A_2, synthesis is augmented in renal cortical slices from rats injected with gentamicin. These three studies indicate that stimulation of prostaglandin synthesis via the cyclooxygenase pathway occurs during the course of gentamicin administration so that this mechanism may have contributed to some extent to the decline of arachidonic acid that we observed in the renal cortex of gentamicin-treated rats. However, it should be appreciated that in the renal cortex the enzymes involved in prostaglandin synthesis are localized to certain cell specific sites, i.e. glomerular mesangial and epithelial cells, endothelial cells of the renal arterial vasculature and cortical collecting tubule epithelium [48]. Given the fact that proximal tubular epithelium constitutes the overwhelming mass of renal cortex, it follows that even maximum rates of prostaglandin synthesis by those renal cortical cells endowed with the necessary synthetic machinery could not account for the 48% fall of arachidonic acid that we observed in our experiments. We infer, therefore, that the decline of arachidonic acid reflects primarily its consumption due to free radical attack. Furthermore, the fact that these changes occur early in the course of gentamicin administration and precede the appearance of derangements in proximal tubular transport [3, 41, 42] raises the possibility that lipid peroxidation is a proximal event in the injury cascade of aminoglycoside nephrotoxicity.

The generation of free radicals occurs as a natural by-product of oxidative metabolism [29]. These reactive species are normally detoxified by several enzyme systems including superoxide dismutase, catalase and glutathione peroxidase [29, 49]. Lipid peroxidation can result as a consequence of a primary increase in the generation of free radicals or as a consequence of a primary decrease in the activity of one or more of these antioxidant systems [29]. In the present study we obtained evidence in support of the latter possibility. The initial change we detected was a depression of catalase activity first evident after the second dose of gentamicin. After the fourth dose of gentamicin, we observed a decline of total glutathione and a shift from reduced to oxidized glutathione, a response pattern consistent with increased oxidative stress such as lipid peroxidation [40]. Superoxide dismutase activity, in contrast, was highly variable and showed no consistent change.

Catalase is found primarily in peroxisomes [30]; a small fraction may be present in the cytoplasm [50]. It catalyzes the conversion of hydrogen peroxide, generated by a variety of oxidases, to water and molecular oxygen [30]. Hydrogen peroxide can react with metal chelates via the Fenton reaction [29] and generate hydroxyl radical, a highly reactive species which can oxidize a wide variety of organic compounds including polyunsaturated fatty acids [29]. We postulate that gentamicin in some manner depresses catalase activity and that this results in the accumulation of hydrogen peroxide which exceeds the metabolic capacity of the cell's residual peroxidase systems including glutathione peroxidase and leads to increased generation of hydroxyl radical and peroxidation of lipids. The observation that the onset of the depression of catalase activity appeared to coincide temporally with the onset of the decline in polyunsaturated fatty acids is consistent with this hypothesis. In addition to depressing catalase activity, it is possible that gentamicin also depresses the activity of glutathione peroxidase or reductase which would magnify the propensity for lipid peroxidation.

The mechanism by which gentamicin administration caused depression of catalase activity in our study remains to be determined. In preliminary studies we have demonstrated that preincubation of a 700 *g* fraction prepared from a homogenate of rat renal cortex in medium containing gentamicin (final concentration 1.5 mM) resulted in a 59% decrease in catalase activity. This concentration of gentamicin can readily be achieved in rat renal cortex after two injections of drug at 100 mg/kg body weight [41]. Thus, these observations allow consideration of the possibility that gentamicin may directly inhibit catalase. It should be emphasized that this mechanism implies an interaction between gentamicin and peroxisomes. Up to now, however, it has generally been held that aminoglycosides are transported across the apical membrane of proximal tubular cells by adsorptive endocytosis following which they are shuttled directly into lysosomes [51]. If subsequent studies establish that the depression of catalase is due to a direct effect of gentamicin, then it will necessitate revision of current concepts concerning proximal tubular cell transport and subcellular distribution of aminoglycoside antibiotics.

In summary, the results of our studies demonstrate that lipid peroxidation occurs early in the course of gentamicin administration. We postulate that inhibition of catalase activity is responsible for initiating free radical attack of polyunsaturated fatty acids. The early onset of these changes raises the possibility that lipid peroxidation is a proximal event in the injury cascade which eventuates in proximal tubular cell injury.

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