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FAILURE OF GENTAMICIN TO ELEVATE CELLULAR MALONDIALDEHYDE CONTENT OR INCREASE GENERATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES IN PRIMARY CULTURES OF RENAL CORTICAL EPITHELIAL CELLS

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Abstract—The role of lipid peroxidation in gentamicin cytotoxicity was assessed in primary cultures of renal cortical epithelial cells. The cellular content of malondialdehyde (MDA), one of the end products of lipid peroxidation, was determined, and the intracellular generation of reactive oxygen species, one of the events commonly occurring at the beginning of the process of lipid peroxidation, was estimated. Exposures to gentamicin were chosen that preceded or accompanied overt toxicity. MDA was determined by the thiobarbituric acid reactive substances assay. Intracellular generation of reactive oxygen species was estimated by quantitating the fluorescence of 2′,7′-dichlorofluorescein (DCF′), a fluorophore formed by the reaction of the deacetylation product (2′,7′-dichlorofluorescin, DCF) of the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFDA) with reactive oxygen species. Neither elevated MDA content nor intracellular generation of reactive oxygen species was observed in primary cultures of renal cortical epithelial cells treated with gentamicin, using exposures which preceded or accompanied overt toxicity. We conclude that lipid peroxidation does not play a role in gentamicin cytotoxicity.

Previous investigations of the effect of gentamicin on lipid peroxidation have employed *in vivo* models [1–3]. In the present study, the role of lipid peroxidation in the cytotoxicity of gentamicin was assessed in a primary culture system of rat renal cortical epithelial cells. This model system is particularly appropriate for the study of gentamicin, a nephrotoxin which has been characterized as having a direct toxic action on the proximal tubular cell [4, 5], as opposed to causing deleterious nervous, endocrine or hemodynamic alterations. The cell culture model is uniquely suited for studying direct toxic effects on cells as these influences are removed from the model.

In the present study, exposures to gentamicin which preceded or accompanied cellular injury were chosen. Injury was estimated by determining gentamicin-induced release of cytosolic lactate dehydrogenase (LDH) [6]; overt injury was defined as significant release of LDH. By utilizing concentrations of gentamicin and durations of exposure which preceded injury (2 mM, 6–24 hr; 3–4 mM 6–12 hr) as well as exposures that accompanied overt cellular injury (3–4 mM, 24 hr), we sought to reveal the events which lead to cytotoxicity.

The extent of lipid peroxidation in gentamicin-treated cells was estimated by determining the cellular content of malondialdehyde (MDA), one of the end products of lipid peroxidation. In addition, the intracellular generation of reactive oxygen species, a common early event in lipid peroxidation, was estimated by quantitating the fluorescence of 2′,7′-dichlorofluorescein (DCF′), the fluorophore generated when reactive oxygen species oxidize the deacetylation product (2′,7′-dichlorofluorescin, DCF) of the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFDA).

The results presented here demonstrate that gentamicin did not cause lipid peroxidation, or the intracellular generation of reactive oxygen species, in exposures which preceded or accompanied significant cellular injury.

MATERIALS AND METHODS

Preparation of renal cortical epithelial cultures. Primary cultures were prepared by a method described previously [7], except that in studies employing DCFDA, epithelial cells were grown in culture dishes containing sterile 24.5 mm circular coverslips. In studies of MDA content, cells were grown in 35 mm culture dishes (Falcon). Cells were grown in culture for 3 days prior to drug treatment, a period which permits the cells to recover from the trauma of isolation. Following the 48-hr medium change, the medium was changed every 24 hr. In experiments employing DCFDA, fluorescence microscopy and a low power objective (25×), it was necessary to plate the cell suspension more densely than indicated in Smith *et al.* [7]. As previously indicated, the degree of confluency at day 3 depended on plating density; to easily find low power

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fields filled with cells, it was advantageous to increase plating density by 20%.

Drugs and chemicals. Gentamicin sulfate was the gift of the Schering Corp. Gentamicin sulfate was dissolved in complete culture medium, and the pH was returned to 7.4 with KOH. All other chemicals were purchased from the Sigma Chemical Co.

Assay of thiobarbituric acid reactive substances. The MDA content of the cellular monolayer was assayed by a modification of the method of Buege and Aust [8]. Cells were treated in complete medium lacking phenol red because phenol red absorbs at the wavelength used in this determination. At the end of the treatment period, the medium was aspirated, and 1 mL of distilled water was added to each dish. The monolayer was scraped thoroughly with a teflon policeman, and the suspension of disrupted cells was transferred to a 15-mL test tube with a screw cap closure. One milliliter of 10% (w/v) trichloroacetic acid and 1 mL of 0.5% thiobarbituric acid were added to each tube. The tubes were closed tightly and placed in a boiling water bath for 15 min. The tubes were cooled after boiling and then centrifuged at 2000 *g* for 10 min. The supernatant fraction was aspirated, and the absorbance was determined at 532 nm in a Beckman DU-40 Spectrophotometer. The spectrophotometer was blanked with a solution that contained all the reagents, but without the disrupted cell suspension.

Use of DCFDA. DCFDA has been used with cells in culture to determine relative changes in the intracellular generation of reactive oxygen species [9]. The rationale for the use of DCFDA has been described by Bass *et al.* [10]. Briefly, DCFDA is a non-fluorescent compound which diffuses across the plasma membrane. DCFDA is deacetylated intracellularly by cytosolic esterase(s) to form the non-fluorescent, intracellularly trapped species, DCF. DCF reacts with intracellular reactive oxygen species to form DCF′, a fluorescent intracellularly trapped compound whose fluorescence is proportional to the intracellular concentration of reactive oxygen species.

Loading with DCFDA. Thirty minutes prior to the end of the specified treatment period with gentamicin, *tert*-butylhydroperoxide or cephaloridine, cells were loaded with DCFDA by adding an aliquot of 5 mM DCFDA in ethanol to the culture dish. The final concentration of DCFDA was 5 μM. The concentration of ethanol after the addition was 0.1%.

Rinsing the monolayer to remove uninternalized probe. After the period of concurrent treatment with gentamicin and loading with DCFDA, the medium was aspirated and the monolayer was rinsed three times with a prewarmed (37°) *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffered Krebs-Hensleit buffer (HBKHB) of the following composition (in mM): NaCl (110), KCl (5), $CaCl_2$ (1.25), $MgSO_4$ (1), $NaHCO_3$ (8), Na_2HPO_4 (0.5), KH_2PO_4 (0.5), glucose (10), HEPES (20) pH 7.4. The monolayer was maintained in this physiological buffer until the end of the experiment.

Quantitation of the fluorescence of DCF′. The coverslip with attached cells was fitted into a Dvorak–Stotler chamber (Nicholson Precision Instruments). The chamber was flushed and filled with 1 mL of prewarmed HBKHB, and placed on an inverted Zeiss IM35 microscope. A field of cells with epithelial morphology was located using phase contrast illumination (25× objective).

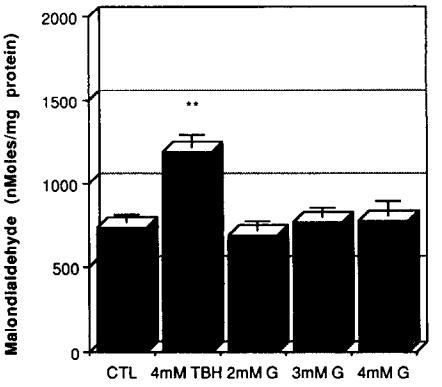

Fig. 1. MDA levels in cultured kidney cortical epithelial cells after 24 hr of treatment with gentamicin (G) or *tert*-butylhydroperoxide (TBH). CTL = control. Values are means ± SEM (N = 3–5, in duplicate cultures). Key: (**) significantly different from controls, $P \leq 0.01$.

A SPEX fluorolog 2 provided excitation illumination. To minimize the intensity of excitation illumination, slid widths were set to 1 mm. A light tight cover was placed over the chamber on the microscope stage and an excitation scan centred at 488 nm was performed. Epifluorescence from the sample (passing through a fluorescein filter set) was quantitated with a photometer. All values obtained were corrected for autofluorescence. The results were quantitated as the intensity of the emission signal with 488 nm excitation. After the excititation scans, the monolayer was observed with phase contrast illumination to ensure that focus was maintained and that the stage had not moved.

Statistical analysis. Analysis of variance and Scheffe's method of post-hoc comparisons were used to determine the significance of differences between mean values of treated and control groups. $P \leq 0.05$ was used as the level of statistical significance.

RESULTS

The effect of gentamicin on lipid peroxidation, estimated by determination of cellular MDA content in cultured kidney cells, is shown in Figs. 1–3. The effect of 24-hr exposures to gentamicin (2–4 mM) is shown in Fig. 1. The effect of 12-hr exposures is shown in Fig. 2. The result of 6-hr exposures to gemtamicin is shown in Fig. 3. While the positive control, *tert*-butylhydroperoxide, elevated the cellular content of MDA in every case, exposure to gentamicin had no effect.

Positive controls for generation of reactive oxygen species are shown in Fig. 4. Two compounds which have been shown to cause the intracellular generation of reactive oxygen species were employed. Cephaloridine (3 mM, 1 hr), a well-known nephrotoxin, caused a modest increase in DCF oxidation, while *tert*-butylhydroperoxide (500 μM, 1 hr) caused a significant increase in DCF oxidation.

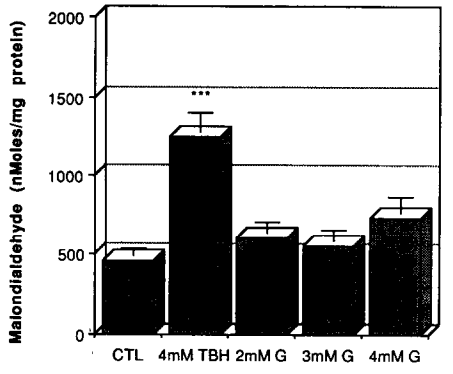

Fig. 2. MDA levels in cultured kidney cortical epithelial cells after 12 hr of treatment with gentamicin (G) or *tert*-butylhydroperoxide (TBH). CTL = control. Values are means ± SEM (N = 3–5, in duplicate cultures). Key: (***) significantly different from controls, $P \leq 0.001$.

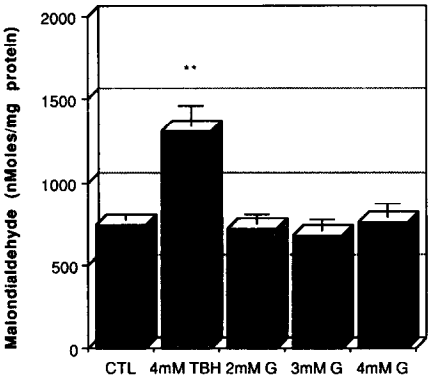

Fig. 3. MDA levels in cultured kidney cortical epithelial cells after 6 hr of treatment with gentamicin (G) or *tert*-butylhydroperoxide (TBH). CTL = control. Values are means ± SEM (N = 3–5, in duplicate cultures). Key: (**) significantly different from controls, $P \leq 0.01$.

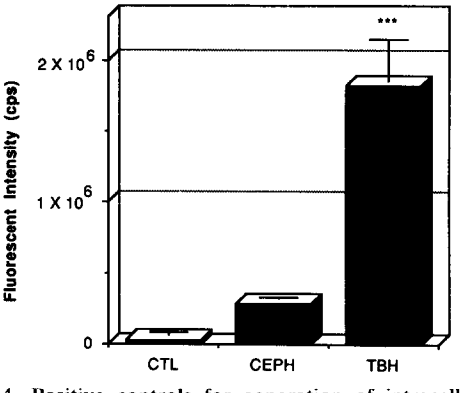

Fig. 4. Positive controls for generation of intracellular reactive oxygen species in cultured kidney cortical epithelial cells. Abbreviations: CTL, control; CEPH, cephaloridine (3 mM, 1 hr); and TBH, *tert*-butylhydroperoxide (500 μM, 1 hr). Values are means ± SEM (N = 3 in duplicate cultures). Key: (***) significantly different from controls, $P \leq 0.001$.

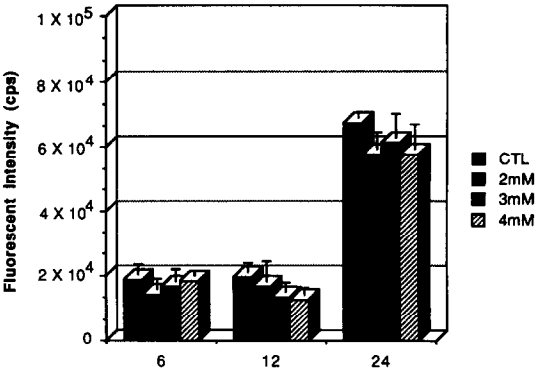

Fig. 5. Lack of oxidation of 2′,7′-dichlorofluorescin (DCF) in gentamicin-treated cells. Values are means ± SEM (N = 3 in duplicate cultures).

The failure of gentamicin to oxidize DCF is shown in Fig. 5. The slight elevation of DCF′ fluorescence in the 24-hr group may represent a difference in autofluorescence, but is substantially lower than DCF′ fluorescence in either of the positive controls, and not different from the 24-hr control values.

To be certain that treatment with gentamicin was not suppressing the fluorescence of DCF′, either by causing DCF′ to leak from the cells or by preventing the deacetylation of DCFDA, we exposed cells that had been treated with gentamicin and loaded with DCFDA to *tert*-butylhydroperoxide (500 μM, 1 hr). An increase in fluorescence, similar to that shown in TBH-treated cells was observed (data not shown), which was consistent with gentamicin not affecting the fluorescence of DCFDA.

DISCUSSION

Several investigators have studied the potential of gentamicin to generate free radicals and produce lipid peroxidation. Walker and Shah [11] demonstrated that gentamicin increases the production of hydrogen peroxide by isolated renal cortical mitochondria in a dose-dependent manner. Subsequently, these same investigators, employing an *in vivo* model, found that hydroxyl radical scavengers and iron chelators prevented gentamicin-induced deterioration of renal function and histological damage [1]. In addition, a hydroxyl radical scavenger prevented lipid peroxidation, as evidenced by a dramatic drop in renal cortical MDA content. A second group of researchers also found evidence of gentamicin-induced lipid peroxidation *in vivo*. Ramsammy *et al.* [3] demonstrated an elevated renal cortical MDA content, a significant shift from polyunsaturated to saturated fatty acids, a decline in catalase activity, and reductions in total and reduced glutathione. However, in a subsequent manuscript, this group found that while gentamicin does produce an increase in lipid peroxidation in the renal cortex, and an antioxidant does inhibit gentamicin-induced lipid peroxidation, the antioxidant does not prevent toxicity caused by gentamicin [2], in contrast to the

findings of Walker and Shah, who employed a hydroxyl radical scavenger [1]. Ramsammy *et al.* [2] concluded that lipid peroxidation was not a primary cause of gentamicin toxicity.

In the present study, the extent of lipid peroxidation, estimated by measuring levels of MDA in the cellular monolayer, was not altered by treatment with gentamicin (2–4 mM, 6–24 hr), regardless of whether or not these exposures caused significant LDH release. (As previously reported [6], significant LDH release occurred following exposure to 3–4 mM gentamicin for 24 hr.) The positive control, *tert*-butylhydroperoxide, reliably elevated this measure of lipid peroxidation in every case. Because no elevation of MDA content was observed when cell cultures of cortical tubular epithelium were exposed to gentamicin, in exposures that precede and accompany significant LDH release, it seems likely that the increases in whole kidney MDA content observed by Ramsammy *et al.* [2, 3] and Walker and Shah [1] came from a source other than the cortical tubular epithelium.

One of the earliest events in the process of lipid peroxidation is often the generation of reactive oxygen species, and the generation of these species was assessed using the fluorescent probe DCFDA. Only the amplitude of the excitation spectra changes in the presence of reactive oxygen species, so in this regard the probe is limited compared to fluorescent probes (e.g. fura-2) for which a ratio of fluorescences may be used to calculate the intracellular concentration of the reactants of interest. Although it does not permit the molar quantitation of reactive oxygen species, it does permit the qualitative assessment of intracellular reactive oxygen species, and it is the major fluorescent probe available for such measurements. In the present study, gentamicin (2–4 mM, 6–24 hr) did not increase the fluorescence of DCF′ above control levels.

In summary, our results are not in agreement with those of Walker and Shah [1, 11] who found evidence for hydroxyl radical and hydrogen peroxide mediation of gentamicin nephrotoxicity. Our results are consistent, however, with the conclusions of Ramsammy and coworkers [2], who concluded that lipid peroxidation caused by gentamicin is a consequence, and not a cause, of gentamicin nephrotoxicity. Our results indicate that gentamicin, while cytotoxic to primary cultures of renal cortical epithelial cells, does not elevate the cellular content of MDA, nor does it cause increased intracellular generation of reactive oxygen species. Thus, it would seem that lipid peroxidation does not play a role in gentamicin-induced cytotoxicity in this primary culture system and that lipid peroxidation observed by others *in vivo* came from a source other than the cortical epithelial cells of the renal tubule.

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