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RAPID COMMUNICATION

FORMATION OF FREE RADICALS BY GENTAMICIN AND IRON AND EVIDENCE FOR AN IRON/GENTAMICIN COMPLEX

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Abstract - Participation of free radicals in the adverse renal and cochlear side effects of aminoglycoside antibiotics is controversial. We measured the production of free radicals by gentamicin *in vitro* through the oxidation of arachidonic acid. Gentamicin alone (0.05 to 10 mM) did not cause lipid peroxidation. However, it dramatically promoted radical formation in the presence of iron salts. Peroxidation was maximal at 1 mM gentamicin plus 0.1 mM Fe(II)/Fe(III) (0.05 mM $FeSO_4$ and $FeCl_3$ each). At these iron concentrations, peroxidation was not significant in the absence of gentamicin. Since chelators can enhance iron-catalyzed oxidations, this finding suggested that gentamicin-dependent radical formation was based upon iron chelation. This hypothesis was tested by measuring the influence of gentamicin on the oxidation of salicylate by Fe-EDTA complexes, a reaction that is inhibited by competing iron chelators. Gentamicin was a concentration-dependent inhibitor. In contrast, concentrations of gentamicin as high as 50 mM did not interfere with iron-independent salicylate oxidation. These results suggest that gentamicin acts as an iron chelator, and that the iron-gentamicin complex is a potent catalyst of free radical formation.

Key words: gentamicin; ototoxicity; iron; lipid peroxidation; iron chelation; free radicals

Aminoglycosides, such as gentamicin, are extremely efficacious antibiotics, particularly against gram-negative bacteria. Their bactericidal action involves a series of distinct steps including binding to ribosomes and inhibiting protein synthesis [1]. Aminoglycosides also have toxic side effects on the kidney and the inner ear with well documented pathologies and pathophysiologies [2-4]. Although several hypotheses have been advanced [5, 6], the biochemical mechanisms for this nephro- and ototoxicity are not understood.

We have recently documented the existence of a cytotoxic form of gentamicin [7, 8] and suggested that the formation or action of this compound may involve free radicals [9, 10]. The notion of a free radical mechanism of aminoglycoside toxicity, however, is controversial. *In vivo* evidence is indirect and contradictory. While the radical scavenger WR-2721 attenuates the ototoxicity of the aminoglycoside kanamycin in guinea pigs [11], another radical scavenger, N-acetylcysteine, is ineffective [12]. Likewise, gentamicin increases renal lipid peroxidation, but this effect is only considered an epiphenomenon of nephrotoxicity [13]. Finally, to the best of our knowledge, there is no *in vitro* evidence for any mechanism by which gentamicin could catalyze free radical reactions.

Aminoglycosides are considered to be redox-inactive compounds [14]. Therefore, conversion to a redox-active form may require the participation of transition metals, such as iron. At physiological pH, iron-catalyzed oxidation is greatly accelerated by chelators [15]. Indeed, iron supplementation enhances gentamicin nephrotoxicity in rats [16, 17], and the pathology of iron overload shares characteristics with aminoglycoside nephrotoxicity [18]. Interestingly, the iron chelator deferoxamine works synergistically with gentamicin to increase its antimicrobial efficacy [19] and, by itself, can be ototoxic in guinea pigs [20]. Another aminoglycoside, streptomycin, forms coordinate complexes with cobalt, nickel, copper, and calcium salts [21], but a complex with iron has not been demonstrated. The present study investigated oxidation of arachidonic acid by iron and gentamicin in a non-enzymatic system and gives evidence that such oxidation may occur through the formation of an iron-gentamicin complex.

MATERIALS AND METHODS

Lipid peroxidation

Arachidonic acid peroxidation was monitored spectrophotometrically by measuring conjugated diene levels according to Buege and Aust [22]. Incubations were carried out at 37° for 90 min. Unless stated otherwise, the reaction mixtures (200 μL) contained 0.25% arachidonic acid (v/v), 10 mM sodium phosphate (pH 7.4), 50 μM $FeCl_3$, 50 μM $FeSO_4$, and either 1 mM gentamicin sulfate (previously adjusted to pH 7.4 with NaOH) or an equivalent amount of

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sulfate (2.5 mM sodium sulfate). Reactions were stopped by the addition of 1 mL of chloroform:methanol (2:1). Following vortexing and centrifugation, the organic phase (lower layer) was removed and dried at 45° under a stream of N_2. Cyclohexane (200 μL) was then added to the lipid residue, and absorbances were read at 235 nm. A molar extinction coefficient of 2.52 x 10^4 $M^{-1}cm^{-1}$ for the conjugated diene was used in calculations [22].

Salicylate oxidation

The Fe-EDTA catalyzed oxidation of salicylate to dihydroxybenzoate was measured according to Halliwell and Gutteridge [23]. Incubations were carried out at 25° for 90 min. Salicylate oxidation by Fe-EDTA was initiated by either enzymatic superoxide generation, or the addition of H_2O_2 and ascorbate. In the enzymatic generation of superoxide, the reaction mixtures (2 mL) contained 2.5 mM salicylate, 0.3 mM EDTA, 0.1 mM $FeSO_4$, 0.2 mM hypoxanthine, and 150 mM K_2HPO_4 (adjusted with HCl to pH 7.4). The reactions were started by the addition of 40 μL of xanthine oxidase (0.4 enzyme units/mL). In the other design, the reaction mixtures (2 mL) contained 2.5 mM salicylate, 0.3 mM EDTA, 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, 150 mM K_2HPO_4 (adjusted with HCl to pH 7.4), and the reaction was started by the addition of 200 μL of 1 mM H_2O_2.

The effect of gentamicin on the iron-independent breakdown of H_2O_2 was measured using UV light as the catalyst. The reaction mixtures (2 mL) contained 2.5 mM salicylate, 0.1 mM H_2O_2, 150 mM K_2HPO_4 (adjusted with HCl to pH 7.4) and either 50 mM gentamicin sulfate or 250 mM sodium sulfate. The disproportionment of H_2O_2 was catalyzed by exposure to short-wave UV light for 30 min at 0°.

All reactions were stopped by the addition of 80 μL of concentrated HCl, 0.5 g NaCl (solid), and 4 mL of chilled diethyl ether. Following vortexing, 3 mL of the ether layer was dried at 40° under a stream of N_2. Chilled water (250 μL) was then added to the residue, followed by (in order) 0.125 mL of 10% (w/v) trichloroacetic acid dissolved in 0.5 M HCl, 0.25 mL of 10% (w/v) sodium tungstate, and 0.25 mL of freshly prepared 0.5% (w/v) $NaNO_2$. After 5 min, 0.5 mL of 0.5 M NaOH was added, and absorbances were read at 510 nm. A molar extinction coefficient for dihydroxybenzoate of 3.25 x 10^6 $M^{-1}cm^{-1}$ was used in calculations [23].

All chemicals were purchased from the Sigma Chemical Co., St. Louis, MO, with the exception of gentamicin sulfate, which was a gift from the Schering Corp., Bloomfield, NJ.

RESULTS

Lipid peroxidation

Conjugated diene formation from arachidonic acid, a measure of free radical activity [22], was negligible with either iron or gentamicin alone. However, it increased to approximately 160 μM after 90 min in their combined presence (Fig. 1).

In the presence of 1 mM gentamicin, peroxidation approached saturation at 0.1 mM iron salts. Conversely, in the presence of 0.1 mM iron salts, peroxidation reached a maximum near 1 mM gentamicin. No increase in peroxidation was observed when sodium sulfate replaced gentamicin sulfate. The biphasic effect on lipid peroxidation by gentamicin is consistent with the behavior of iron chelators, which promote iron-dependent oxidation at low molar ratios to iron, and inhibit it at high ratios [24].

Phosphate buffer, present in these incubations, is a weak iron chelator and thus a possible confounding factor. To test its effect on the peroxidation assay, gentamicin and iron salts were incubated with arachidonic acid in either 10 mM Tris/HCl buffer, pH 7.4, or in the absence of buffer with the pH of the stock solutions carefully adjusted to 7.4 prior to incubation. In the absence of phosphate, gentamicin significantly enhanced lipid peroxidation as before, and the kinetics seemed somewhat accelerated. In Tris buffer, 1 mM gentamicin plus 0.1 mM iron salts produced 111 ± 6 μM conjugated diene in 30 min while in the absence of buffer 116 ± 7 μM was produced. Sodium sulfate controls exhibited negligible conjugated diene formation under both conditions.

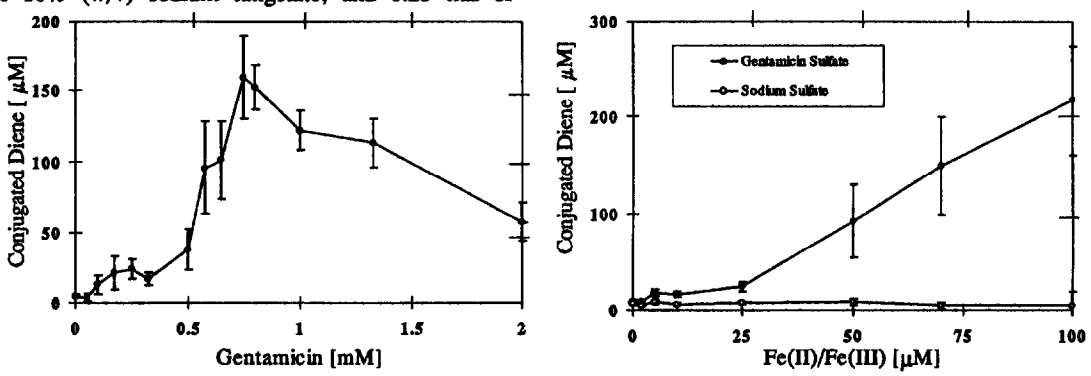

Fig. 1. Arachidonic acid peroxidation in response to iron and gentamicin. Diene concentration was determined as described in Materials and Methods. Concentrations of gentamicin sulfate were varied in the presence of 0.1 mM Fe(II)/Fe(III) (left panel), or Fe(II)/Fe(III) was varied in the presence of 1 mM gentamicin (right panel). Data points are the means ± SEM of 4-8 experiments.

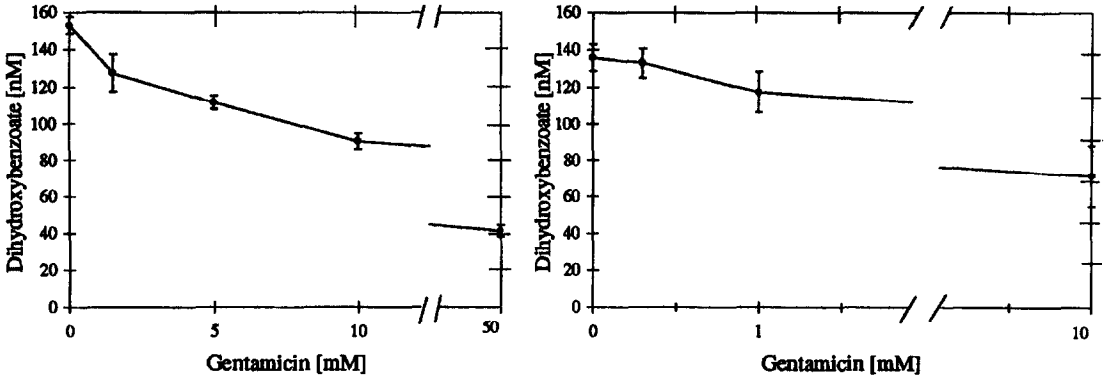

Fig. 2. Inhibition of Fe-EDTA catalyzed salicylate oxidation by gentamicin sulfate. The oxidation of salicylate by either H_2O_2, ascorbate, and Fe-EDTA (left panel) or superoxide and Fe-EDTA (right panel) was measured by the formation of dihydroxybenzoate (see Materials and Methods). Data points are the means ± SD of 3-5 experiments.

Salicylate oxidation

Iron chelators, such as deferoxamine, inhibit salicylate oxidation by Fe-EDTA through competition with iron and formation of iron complexes of greater stability against reduction by ascorbate or superoxide [23]. To determine if gentamicin acted like such an iron chelator, salicylate oxidation was measured by the production of dihydroxybenzoate (Fig. 2). Gentamicin sulfate inhibited the oxidation of salicylate in a concentration dependent fashion regardless of whether the reaction proceeded from H_2O_2, ascorbate, and Fe-EDTA (Fig. 2, left panel) or from the enzymatic generation of superoxide in the presence of Fe-EDTA (Fig. 2, right panel).

In contrast, gentamicin did not interfere with salicylate oxidation under conditions where H_2O_2 disproportionment was catalyzed by UV light rather than iron catalysis. Dihydroxybenzoate formation in the presence of 50 mM gentamicin sulfate (44 ± 11 nM, mean ± SEM, N = 5) did not differ significantly from controls containing sodium sulfate (47 ± 10 nM).

DISCUSSION

The results indicate that gentamicin promotes the oxidation of arachidonic acid in the presence of iron salts. To the best of our knowledge, this is the first direct demonstration of free radical production by an aminoglycoside. Furthermore, our data suggest that gentamicin catalyzes this reaction by forming a gentamicin-iron complex. This is evident from the fact that gentamicin inhibits Fe-EDTA-catalyzed but not iron-independent salicylate oxidation.

In a mechanism for the peroxidation of arachidonic acid, oxygen is implied. We propose that gentamicin, iron, and a species of oxygen form a ternary complex which facilitates electron transfers. Such an action of gentamicin would be analogous to that of bleomycin, a structurally unrelated, non-aminoglycoside antibiotic [25]. The iron-bleomycin species combines with oxygen to form "active bleomycin" through a one-electron transfer. A second binding domain on bleomycin attaches to DNA, allowing reactive oxygen to attack the DNA strands. Gentamicin may similarly form "active gentamicin." This species may likewise have specific intracellular targets, such as phosphatidylinositol 4,5-bisphosphate, for which aminoglycoside antibiotics have a selective and high affinity [26, 27].

Free radicals have been implicated in a variety of pathologies and drug actions [28, 29]. Whether the oto- and nephrotoxicities of aminoglycosides are in fact mediated by such a mechanism remains to be established. However, it seems likely that formation of an "active gentamicin" is the fundamental reaction behind the previously reported enzymatic conversion of gentamicin to a cytotoxin [7-10]. The enzyme fraction may supply the necessary iron and reducing equivalents. Recent results from this laboratory on the prevention of gentamicin-induced ototoxicity by radical scavengers [10] or iron chelators [30] strongly support such a notion. The mechanism proposed here would provide a rational explanation for a free radical mechanism involved in aminoglycoside toxicity.

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