

INHIBITION OF RENAL ORNITHINE DECARBOXYLASE BY AMINOGLYCOSIDE ANTIBIOTICS *IN VITRO*

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Abstract—The inhibition of renal ornithine decarboxylase (ODC) by aminoglycoside antibiotics was characterized in the postmitochondrial fraction of a kidney homogenate from adult pigmented guinea pigs. Enzymatic activity was defined as the rate of decarboxylation of [14 C]ornithine sensitive to a specific ODC inhibitor, α -difluoromethylornithine (DFMO). The K_m for ornithine was $61 \pm 32 \mu\text{M}$. There were two forms of the enzyme with respect to their affinity for pyridoxal phosphate (PLP): (I) $K_m = 2.1 \pm 1.8 \mu\text{M}$; (II) $K_m = 36.2 \pm 12.7 \mu\text{M}$. Putrescine, a known ODC inhibitor, acted competitively on the renal enzyme with $K_i = 1.7 \pm 1.4 \text{ mM}$. Aminoglycoside antibiotics inhibited ODC by an uncompetitive mechanism with inhibitor constants of comparable magnitude: neomycin, $K_i = 1.3 \pm 0.1 \text{ mM}$; gentamicin, $K_i = 1.6 \pm 0.1 \text{ mM}$; kanamycin, $K_i = 1.9 \pm 0.2 \text{ mM}$; and netilmicin, $K_i = 1.7 \pm 0.2 \text{ mM}$. Neomycin inhibited both forms of the enzyme (low and high affinity for PLP) uncompetitively with similar inhibitor constants (1.5 ± 0.3 and $1.8 \pm 0.4 \text{ mM}$ respectively), suggesting a single mechanism of action. Inhibition of ODC suggests that aminoglycoside-polyamine interactions may be an important component of the sequence of biochemical events associated with aminoglycoside toxicity.

Aminoglycoside antibiotics such as neomycin and gentamicin and the polyamines putrescine, spermidine and spermine not only share chemical features as polycations but also a number of pharmacological actions. Discrete interactions between aminoglycosides and polyamines which may relate to the mechanism of aminoglycoside toxicity have been described in bacteria as well as mammalian tissues. For example, spermidine and the aminoglycosides competitively inhibit binding of dihydrostreptomycin (DHS) to cell wall teichoic acids isolated from various gram-positive eubacteria [1]. Spermine and spermidine share renal binding sites with aminoglycosides [2], and the aminoglycosides compete for active polyamine transport in both the kidney [3] and the inner ear [4].

Biochemical studies to elucidate nephrotoxic and ototoxic actions of aminoglycosides have provided evidence for a multi-step model of toxicity which proposes an initial interaction between the drugs and the plasma membrane, followed by an energy-dependent uptake process competitive with polyamines, and finally intracellular binding [4-6]. To characterize further the potential role of aminoglycoside interactions with polyamines in this injury cascade, we studied the effects of aminoglycosides on the activity of renal ornithine decarboxylase.

Ornithine decarboxylase (ODC) is a key enzyme in the polyamine biosynthetic pathway and subject to feedback-inhibition by its reaction product putrescine, as well as by the other polyamines spermidine and spermine [7]. In view of the known interactions between the polyamines and aminoglycosides, it is plausible that the aminoglycosides may affect this enzyme and, thus, cellular mechanisms regulated by polyamines.

METHODS

Kidneys from pigmented guinea pigs (200-250 g, Murphy Breeding Laboratories, Plainsfield, IN) were homogenized in 50 mM sodium *N*-2-hydroxyethyl-1-piperazine-*N'*-2-ethane sulfonic acid (HEPES), 2 mM dithioerythritol (DTE) and 50 μM pyridoxal phosphate (PLP), pH 7.2, using a Polytron (Brinkmann, Switzerland) for 10 sec. No PLP was added to the homogenization in experiments where the final PLP concentrations were varied. The homogenate (20%, w/v) was centrifuged at 13,000 *g* for 20 min at 4°, and the supernatant fraction was frozen until assayed for ODC activity by a modification of the method of Russell and Snyder [8]. Aliquots of the supernatant fraction were incubated in test tubes with separate reservoirs for the incubation medium and H_2SO_4 and fitted with stoppers holding filter paper disks saturated with 15 μl of 40% KOH. Incubation was for 60 min at 37° in a total reaction volume of 100 μl containing 50 mM HEPES, 2 mM DTE, 0.1 mM EDTA, and 20 μg bovine serum albumin (BSA), pH 7.2. Ornithine concentration was 100 μM (L-[1- 14 C]ornithine, 52.4 mCi/mmol; New England Nuclear, Boston, MA) and PLP, 50 μM , except when the K_m for these substances or the K_i values for the aminoglycosides were determined. In those experiments, ornithine was varied from 25 to 100 μM , and PLP from 0.5 to 100 μM . Reactions were terminated by combining 200 μl of 4 N H_2SO_4 with the incubation medium and post-incubating for 30 min to allow for complete liberation of $^{14}\text{CO}_2$ and absorption onto the KOH-saturated filter. Radioactivity on the filter disks was quantitated by liquid scintillation counting. Control incubations to account for non-specific or nonenzymatic decarboxylation were performed in each set of experiments by adding 500 μM α -difluoromethylornithine (DFMO), a specific, irre-

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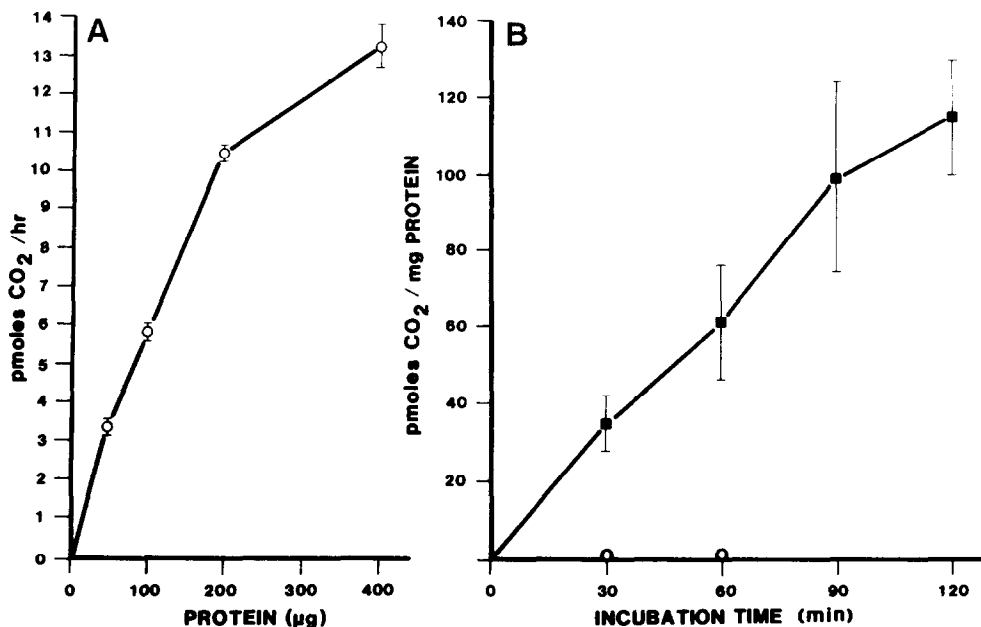


Fig. 1. (A) Effects of protein concentration on the rate of CO₂ formation. Experiments were carried out as described in Methods, and the protein concentration in the assay was varied as indicated. Individual points represent means \pm SD of three incubations each. (B) CO₂ formation as a function of the time of incubation. Experiments were carried out as described in Methods with incubation times varied as indicated. Points represent averages of two incubations each. Open circles denote experiments in the presence of DFMO (500 μ M).

versible inhibitor of ODC, or by substituting BSA (20 μ g) for the enzyme fraction. Protein was determined with the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

The inhibition by neomycin (Fig. 2, insert) and all other compounds was examined by linear regression analysis of Lineweaver-Burk plots using the Enzpack analytical software (Elsevier Biosoft, Amsterdam). Inhibitor constants were calculated according to Bergmeyer [9] from assays with various inhibitor concentrations.

DFMO was a gift from the Merrell Dow Research Institute (Cincinnati, OH). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

Initial experiments demonstrated that the formation of CO₂ from [¹⁴C]ornithine by the renal postmitochondrial fraction was essentially linear with protein concentrations up to 200 μ g in the assay (Fig. 1A) and incubation times up to 2 hr, the longest time tested (Fig. 1B). Enzymatic activity was abolished when 100–500 μ M DFMO was added. An incubation time of 60 min and protein concentrations below 200 μ g were selected for all subsequent studies.

Since previously reported data on renal ODC come from studies on the rat, mouse or hamster, kinetic parameters were determined for the enzyme preparation from the guinea pig. The analysis of the PLP dependence of the reaction indicated two K_m values for PLP: (I) $2.1 \pm 1.8 \mu$ M and (II) $36.2 \pm 12.7 \mu$ M with corresponding V_{max} values of

22.8 ± 7.8 (I) and 109.7 ± 38.1 pmol CO₂ · hr⁻¹ · mg⁻¹ (II). In contrast, a single K_m of $61 \pm 32 \mu$ M was evident for ornithine. For the standard assay conditions, V_{max} ranged from 110 to 142 pmol CO₂ · hr⁻¹ · (mg protein)⁻¹ for different preparations of the 13,000 g supernatant fraction.

The addition of neomycin to the assay induced a dose-dependent decrease of ODC activity with a half-maximal inhibition in the millimolar range (Fig. 2). Neomycin had no effect on the blank values, i.e. the decarboxylation in the presence of DFMO or with BSA only. The aminoglycoside antibiotics neomycin, kanamycin, gentamicin, and netilmicin all inhibited ODC in an uncompetitive manner (Fig.

Table 1. Inhibition of ornithine decarboxylase

Agent	K_i (mM)	(N)	Type of inhibition
Neomycin	1.3 ± 0.1	(4)	Uncompetitive
Gentamicin	1.6 ± 0.1	(3)	Uncompetitive
Netilmicin	1.7 ± 0.2	(5)	Uncompetitive
Kanamycin	1.9 ± 0.2	(4)	Uncompetitive
Putrescine	1.7 ± 1.4	(3)	Competitive

Values represent means \pm SD of (N) independent experiments. In each experiment, duplicate or triplicate incubations were carried out at each of four different ornithine concentrations (25–100 μ M). Intercepts were determined by linear regression analysis of Lineweaver-Burk plots. Correlation coefficients for the individual analyses were: ornithine in the absence of inhibitors, >0.99 ; putrescine and gentamicin, >0.94 ; neomycin, 0.85 to 0.93; kanamycin, 0.81 to 0.98; and netilmicin, 0.78 to 0.99.

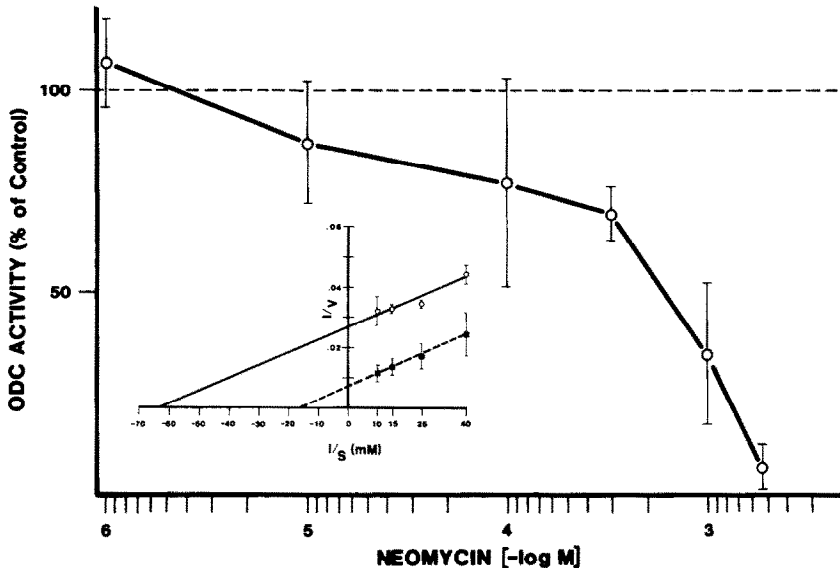


Fig. 2. Inhibition of ornithine decarboxylase activity by neomycin. Experiments were carried out as described in Methods with neomycin added as indicated. Values are expressed as percent of control ODC activity (in the absence of drug) and represent means \pm SD of four to six incubations each. Control ODC values were 61.8 ± 14.6 pmol $\text{CO}_2 \cdot \text{hr}^{-1} \cdot (\text{mg protein})^{-1}$, $N = 21$. Inhibition by 0.5 mM neomycin and above was significant ($P < 0.01$, Student's unpaired t -test). Insert: Lineweaver-Burk analysis of ODC inhibition by neomycin. Experiments were conducted as described in Methods with various concentrations of ornithine (25–100 μM) in the absence (\blacksquare) and presence (\circ) of 1 mM neomycin. $V =$ pmol $\text{CO}_2 \cdot \text{hr}^{-1} \cdot (\text{mg protein})^{-1}$; $S =$ [ornithine, mM].

2, insert; Table 1) with similar inhibitor constants ranging from $K_i = 1.3 \pm 0.1$ mM (neomycin) to $K_i = 1.9 \pm 0.2$ mM (kanamycin). The two forms of the enzyme with respect to their apparent K_m for PLP were both inhibited uncompetitively by neomycin with K_i values that did not differ significantly from the K_i determined above against various ornithine concentrations: (I) $K_i = 1.5 \pm 0.3$; (II) $K_i = 1.8 \pm 0.4$ mM. Putrescine inhibited ODC competitively with $K_i = 1.7 \pm 1.4$ mM.

DISCUSSION

The characteristics of guinea pig renal ODC from the soluble, postmitochondrial supernatant fraction agree well with those from other species: the K_m for ornithine (61 μM) is within the range of other reported K_m values (60–200 μM) and the enzyme is inhibited by 0.1 to 0.5 mM DFMO [7, 10]. Competitive inhibition by putrescine is also in agreement with the effects of putrescine on ODC in other tissues [7]. The presence of two K_m values for PLP suggests that the renal enzyme exists in two forms which are distinguished by their affinity to PLP but not ornithine. Two forms of ODC with respect to their K_m for PLP have been reported for *Physarum polycephalum* [11], Swiss 3T3 mouse fibroblasts [12] and *Dictyostelium discoideum* [13].

The salient point of this study is the finding that aminoglycoside antibiotics inhibited renal ODC. Nephrotoxicity and ototoxicity of these drugs have been recognized for over 40 years; however, only recently has a plausible hypothesis been proposed to explain the complex sequence of events involved in the development of toxicity [6]. Inhibition of ODC

may play an important role in this process by interfering with critical control mechanisms in the physiology of the target cells since the polyamines serve as important effectors in cellular growth, maintenance, and repair processes [7]. Points in favor of a role of this action in the expression of toxicity are the facts that neomycin has also been shown to inhibit cochlear ODC [14] and that the inhibitor constants for the aminoglycosides are in the low millimolar range for the renal enzyme. Such concentrations can be achieved *in vivo* in the kidney, particularly the cortex, following systemic administration of the drugs [15–18].

On the other hand, ODC is a ubiquitous enzyme and thus is a potential target of these drugs in other tissues as well. It has been suggested previously that the selective toxicity of the aminoglycosides is a result of several discrete biochemical interactions [6]. These include an active uptake process in competition with the polyamines, and disturbance of cell function through inhibition of polyphosphoinositide metabolism. In addition, specificity may also partially be attributed to the fact that renal aminoglycoside concentrations are several orders of magnitude higher than those found in other tissues [19]. This means that ODC is only inhibited by aminoglycosides if high enough intracellular drug concentrations are reached. The observation that the respective inhibitor constants of the aminoglycosides for ODC do not correlate directly with the rank order of nephrotoxicity (or for that matter ototoxicity) of these drugs [18] is in good agreement with the notion that inhibition of ODC may represent a major component of a complex injury cascade [6] rather than a single determining step. Inhibition of ODC,

however, may constitute a crucial mechanism for the lethal effects of these drugs on susceptible cells.

Induction of ODC has been shown to be an essential component in compensatory cellular responses to injury. Enzyme activity and intracellular polyamine levels increase following cellular damage by a variety of noxious stimuli including toxic drugs [7, 8, 20], apparently in order to adapt protein synthesis to repair processes. Inhibition of ODC by the aminoglycosides would then provide a potential mechanism by which cellular recovery is blocked. Such a block may well represent a final biochemical insult leading to death of susceptible cells.

The question then arises as to the mechanism by which aminoglycosides inhibit ODC. Although our experiments did not directly address this problem, the uncompetitive inhibition suggests a potential site of action. Uncompetitive inhibition is an attack at the enzyme-substrate complex, in this case ODC-PLP-ornithine. PLP has been shown to react *in vitro* with the polyamine spermine and the aminoglycoside gentamicin [21, 22] which appears to form a Schiff base and ring structure [23] with this coenzyme. The lethal effects of these drugs in the rat *in vivo* can also be antagonized by PLP [23]. Since ODC has an absolute requirement for PLP [24], such an aminoglycoside action would be an example of modulation of catalytic activity through coenzyme inhibition [11].

Our results thus suggest ODC as a target of aminoglycosides in susceptible cells. Inhibition of cellular repair mechanisms mediated by ODC may be an essential component in the complex mechanisms of aminoglycoside-induced nephrotoxicity and ototoxicity.

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