The Renal Mitochondrial Toxicity of Beta-Lactam Antibiotics: \textit{In Vitro} Effects of Cephaloglycin and Imipenem

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\textbf{ABSTRACT}

The nephrotoxic beta-lactam antibiotics cephaloridine, cephaloglycin, and imipenem produce irreversible injury to renal mitochondrial anionic substrate uptake and respiration after 1 to 2 h of \textit{in vivo} exposure. Toxicity during \textit{in vitro} exposure is nearly identical but is immediate in onset and is reversed by the mitochondria being washed or the substrate concentrations being increased. A model of injury that accounts for these findings proposes that the beta-lactams fit carriers for mitochondrial substrate uptake, causing inhibition that is initially reversible and becomes irreversible as the antibiotics acylate the transporters. These studies were designed to create an environment of prolonged \textit{in vitro} exposure, first, to determine whether toxicity becomes irreversible with time and, second, to study the molecular properties of toxicity. Respiration with and the uptake of succinate and ADP were measured in rabbit renal cortical mitochondria exposed for 2 to 6 h to 300 to 3,000 \textmu g of cephalaxin (nontoxic) or cephaloglycin or imipenem (nephrotoxic) per mL and then washed to remove the antibiotic. \textit{In vitro} cephaloglycin reduced respiration only slightly and was therefore not studied further. Cephaloglycin and imipenem irreversibly reduced both respiration and succinate uptake. ADP uptake was unaffected by cephaloglycin and was slightly reduced by imipenem. Finally, cilastatin, which prevents the tubular necrosis produced by imipenem \textit{in vivo}, reduced its mitochondrial toxicity \textit{in vitro}. It is concluded that the pattern of \textit{in vitro} injury of the nephrotoxic beta-lactams to mitochondrial substrate uptake and respiration evolves in a time-dependent and concentration-dependent manner, consistent with the proposed model of acylation and inactivation of substrate transporters, and that the protective action of cilastatin against imipenem occurs at least partly at a subcellular level.

Key Words: Beta-lactams, cephalosporins, carbapenems, nephrotoxicity, mitochondria

Among the beta-lactam antibiotics, several cephalosporins (1) and several carbapenems (2–4) produce acute proximal tubular necrosis when given in large doses. Beta-lactam injury, studied most thoroughly with the cephalosporin cephaloglycin, may occur at therapeutic doses under conditions of risk, such as combined administration with aminoglycosides (5), renal ischemia (6), and endotoxemia (7).

Studies in this laboratory have demonstrated two early functional insults of the nephrotoxic beta-lactams in renal cortical mitochondria—reduced substrate uptake and reduced respiration—and provided evidence that these effects are pathogenically interrelated. Irreversible injury is seen after \textit{in vitro} exposure for 1 to 2 h to toxic doses of cephaloglycin (8), cephaloridine (9), and imipenem (10)—all of which are nephrotoxic—but not cephalaxin, which is not nephrotoxic (11). In these studies, we exposed renal cortical mitochondria separately to cephaloglycin and imipenem \textit{in vitro} to determine: (i) whether the immediate \textit{in vitro} toxicity of these antibiotics, which is reversed by increasing substrate concentrations or by washing the mitochondria (12), resembles \textit{in vivo} toxicity in becoming irreversible with time, and (ii) whether cilastatin, which reduces the nephrotoxicity, tubular cell uptake, and intracellular hydrolysis of imipenem (2), can protect against the toxic beta-lactams at a subcellular level.

\textbf{MATERIALS AND METHODS}

Except where otherwise indicated, reagents were purchased from the Sigma Chemical Corporation (St. Louis, MO). The following isotopes were used: [2,3\textsuperscript{14}C]sucrose (42 mCi/mmol) and [U-\textsuperscript{14}C]sucrose (560 mCi/mmol), obtained from Amersham Corp (Arlington Heights, IL); [2,8\textsuperscript{3}H]adenosine 5'-diphos-
Mitochondrial Isolation

Female New Zealand White rabbits weighing 1.6 to 2.4 kg (Nitarbell Rabbity, Hayward, CA) were allowed free access to food (Wayne 15% Rabbit Ration; Allied Mills, Chicago, IL) and water until the morning of study. Untreated animals were killed by decapitation, and their kidneys were immediately removed. Renal cortical mitochondria were prepared as described by Johnson and Lardy (13) in an isolation medium (pH 7.4) containing 260 mM sucrose, 5 mM Tris-HCl, and 0.2 mM EGTA. To maintain comparable conditions for measurement of respiration and substrate uptake, all studies of mitochondrial function used a standard pH 7.4 respiration medium: 220 mM sucrose, 20 mM Tris-HCl, 10 mM sodium phosphate, and 5 mM potassium chloride—plus the substrates, inhibitors, and tracers indicated in individual protocols below.

Preparation of Renal Dehydropeptidase

Imipenem is hydrolyzed in the proximal tubular cell to antimicrobially inactive products by a membrane-bound dehydropeptidase (DHP) (2). Cilastatin, the DHP inhibitor marketed in combination with imipenem to block its renal metabolism, also reduces the tubular cell uptake and secretion and the tubular cell toxicity of imipenem (2). Because the mitochondrial preparations could possibly be contaminated by trace quantities of this enzyme and because the enzyme could possibly convert imipenem to a toxic intermediate during hydrolysis, DHP was prepared from renal cortex as described by Kropp and associates (14) for use in in vitro studies with imipenem.

In Vitro Exposure to the Beta-Lactams

Cephalexin and cephaloglycin (supplied by the Lilly Research Laboratories, Indianapolis, IN) were dissolved in 1 mEq of sodium bicarbonate per mL, and imipenem (supplied by the Merck Sharp & Dohme Research Laboratories, Rahway, NJ) was dissolved in a 10 mM sodium phosphate buffer (pH 7.0), at concentrations of 40 to 100 mg of antibiotic base per mL. These diluents, required because of the solubility characteristics of the individual antibiotics, had no measurable effects on the pH values of the incubation media in the quantities used.

Separate samples of mitochondria were incubated at 4°C for 0 to 6 h in 1 mL of isolation medium containing 1 mg of mitochondrial protein plus 0 to 3,000 μg of cephalexin, cephaloglycin, imipenem, or the appropriate quantity of their vehicles. At the appropriate times, the suspensions were centrifuged at 15,000 × g for 5 min, the supernatant was discarded, and the pellet suspended in cold isolation medium and recentrifuged to provide the final washed mitochondria for measurements of uptake and respiration. Previous studies at incubation temperatures higher than 4°C showed too much deterioration of function in controls to allow meaningful study of the effects of the beta-lactams.

Individual Measurements

Respiration. Oxygen consumption was measured in mitochondria at 20°C with a Clarke platinum electrode assembly (Gibson Medical Electronics, Inc., Middleton, WI) in 1.7 mL of respiration medium containing 1.7 mg of mitochondrial protein (15). As in previous studies, succinate was used as the metabolic substrate, at a concentration of 10 mM, and 5 μg of rotenone per mL (12.7 μM) was added to the respiration medium to block electron transport proximal to succinate entry into the respiratory chain. State 3 respiration was measured in the presence of 0.125 mM ADP.

Uptake. The net uptakes of succinate and ADP were studied by the method of sieve filtration (16). For measurement of succinate uptake, the incubation medium contained 1.2 × 10⁻⁶ M [¹⁴C]succinic acid (0.05 μCi/mL) without ADP; incubation and rinsing media contained 5 μg of antimycin A per mL to block succinate metabolism. For the study of ADP uptake, the incubation medium contained 4.4 × 10⁻¹⁰ M [³H]ADP (0.01 μCi/mL) with no substrate. In each case, mitochondria (0.5 mg of protein) were incubated at 20°C in 2 mL of respiration medium for 5 min (succinate) or 1 min (ADP)—times previously established to produce steady-state levels of the two compounds (8).

The suspensions were then trapped on Millipore DAWP 025 00 (0.65-nm) filters (Millipore Corp., Bedford, MA) with a Hoeffer model FH 225V 10 Place Manifold (Hoeffer Scientific Instruments, San Francisco, CA) and washed twice with 5 mL of ice-cold respiration medium. Two washes were established in preliminary studies as necessary to clear contaminating extramitochondrial isotope (using [¹⁴C]succinate) while causing minimal reduction of transported substrate.

Samples were placed in Aquasol Universal LSC, (Dupont, NEN Research Products) overnight to allow clarification of the filters and were then counted in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Inc., Mountain View, CA). Mitochondrial succinate or ADP was calculated from the total counts per filter and identically quenched standards of known specific activity. Contamination by extramitochondrial medium, tested by occasional
sampling of mitochondria incubated with $[^{14}C]$sucrose, was consistently small, equivalent to an average of approximately 1% of substrate counts.

For measurement of intramitochondrial water content, mitochondrial pellets were suspended for 5 min at 20°C in respiration medium containing trace quantities of $[^{3}H]$water and $[^{14}C]$sucrose and were then recentrifuged for 5 min at 15,000 $\times$ g. Separate samples of the recentrifuged pellets were counted or assayed for protein, and the intramitochondrial water content per gram of protein was determined as the difference between the total water and sucrose spaces.

Efflux. The washout of succinate from normal and beta-lactam-intoxicated mitochondria was measured by incubating separate samples (1 mg of protein) with the isotope for 5 min and then trapping the suspensions on Millipore filters and subjecting them to three different times of washing, ranging from 3 to 9 min, by continuous application of 20°C respiration medium and by counting the radioactivity remaining on the filters. Efflux rates were calculated by the method of least squares as the slopes of the logarithms of concentration against time.

Analytical

All data are presented as means ± SE. Statistical comparisons were made by Student’s $t$ test, where single measurements were made with accompanying controls, or by an analysis of variance, where multiple comparisons were made. Differences were judged to be significant where $P$ was <0.05 or where confidence levels were >95%.

RESULTS

Time and Concentration of Exposure

Peak renal cortical concentrations of the cephalosporins after single nephrotoxic doses are in the range of 1,000 to 3,000 $\mu$g/mL (1), and nephrotoxic injury develops over 1 to 5 h (17). Imipenem has not been studied in enough detail to provide comparable values. Figure 1 shows the pattern of evolution of irreversible mitochondrial respiratory injury over these ranges of time and concentration with in vitro exposure. Respiratory rates in the control mitochondria were reduced by incubation with the two antibiotic vehicles slightly and to a similar degree. Therefore, all controls at each time and concentration were averaged. There was a small progressive deterioration of function control mitochondria exposed to the antibiotic vehicles for 0 to 6 h (Figure 1A) and exposed briefly (zero-time) to increasing concentrations of the beta-lactams (Figure 1B). Although mitochondria incubated in 3,000 $\mu$g of cephalaxin per mL showed progressively lower rates of respiration than did controls over 0 to 6 h of exposure, the same concentrations of the nephrotoxic beta-lactams were significantly more toxic (Figures 1A and 2). Four hours of exposure to cephaloglycin or imipenem caused progressively greater reductions of respiration as concentrations were increased from 300 to 3,000 $\mu$g/mL (Figure 1B).

Respiration with and the Transport of Succinate

Respiration. The effects of cephalaxin, cephaloglycin and imipenem on state 3 respiratory rates (Figure 2) revealed significantly greater in vitro injury from the nephrotoxic beta-lactams than in the control or cephalaxin-exposed mitochondria.

Net Uptake. In parallel studies of succinate uptake (Figure 3), both cephaloglycin and imipenem reduced transport significantly and to a greater degree than they affected respiration. This greater effect on uptake is also seen with in vitro toxicity (8–10), and after ischemic injury (11), and is thought to occur...
Beta-Lactam Nephrotoxicity

Figure 2. The in vitro toxicity of beta-lactam antibiotics to mitochondrial respiration. Renal cortical mitochondria were incubated at 4°C for 4 h in isolation medium containing 3,000 μg of cephalaxin (Clx), cephaloglycin (Cgl), or imipenem (Imip) per mL, centrifuged, and washed. State 3 rates are presented. Data are means ± SE (control [Cntrl] N = 22; experimental N = 8 each). Significance levels by analysis of variance comparing injured with control mitochondria >97.5% (•) or >99.9% ($). The reduction of respiration after cephalaxin was significantly less than that produced by cephaloglycin (>99.9%) or imipenem (>97.5%).

Figure 3. In vitro toxicity of beta-lactam antibiotics to mitochondrial succinate uptake. Mitochondria were incubated with the appropriate vehicle (Control) or with 3,000 μg of cephaloglycin (Cgl) or imipenem (Imip) per mL for 4 h at 4°C and were then centrifuged, washed, and incubated for 5 min in a 20°C bath containing 1.2 × 10^{-6} M succinate. Data are presented as means ± SE (N = 18 to 20 each). P values comparing beta-lactam-intoxicated with control mitochondria <0.001 ($).

Figure 4. Effects of beta-lactam antibiotics on mitochondrial ADP uptake. Mitochondria were incubated with the appropriate vehicle (control, Cntrl) or with 3,000 μg of cephaloglycin (Cgl) or imipenem (Imip) per mL for 4 h at 4°C and were then centrifuged, washed, and incubated for 1 min in a 20°C bath containing 4.4 × 10^{-6} M ADP. Data are presented as means ± SE (N = 24 each). Significance level by analysis of variance comparing experimental with control mitochondria >99.9% ($).

Because substrate uptake is only partly rate limiting at the millimolar concentrations needed for studies of oxygen consumption.

Efflux. Slopes of succinate efflux in control, cephaloglycin-intoxicated, and imipenem-intoxicated mitochondria of 0.055 ± 0.008, 0.076 ± 0.006, and 0.065 ± 0.010 (N = 12 each), respectively, were comparable to the same measurements after in vivo exposure (8) and were not significantly different from one another. This indicates that, as is the case with in vivo toxicity, the in vitro effect of the beta-lactams on net succinate uptake is largely or wholly a result of reduced substrate entry.

ADP Uptake

Cephaloglycin had no significant effect on ADP transport (Fig. 4); imipenem reduced ADP uptake significantly but less dramatically than it decreased the uptake of succinate. These findings are similar to the in vivo effects of the beta-lactams on ADP transport (8, 10, 11).

Effects of Cilastatin

This inhibitor of imipenem nephrotoxicity reduced both the respiratory toxicity (Figure 5) and the toxicity to the succinate uptake (Figure 6) of imipenem. Coincubation with DHP (14) eliminated the in vitro respiratory toxicity of imipenem (state 3 rate after 4 h of exposure to 3,000 μg of imipenem per mL plus 0.5 mg of DHP protein per mL = 153 ± 4 [N = 8]), suggesting that hydrolysis of the antibiotic does not...
were negligible. The following molecular mechanism has been proposed to explain the respiratory toxicity of the cephalosporins (1). All cephalosporins, whether toxic or not, fit the carriers for mitochondrial anionic substrate uptake. In the intact kidney, where natural substrates are abundant, this causes limited or transient respiratory inhibition and no tubular necrosis. In vivo toxicity, measured in mitochondria isolated 1 to 2 h after antibiotic administration, is seen only with the intracellularly sequestered and reactive (i.e., nephrotoxic) cephalosporins that acylate these carriers to cause irreversible injury to substrate uptake.

This model was derived from several observations. In vitro exposure of normal renal mitochondria to toxic or nontoxic cephalosporins produces an immediate inhibition of respiration that is overcome either by the mitochondria being washed or by the metabolic anionic substrate concentrations being increased (12). In vivo respiratory toxicity, however, is specific to the cephalosporins that cause tubular necrosis (18, 21), evolves more slowly (18, 21), and is irreversible (12). Acylation by the beta-lactams of functionally important membrane bound proteins, caused by the cephalosporins (17) follows a pattern that closely resembles acute ischemic injury (20).

DISCUSSION

Several lines of evidence support a pathogenic role of mitochondrial injury in cephalosporin nephrotoxicity. Toxicity to respiration with different substrates is produced in vitro by nephrotoxic but not by nontoxic cephalosporins (18), develops as early as 1 h after antibiotic administration (18), and is augmented at this early time by an aminoglycoside regimen that potentiates nephrotoxicity (5). Exposure of cortical slices to cephaloridine decreases their ATP content by 1.5 h (19), and the ultrastructural damage

Figure 6. Effects of cilastatin on the toxicity of imipenem to mitochondrial succinate uptake. Renal cortical mitochondria were incubated at 4°C for 4 h in isolation medium containing the appropriate vehicle or 3,000 μg of imipenem per mL, 3,000 μg of cilastatin per mL, or imipenem plus cilastatin (Imip + Cst) and were then centrifuged, washed, and incubated for 5 min in a 20°C bath containing 1.2 × 10^{-6} M succinate. Data are presented as means ± SE (N = 12 each). Significance levels by analysis of variance comparing injured with control mitochondria >99% (asterisk) or >99.9% (dagger). Uptake was significantly greater in mitochondria incubated with imipenem plus cilastatin than in those incubated with imipenem alone (>99.9%).
the mechanism by which they exert their antibacterial action (22,23), occurs in tubular cell mitochondria (24). Cephaloglycin and cephaloridine, the most nephrotoxic of the cephalosporins, are among the most sequestered and most reactive protein acylators, while cephalxin and the penicillins, which have little or no nephrotoxic potential, are among the least reactive (18,21,25,26).

Substrate entry into mitochondria exposed to nephrotoxic doses of cephaloglycin or cephaloridine in vivo is reduced (8,9), while mitochondrial substrate transport is unaffected by much larger doses of cephalxin (11). Recent work with imipenem, which is also a highly reactive protein acylator (22), has shown essentially identical in vivo effects on mitochondrial transport and respiration (10).

It should follow from this model of toxicity that in vitro beta-lactam injury to mitochondrial substrate uptake and respiration becomes irreversible as acylation of the transporters evolves. Previous efforts at prolonged in vitro incubation were confounded by the deterioration of function in control mitochondria. These studies were therefore done at 4°C, which preserves function in controls incubated for 2 to 6 h. In addition to testing the proposed model of toxicity, isolated mitochondria allowed the study of protection by cilastatin without concern about its effects on the uptake or the metabolism of imipenem by the tubular cell.

As has been found with in vivo beta-lactam exposure, cephaloglycin and imipenem caused irreversible injury to both respiration and succinate entry into the mitochondrion (Figures 1–3) while producing less injury to ADP uptake (Figure 4). In vitro toxicity developed over ranges of time (2 to 6 h) and concentration (300 to 3,000 μg/mL) above those leading to similar effects in vivo (1 to 2 h and ~1,000 μg/mL) (18,21). This requirement for greater time and concentration may have been a result of the low temperatures needed for these studies.

Cilastatin reduced imipenem’s in vitro toxicity to respiration and uptake (Figures 5 and 6), indicating a protective action independent of its inhibitory effect on tubular cell transport of the antibiotic. The more severe imipenem-induced injury to uptake than to respiration, which is thought to occur because substrate transport is only partly rate limiting at the millimolar concentrations of substrate needed for studies of oxygen consumption, may be the reason for cilastatin’s less complete protection of transport.

Simultaneous in vitro incubation of DHP and imipenem reduced the direct mitochondrial toxicity of the carbapenem, possibly as a result of hydrolysis of the antibiotic. Regardless of the mechanism, protection against imipenem by DHP suggests that hydrolysis of the antibiotic does not contribute to its mitochondrial toxicity and rules against a protective ac-

![Figure 7. Structures of penicillins, cephalosporins, carbapenems, and dehydropeptides, including cilastatin.](image-url)


