Subcellular Distribution of Daptomycin Given Alone or with Tobramycin in Renal Proximal Tubular Cells

DENIS BEAUCHAMP,* PIERRETTE GOURDE, MARIE SIMARD, AND MICHEL G. BERGERON
Laboratoire et Service d'Infectiologie, Centre de Recherche du Centre Hospitalier de l'Université Laval, and Département de Microbiologie, Faculté de Médecine, Université Laval, Québec, Québec, Canada G1V 4G2

Received 1 July 1993/Returned for modification 10 October 1993/Accepted 15 November 1993

Previous studies in experimental animals showed that daptomycin, a lipopeptide antibiotic, protects against aminoglycoside nephrotoxicity (C. A. Wood, H. C. Finkbeiner, S. J. Kohlhepp, P. W. Kohnen, and D. N. Gilbert, Antimicrob. Agents Chemother. 33:1280–1285, 1989; D. Beauchamp, M. Pellerin, P. Gourde, M. Pettigrew, and M. G. Bergeron, Antimicrob. Agents Chemother. 34:139–147, 1990). In order to better understand the mechanism involved in this protective effect, the subcellular distribution of daptomycin was investigated in the proximal tubular cells of animals treated with daptomycin alone or in combination with tobramycin. A first group of female Sprague-Dawley rats received a single intravenous injection of daptomycin at a dose of 100 mg/kg of body weight and were killed at 10 min, 1 h, or 24 h after the injection. Other groups of rats were treated during 10 days with saline (NaCl, 0.9%), tobramycin at dosages of 20 mg/kg/12 h, daptomycin at dosages of 10 mg/kg/12 h, or the combination tobramycin-daptomycin at the same dosages. At the time of sacrifice, the renal cortex of the right kidney of each animal was dissected, and small blocks of tissue were fixed, dehydrated, and embedded in Araldite 502 epoxy resin. The subcellular distribution of daptomycin and tobramycin was determined on ultrathin sections by immunogold labeling. Ten minutes after the injection of daptomycin alone, gold particles were seen over the brush border membrane and on the membranes of the endocytic vacuoles of proximal tubular cells. One hour after the injection, a similar distribution was seen and numerous gold particles were found over the lysosomes of proximal tubular cells. After 24 h, daptomycin was seen essentially inside the lysosomes of proximal tubular cells. Daptomycin was also found inside the lysosomes of proximal tubular cells in animals treated with daptomycin alone or in combination with tobramycin. However, daptomycin was seen over the myeloid bodies inside the lysosomes of proximal tubular cells in the renal cortices of animals treated with the combination tobramycin-daptomycin after 10 days of treatment. The double labeling showed daptomycin and tobramycin inside the lysosomes of proximal tubular cells. The results suggest that daptomycin might protect against aminoglycoside nephrotoxicity by interfering with the interaction between the aminoglycoside and phospholipids inside the lysosomes of proximal tubular cells.

Daptomycin is a lipopeptide antibiotic that is active against methicillin-resistant staphylococci and other clinically important aerobic, facultative, and anaerobic gram-positive bacteria. Wood et al. (11) and Beauchamp et al. (3) recently showed that daptomycin protects experimental animals against tobramycin nephrotoxicity, while vancomycin increases the renal toxicity of aminoglycosides (3, 12). The protective effect of daptomycin was observed in the presence of either higher (11) or similar (3) levels of accumulation of tobramycin in the renal cortex.

The subcellular distribution of aminoglycosides has previously been investigated in the proximal tubular cells of experimental animals by cell fractionation (5), autoradiography (4, 9), and immunogold labeling (1, 2). It has also been observed that gentamicin remains associated with lysosomes throughout 9 days (5) or 10 days (1) of treatment.

The renal tissue and cellular distributions of daptomycin injected alone or in combination with an aminoglycoside are not well-known. The subcellular distribution of daptomycin injected alone or in combination with tobramycin was thus investigated in the renal cortical cells by immunogold labeling to better understand the mechanism of the interaction of daptomycin with an aminoglycoside.

MATERIALS AND METHODS

Animals and treatment. Female Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Montréal, Québec, Canada) weighing between 175 and 200 g were used. They had free access to food and water throughout the experiment. A first group of 15 rats was anesthetized with pentobarbital sodium (45 mg/kg of body weight), and a polyethylene catheter (PE-50; Intramedic, Clay Adams, Parsippany, N.J.) prefilled with 0.9% NaCl was inserted into the right jugular vein. The other end of the catheter was guided subcutaneously until it emerged through a hole made through the skin of the neck and was secured. After recovering from anesthesia, rats were returned to their cages and were left undisturbed overnight. On the following day, the animals were given, through the catheter, daptomycin (kindly donated by Eli Lilly & Co. Scarborough, Ontario, Canada) at a dose of 100 mg/kg and were killed 10 min (n = 4), 1 h (n = 4), or 24 h (n = 4) after the injection. At each time of sacrifice, one saline-injected rat was also killed as a control. Other groups of 15 rats each were treated with saline (n = 4) or daptomycin at dosages of 10 mg/kg/12 h subcutaneously (n = 4), tobramycin at dosages of 20 mg/kg/12 h intraperitoneally (n = 4), or the combination

* Corresponding author. Mailing address: Service d'Infectiologie, Centre de Recherche du Centre Hospitalier de l'Université Laval, 2705 Boulevard Laurier, Ste-Foy, Québec, Canada G1V 4G2. Phone: (418) 654-2705. Fax: (418) 654-2715.
tobramycin-daptomycin (n = 4) at the same dosages during 10 days. Animals were killed on day 11.

**Tissue sampling and fixation.** All animals were killed by decapitation and were bled. A midline abdominal incision was made, the right kidney of each animal was removed and bisected, and the renal cortex was dissected. A small piece of cortex was cut into small blocks of 1 mm³, and the blocks were placed into drops of 0.5% phosphate-buffered glutaraldehyde and were left overnight in the same fixative at 4°C. The blocks were then washed in 0.1 M phosphate buffer (pH 7.4), dehydrated in ascending grades of ethanol (50 up to 99%), and embedded in Araldite 502 epoxy resin (J. B. Em Services Inc., Pointe-Claire, Québec, Canada). Ultrathin sections (silver to light gold) were cut with an ultramicrotome (Ultracut S; Reichert-Jung; Leica Instruments GmbH, Montréal, Québec, Canada), mounted on nickel grids, and processed for immunocytochemistry.

**Immunogold labeling.** To determine the subcellular localization of daptomycin and tobramycin, the protein A-gold complex (7, 8) and antibody-gold complex (10) techniques were used. Each grid was first floated on a drop of phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.) for 20 min. Each grid was then placed on a drop of rabbit-anti-daptomycin antibody (Berkley Antibody Company [Babco], Richmond, Calif.) diluted 1/1,000 to 1/1,500 or sheep anti-tobramycin antibody (CortexBiochem, San Leandro, Calif.) diluted 1/20 to 1/200. These dilutions gave optimal results and low levels of background activity. Incubations with antisera were carried out for 60 min at room temperature in a moist chamber. The sections were then rinsed with PBS to remove unbound antibody and were incubated for 30 min on a drop of goat anti-rabbit-gold (diameter, 30 nm; Cederlane Laboratories Ltd., Hornby, Ontario, Canada) diluted 1/10 for rabbit anti-daptomycin antibody or onto a drop of protein A-gold (diameter, 15 nm) complex diluted 1/10 (Cederlane Laboratories) for sheep anti-tobramycin antibody. At the end of the incubation, the sections were washed twice with PBS, rinsed with distilled water, and dried. The double labeling was performed on opposite sides of the grid to eliminate nonspecific interactions. Staining of the grids with uranyl acetate and lead citrate was performed before they were examined with a Jem-JEM 1010 electron microscope (Soquelec, Montréal, Québec, Canada) at 60 kV.

**Immunogold controls.** Several concurrent control experiments for assessing the specificity of the immunolabeling were performed as follows: (i) incubation with protein A-gold or goat anti-rabbit-gold alone to identify the nonspecific absorption of each into the sections, (ii) absorption of each antibody with its specific antigen before the labeling protocol to verify the specificity of the antigen-antibody interaction, (iii) incubation with unlabeled protein A before applying the protein A-gold complex to verify the specificity of the immunoglobulin G-protein A interaction, (iv) replacement of the specific antibody by normal serum, (v) absorption of each antibody with an excess of the opposite antigen before immunogold labeling to verify the cross-reactivity between antibodies, and (vi) immunogold labeling of each antibody on renal cortical tissues from saline-treated rats.

**RESULTS**

Subcellular distribution of daptomycin in animals given a single injection of daptomycin. A first group of rats received a single intravenous injection of daptomycin at a dose of 100 mg/kg, and the subcellular distribution of daptomycin in the renal cortex was determined at 10 min, 1 h, and 24 h after the injection. Ten minutes after the injection, daptomycin was essentially seen over the brush border membrane and on the membrane of endocytic vacuoles of proximal tubular cells (Fig. 1A). One hour after the injection, a similar labeling was observed, but gold particles were also found over the lysosomes of proximal tubular cells (Fig. 1B). Twenty-four hours after the injection, gold particles were specifically concentrated over the lysosomes of proximal tubular cells, while no other subcellular site was labeled (Fig. 1C).

Subcellular distribution of daptomycin in animals treated with daptomycin alone or in combination with tobramycin. A second group of rats was treated subcutaneously with daptomycin at dosages of 10 mg/kg/12 h or with the combination tobramycin-daptomycin at the same dosages during 10 days, and the animals were killed on day 11. Gold particles were essentially seen over the lysosomes of proximal tubular cells in animals treated with daptomycin alone. Gold particles were distributed uniformly throughout the matrix of the lysosomes, as observed previously in the renal cortices of animals treated with a single injection of daptomycin (100 mg/kg) and killed 24 h after the injection. In animals treated with the combination daptomycin-tobramycin, lysosomes containing myeloid bodies were observed in proximal tubular cells, and gold particles were essentially seen over the myeloid bodies inside these lysosomes (Fig. 2A). Gold particles were more numerous in the lysosomes of animals treated with the combination daptomycin-tobramycin than in animals treated with daptomycin alone.

Subcellular distribution of tobramycin in animals treated with tobramycin alone. The subcellular localization of tobramycin in the proximal tubular cells of animals treated with tobramycin alone was recently reported by us, and the present results are consistent with those published previously (2). Lysosomes were the unique subcellular site of accumulation of tobramycin observed after 10 days of treatment. As shown previously, the intralysosomal distribution of tobramycin was different from that of gentamicin. In fact, in animals treated with gentamicin, gold particles were distributed uniformly over the lysosomes (1), while in animals treated with tobramycin, gold particles were concentrated into small areas over the lysosomes (2). No other subcellular site was labeled.

Subcellular distribution of daptomycin and tobramycin in the renal cortices of animals treated with the combination daptomycin-tobramycin. Anti-tobramycin antibodies were labeled with 15-nm-diameter gold particles, while anti-daptomycin antibodies were labeled with 30-nm-diameter gold particles. Gold particles (15 and 30 nm) were seen over the lysosomes of proximal tubular cells (Fig. 2B). Again, daptomycin (30-nm-diameter gold particles) was specifically seen over the myeloid bodies inside the lysosomes in these animals. No other subcellular site was labeled.

**Immunogold controls.** The control experiments proved the high degree of specificity of the labeling. The absorption of the antibody with its antigen resulted in the abolition of the labeling. The labeling was also absent under the other control conditions tested. The absorption of the antibodies with an excess of the opposite antigen did not modify the labeling. Moreover, no labeling was observed when the sections prepared from the renal cortices of animals treated with tobramycin were incubated with rabbit anti-daptomycin antibodies. Similarly, no labeling was observed when sections prepared from the renal cortices of animals treated with daptomycin were incubated with sheep anti-tobramycin antibodies. Finally, no labeling was observed over any subcellular site of untreated control kidneys incubated with the specific antibodies. All
FIG. 1. Subcellular localization of daptomycin in a proximal tubular cell at 10 min (A), 1 h (B), and 24 h (C) following a single intravenous injection of 100 mg/kg. Daptomycin antibodies were labeled with 30-nm-diameter gold particles. L, lysosomes; BBM, brush border membrane; V, endocytic vacuoles; M, mitochondria. Magnifications: ×26,600 (A), ×37,500 (B), and ×41,660 (C).
these observations were similar for all animals of each experimental group.

**DISCUSSION**

Previous studies done in our laboratories showed that animals treated with the combination daptomycin-tobramycin during 10 days had less inhibition of sphingomyelinase activity, less cellular regeneration, and fewer histopathologic changes in the renal cortex than those in animals treated with tobramycin alone (3). Wood et al. (11) also showed that rats given the combination of daptomycin-tobramycin exhibited a lower mean creatinine level in serum, higher mean creatinine clearance values, and less cortical tubular cell regeneration than animals treated with tobramycin alone. No detectable renal injury was observed following treatment with daptomycin alone (3, 11). These changes were observed in the presence of similar (3) or significantly higher (11) levels of tobramycin in the renal cortex.

Several hypotheses were suggested to explain the mechanism by which daptomycin protects proximal tubular cells against aminoglycoside nephrotoxicity. They are a chemical interaction between the aminoglycoside and the protective agent that modifies the aminoglycoside and thereby aborts or attenuates intracellular damage (11); an alteration of the intracellular sites of aminoglycoside toxicity by the protective drug, preventing aminoglycoside target interactions (11); a reduction of tobramycin binding to renal brush border membrane by daptomycin, resulting in a lower toxicity (3); a sequestration of the aminoglycoside within intracellular vacuoles observed in animals treated with daptomycin, preventing the lysosomes from aminoglycoside-induced inhibition of phospholipase activity (3); or a possible interaction between the aminoglycoside and the protective agent within the lysosomes (3).

The subcellular distribution of gentamicin from 10 min to 10 days following a single or multiple injections was recently determined in our laboratory by immunogold labeling (1). Within the first hour following a single injection, the drug was seen over the brush border membrane, on the membrane of endocytic vacuoles, and into the matrixes of small lysosomes located at the apical side of proximal tubular cells. After 10 days of treatment, gentamicin (1) and tobramycin (2) were localized in the lysosomes of proximal tubular cells, their unique subcellular site of accumulation.

The subcellular localization of daptomycin has never been investigated previously. Wood et al. (11) were unable to detect by a high-pressure liquid chromatography assay the presence of daptomycin in renal tissue following 14 days of treatment. Data from our laboratory suggest that daptomycin does not accumulate in the renal cortical tissues of animals given daptomycin alone, but significantly higher levels of daptomycin were measured in the renal cortices of animals treated with the combination daptomycin-tobramycin following 6 to 10 days of treatment (5a).

The present study shows that daptomycin injected alone enters proximal tubular cells at the apical part of these cells and was found in the lysosomes 1 h after the injection. Daptomycin was still detectable in the lysosomes of these cells 24 h after a single injection of 100 mg/kg. The distribution of daptomycin within the lysosomes observed in the renal cortices of animals treated with daptomycin alone was different from that seen in the cortices of animals treated with the combination daptomycin-tobramycin following 10 days of treatment. Daptomycin was distributed uniformly throughout the matrix of lysosomes of proximal tubular cells in animals given daptomycin alone. By contrast, daptomycin was essentially seen over the myeloid bodies in the lysosomes of proximal tubular cells in animals given the combination daptomycin-tobramycin. These results are consistent with recent data suggesting that daptomycin inserts within bilayers of phosphatidylinositol, dimyristoylphosphatidyglycerol, and dipalmitoylphosphatidylcholine, as judged by its influence on the fluidities of these bilayers (6). In fact, Gurnani et al. (6) reported that the modifications of the lipid band associated with the binding of gentamicin to phosphatidylinositol are consistent with a tightening of the lipid network and that the affinity of the aminoglycoside for phosphatidylinositol is slightly increased in the presence of daptomycin, but they did not find any evidence of a direct interaction involving the neutralization of the aspartate groups of daptomycin by gentamicin.

Our results combined with those of in vivo experiments (3, 11) and from infrared spectroscopy of the interaction between daptomycin, gentamicin, and phospholipid bilayers (6) shed more light on the mechanism of the protective effect of daptomycin against aminoglycoside nephrotoxicity. In fact, these studies suggest that the presence of daptomycin within lysosomes of proximal tubular cells is a key factor in the mechanism of protection. One cannot exclude the possibility that an interaction between daptomycin and tobramycin is essential before entry into the lysosomes. The present investigation, which localized daptomycin on myeloid bodies inside the lysosomes of proximal tubular cells, suggests that the insertion of the acyl chain of daptomycin within bilayers of phospholipid previously demonstrated in an in vitro model might occur in vivo. Moreover, the association of daptomycin with intralysosomal phospholipid in aminoglycoside-treated rats is probably responsible for the longer retention of daptomycin in the lysosomes of animals treated with the combination daptomycin-tobramycin compared with that in the lysosomes of animals given daptomycin alone.

We conclude that the intralysosomal accumulation of daptomycin is a key factor involved in its protective effect against aminoglycoside nephrotoxicity. Further experiments must be undertaken to better understand the interaction between daptomycin, aminoglycoside, and phospholipids under the conditions prevailing in the lysosomes, such as a low pH. There is no evidence to explain the effects of daptomycin on the interaction of aminoglycoside and phospholipids that lead to a reduction in the inhibition of phospholipase activities within the lysosomes.

**ACKNOWLEDGMENTS**

This study was supported by The Kidney Foundation of Canada and by the Medical Research Council of Canada (grant MA-10157). Denis Beauchamp is recipient of a F.R.S.Q./Eli Lilly scholarship.
REFERENCES

1. Beauchamp, D., P. Gourde, and M. G. Bergeron. 1991. Subcellular distribution of gentamicin in proximal tubular cells, determined by immunogold labeling. Antimicrob. Agents Chemother. 35:2173–2179.

2. Beauchamp, D., P. Gourde, M. Simard, and M. G. Bergeron. 1992. Subcellular localization of tobramycin and vancomycin given alone and in combination in proximal tubular cells, determined by immunogold labeling. Antimicrob. Agents Chemother. 36:2204–2210.

3. Beauchamp, D., M. Pellerin, P. Gourde, M. Pettigrew, and M. G. Bergeron. 1990. Effects of daptomycin and vancomycin on tobramycin nephrotoxicity in rats. Antimicrob. Agents Chemother. 34:139–147.

4. Bergeron, M. G., Y. Marois, C. Kuehn, and F. J. Silverblatt. 1987. Autoradiographic study of tobramycin uptake by proximal and distal tubules of normal and pyelonephritic rats. Antimicrob. Agents Chemother. 31:1359–1364.

5. Giurgea-Marion, L., G. Toubeau, G. Laurent, J. A. Heuson-Stiennon, and P. M. Tulkens. 1986. Impairment of lysosome-pinocytic vesicle fusion in rat kidney proximal tubules after treatment with gentamicin at low doses. Toxicol. Appl. Pharmacol. 86:271–185.

5a. Gourde, P., L. Lin, M. G. Bergeron, and D. Beauchamp. 1991. Program Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 326.

6. Gurnani, K., H. Khouri, M. Couture, M. G. Bergeron, D. Beauchamp, and D. Carrier. Molecular basis of the inhibition of gentamicin nephrotoxicity by daptomycin: an infrared spectroscopic investigation. Biochem. Biophysica. Acta, in press.

7. Roth, J. 1982. Evaluation of the protein-A gold (pAg) technique for labeling of multiple antigens: preparation of pAg complexes with 3-nm and 15-nm gold particles. Histochem. J. 14:791–801.

8. Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. J. Histochem. Cytochem. 26:1074–1081.

9. Silverblatt, F. C., and C. Kuehn. 1979. Autoradiography of gentamicin uptake by proximal tubule cell. Kidney Int. 15:335–345.

10. Tapia, F. J., I. M. Varndell, L. Probert, J. De Mey, and J. M. Polak. 1983. Double immunogold staining method for the simultaneous ultrastructural localization of regulatory peptides. J. Histochem. Cytochem. 31:977.

11. Wood, C. A., H. C. Finkbeiner, S. J. Kohlhepp, P. W. Kohnen, and D. N. Gilbert. 1989. Influence of daptomycin on staphylococcal abscesses and experimental tobramycin nephrotoxicity. Antimicrob. Agents Chemother. 33:1280–1285.

12. Wood, C. A., S. J. Kohlhepp, D. W. Kohnen, D. C. Houghton, and D. N. Gilbert. 1986. Vancomycin enhancement of experimental tobramycin nephrotoxicity. Antimicrob. Agents Chemother. 30:20–24.