Megalin Deficiency Offers Protection from Renal Aminoglycoside Accumulation*

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Aminoglycosides are antibiotics commonly used to treat life-threatening Gram-negative bacterial infections. However, their use is hampered by their severe nephrotoxicity due to accumulation in renal proximal tubules. Several pathways have been implicated in the renal uptake of aminoglycosides including megalin, an endocytic receptor in proximal tubular cells. Here, we have used mouse models with genetic or functional megalin deficiency to explore the contribution of megalin and other pathways to renal aminoglycoside uptake in vivo. We demonstrate that the uptake of aminoglycosides into the kidney directly correlates with renal megalin activity and is completely eliminated in mice lacking the receptor. Thus, our studies provide unequivocal evidence that megalin is the only major pathway responsible for renal aminoglycoside accumulation and that the receptor represents a unique drug target to prevent aminoglycoside-induced nephrotoxicity in patients.

Aminoglycosides are among the most commonly used antibiotics worldwide. They are active against a wide range of Gram-negative bacteria, including Pseudomonas, Enterobacter, Proteus, and Neisseria species. Because of their effectiveness and the low rate of true resistance, aminoglycosides are often considered the drug of choice to treat life-threatening infections such as bacterial endocarditis, peritonitis, and sepsis, as well as tuberculosis. Their relatively low costs make them attractive, particularly in developing countries. The clinical importance of aminoglycosides is likely to increase in the future due to the rapid rise in pathogens resistant to other classes of antibiotics (1, 2).

The main obstacle in the clinical use of aminoglycosides is their severe nephro- and ototoxicity. Aminoglycosides specifically accumulate in epithelial cells of the renal proximal tubule and in hair cells of the inner ear causing a variety of deleterious effects and eventual cell death (3–9). In recent years, changes in treatment regimens such as single daily dosing and monitoring procedures reduced the risk associated with aminoglycoside therapy. Nevertheless, still up to 10% of patients suffer from the toxic side effects of these antibiotics (1, 2).

When applied systemically, aminoglycosides remain largely inert. They do not adhere to plasma proteins and are eliminated from the body through glomerular filtration. After 24 h, 70–90% of the antibiotic has been excreted into the urine. In general, the tissue penetration of aminoglycosides is low, with the exception of the renal cortex that may absorb up to 5% of the compound. There, aminoglycosides persist for a long time (half-life > 100 h) causing renal damage (10).

Because the accumulation of aminoglycosides in epithelial cells of the proximal tubules is the main factor determining their nephrotoxicity, much attention had been focused on the identification of pathways responsible for cellular aminoglycoside uptake. Fluid phase uptake, adsorptive binding, and clearance by low affinity sites or receptor-mediated endocytosis all have been held responsible for renal aminoglycoside accumulation (7, 8). Among other pathways, megalin, an endocytic receptor expressed on the apical surface of the proximal tubular epithelium, has been implicated in renal aminoglycoside uptake (11, 12). Megalin constitutes the main endocytic pathway for clearance of low molecular weight plasma proteins from the glomerular filtrate (13). In megalin-deficient mice, lack of this uptake pathway results in tubular resorption deficiency and low molecular weight proteinuria (14). Physiological ligands taken up by megalin include insulin (15), transthyretin (16), and carriers for lipophilic vitamins, the vitamin D-binding protein (17) and the retinol-binding protein (18).

Besides the uptake of filtered plasma proteins, megalin may also be responsible for the clearance of xenobiotic compounds from the primary urine. In particular, polybasic substances such as aminoglycosides may interact with abundant negative charges on the extracellular receptor domain thereby gaining entrance to proximal tubular cells (11). Several studies in the rat, an animal model of aminoglycoside-induced nephrotoxicity, have addressed a possible role of megalin in renal aminoglycoside uptake. Møestrup et al. (11) used a specific megalin antagonist, the receptor-associated protein (RAP),1 to block the activity of the receptor in perfused rat proximal tubules. Inhibition of megalin reduced the clearance of gentamicin by ~20% (11). Nagai et al. (19) analyzed the aminoglycoside clearance in rats treated with maleate. This substance causes shedding of megalin from the brush-border surface, thus impairing receptor-mediated ligand uptake. In maleate-treated animals, the tubular clearance of aminoglycosides was decreased to the

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1 The abbreviation used is RAP, receptor-associated protein.
same extent as that of megalin ligands, suggesting uptake via the same receptor pathway (19).

While some experimental evidence links megalin activity with tubular uptake of aminoglycosides in the rat, the quantitative contribution of the receptor to renal aminoglycoside accumulation remained unclear. So far, inhibitors used to interfere with megalin activity were only marginally effective in blocking aminoglycoside clearance (e.g. RAP) or may have had additional unspecific effects on other tubular uptake pathways (e.g. maleate). Furthermore, studies by others suggested that renal aminoglycoside uptake also proceeds from the basolateral surface of the tubules (20) or that uptake is independent of endocytosis (21, 22).

Mice with genetically induced megalin deficiencies represent unique model systems to test a role of megalin in uptake of aminoglycoside into the kidney. Here, we have used two mouse models with induced receptor defects to dissect the quantitative contribution of megalin and other pathways to renal aminoglycoside accumulation. We demonstrate that aminoglycoside uptake into the kidney correlates with renal megalin activity and is absent in mice lacking the receptor (megalin knockout mice). As such, our studies provide clear evidence that megalin is the only major pathway for renal accumulation of aminoglycosides.

**EXPERIMENTAL PROCEDURES**

**Gentamicin Turnover in Mice—**Wild type and megalin-deficient mice (C57BL/6J × 129SvJ hybrids) were bred in house; RAP-deficient mice (C57BL/6J) were purchased from the Jackson Laboratories (www.jax.org). [3H]Gentamicin (55.1 μCi/mmol) was obtained from Amersham Biosciences, Inc. (www.apbiotech.com); gentamicin was purchased from Sigma (www.sigma-aldrich.com). For application in mice, [3H]gentamicin was diluted in 50 mM Tris, 150 mM NaCl, pH 7.4, and injected intraperitoneally or intravenously (data not shown).

**Autoradiography—**[3H]Gentamicin was injected intraperitoneally into laboratory animals at a dose of 2.2 × 10^6 dpm/g body weight (rats) and 7.2 × 10^6 dpm/g body weight (mice). After 24 h, the kidneys were fixed by retrograde perfusion through the abdominal aorta using 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, postfixed, dehydrated and embedded in Epon. Unless indicated otherwise, the tissues were prepared for light microscope autoradiography on 1-μm Epon sections using Ilford

**RESULTS**

Previous studies on the renal metabolism of aminoglycosides have mainly focused on the rat because this animal model exhibits similar susceptibility to aminoglycoside-induced nephrotoxicity as patients (3, 24–27). In contrast, for reasons largely unknown mice are resistant to aminoglycoside nephrotoxicity (24, 25). To ensure that murine resistance to nephrotoxicity was not due to differences in the renal handling of aminoglycosides as compared with patients and rats, we initially characterized the metabolism of gentamicin in wild type mice. We injected [3H]gentamicin intraperitoneally into the animals and determined the distribution of the tracer in plasma, urine, and tissues 24 h later. As seen in Fig. 1, 10% of the tracer was recovered in the kidneys, whereas 45% was found in the urine. No significant amounts of radioactivity were detected in any other tissue. Thus, the remainder of the tracer was most likely lost in the metabolic cages used for urine collection. The renal uptake of [3H]gentamicin was rapid with 10% of the tracer accumulating in the kidneys as early as 30 min after drug administration (Fig. 2A). The radioactivity persisted in the kidneys for more than 24 h and slowly declined thereafter (Fig. 2A). Still after 148 h, 3.4% of the tracer resided in the kidneys (data not shown). The kinetics of renal uptake was identical regardless of whether the tracer was applied intraperitoneally (Fig. 2A) or intravenously (data not shown).
compared the pharmacokinetics of \([3^{\text{H}}]\)gentamicin in wild type (Fig. 4). In contrast, in proximal tubular cells the tracer was more evenly distributed throughout the interior of the cells (Fig. 4A). This observation is consistent with an accumulation of gentamicin in lysosomes in both species because these vesicles are localized in the subapical space in mouse tubules and found deeper in the cytoplasm in the rat (13). The presence of the antibiotic in identical lysosomal compartments of rat and mouse cells was finally confirmed by autoradiography of electron micrographs (Fig. 5). No difference in the subcellular distribution of the tracer was observed between the species (Fig. 5).

So far, our studies have demonstrated that the metabolism and cellular uptake of megalin in mouse exhibits the same characteristics as in humans and rats. Therefore, the mouse constituted an appropriate animal model to study the common molecular pathways involved in renal aminoglycoside accumulation. To determine the quantitative contribution of megalin and other pathways to renal aminoglycoside uptake in vivo, we compared the pharmacokinetics of \([3^{\text{H}}]\)gentamicin in wild type and megalin-deficient mice. In wild type animals, 10.6 ± 0.5% (mean ± S.E.) of the tracer accumulated in the kidneys 24 h after drug administration; 46.9 ± 3.4% was excreted into the urine (Fig. 6). Megalin-deficient mice excreted similar amounts of the tracer (38.2 ± 8.6%), suggesting identical glomerular filtration rates. However, receptor-deficient animals did not exhibit any significant \([3^{\text{H}}]\)gentamicin accumulation in their kidneys (0.6 ± 0.1%, Fig. 6). This effect was not due to a metabolic turnover of aminoglycosides by expression of the neomycin phosphotransferase gene used for megalin gene targeting because mice carrying unrelated gene knockouts were indistinguishable from wild type controls (renin-binding protein knockout, Fig. 6).

To confirm that the rate of aminoglycoside accumulation directly correlated with the amount of megalin in the kidney, we tested the turnover of \([3^{\text{H}}]\)gentamicin in a mouse model of reduced receptor expression. In mice genetically deficient for RAP, the amount of megalin in the kidney is reduced by ~50% as compared with wild type animals (Fig. 7, inset). The reduction in megalin expression is most pronounced when the animals are kept on a C57BL/6J genetic background (29, 30). RAP is a cellular chaperone that blocks binding of ligands to the receptor and is required for proper biosynthesis and intracellular transport of megalin (29, 31). Consequently, in mice lacking this chaperone, the biosynthetic pathway of the receptor is disrupted resulting in decreased expression levels (29, 30). To be able to detect subtle differences in renal aminoglycoside uptake in RAP knockout versus wild type mice, we injected \([3^{\text{H}}]\)gentamicin together with 30 mg/kg unlabeled gentamicin (saturating concentrations, see Fig. 2B). Consistent with a reduction in receptor levels, the amount of \([3^{\text{H}}]\)gentamicin in
Pathway for Renal Aminoglycoside Accumulation

FIG. 6. Accumulation of [3H]gentamicin in kidneys and urine of wild type, megalin−/−, and RnBP−/− mice. Mice either wild type (n = 38) or genetically deficient for megalin (megalin−/−, n = 5) or renin-binding protein (RnBP−/−, n = 7) were given an intraperitoneal dose of [3H]gentamicin (45 μg/kg). Urine was collected for 24 h, and kidney samples were obtained thereafter. The amount of radioactivity recovered in urine and kidneys was quantified. Values are given as mean ± S.E.

FIG. 7. Renal accumulation of [3H]gentamicin in wild type, RAP−/−, and megalin−/− mice. Mice either wild type (n = 15), RAP−/− (n = 5), or megalin−/− (n = 7) were injected intraperitoneally with a mixture of 45 μg/kg [3H]gentamicin and 30 mg/kg unlabeled gentamicin, and the amount of radioactivity in the kidneys was determined 2-4 h later. There was a significant reduction in gentamicin uptake in RAP−/− kidneys as compared with wild type tissues (p < 0.001 by Student’s t test). The inset depicts a semiquantitative Western blot analysis of megalin expression in 1 μg of renal membrane extracts from two wild type (wt) and two RAP-deficient mice/RAP−/−.

RAP−/− kidneys was decreased by ~50% as compared with control tissues (p < 0.001, Fig. 7).

DISCUSSION

Here, we have demonstrated that megalin, an endocytic receptor in the renal proximal tubules, represents the only major pathway for accumulation of aminoglycosides in the mouse kidney. Renal uptake of the antibiotic directly correlates with the levels of receptor activity in mouse models of reduced megalin function (RAP−/−, Fig. 7). No renal uptake of aminoglycosides can be observed in mice completely devoid of the receptor (megalin−/−, Fig. 6). This lack of uptake is a direct consequence of the megalin deficiency and is not caused by unspecified kidney defects because renal functions such as glomerular filtration, urine output, or tubular resorption of electrolytes and other metabolites are normal in megalin−/− animals (14). The identification of an endocytic pathway responsible for cellular uptake of aminoglycosides confirms earlier findings that localized these antibiotics within endosomes and lysosomes of proximal tubular cells (27, 32). In contrast, our results argue against a significant role of other routes such as fluid phase uptake or non-endocytic pathways in entry of aminoglycosides into cells in vivo. Because megalin is exclusively present on the apical membranes of the tubular epithelium (33, 34), in vivo uptake of aminoglycosides from the basolateral surface into tubular cells also seems less likely.

Previous studies on the role of megalin in renal aminoglycoside accumulation focused on the rat as a model system (11, 19). Megalin antagonists were used to interfere with the activity of receptor and to test the consequences for aminoglycoside uptake in vivo. In these studies, RAP reduced the tubular clearance of gentamicin by 20% suggesting a minor role of megalin in renal aminoglycoside accumulation (11). Contrary to the rat, mouse models with induced megalin deficiencies represent the first genetically defined animal models to quantify the contribution of megalin to renal aminoglycoside uptake. In these models, megalin represents the only major pathway for aminoglycoside uptake and lack of the receptor offers protection from renal accumulation of the antibiotic. The moderate effect of RAP on gentamicin uptake observed in the rat is not due to differences in aminoglycoside uptake pathways in this animal model as compared with the mouse. Rather, the ineffectiveness of this inhibitor is explained by its inability to efficiently interfere with gentamicin binding to megalin. Approximately 60–100 molecules of gentamicin bind per megalin molecule whereas RAP occupies only 1–2 ligand binding sites on the receptor. Therefore, the antagonist is largely ineffective in blocking gentamicin binding to megalin.

It is well established that renal uptake of aminoglycosides is the main factor that determines their nephrotoxicity. Thus, aminoglycoside derivatives with reduced tendency to accumulate in the renal cortex also proved less nephrotoxic (35). Given its central role in renal aminoglycoside uptake, megalin activity can be assumed to be a critical factor contributing to aminoglycoside-induced nephrotoxicity. Conceivably, functional megalin deficiency should prevent nephrotoxic side effects of aminoglycoside treatment. Unfortunately, the resistance of mice to aminoglycoside-induced nephrotoxicity precludes testing of this hypothesis in our mouse models. Even after prolonged exposure of wild type mice to high doses of gentamicin, we have not been able to detect major signs of tubular damage. Similar findings were also seen by others (24, 25). The reasons for this resistance remain unknown. To confirm that the insensitivity of mice to aminoglycosides is not due to the existence of different uptake routes, we compared renal gentamicin clearance in mice and rats. All parameters including plasma and renal half-life, tissue distribution, concentration dependence, and saturability of renal uptake were identical in both models. Moreover, gentamicin was detected in the same intracellular compartments (lysosomes) in rat and mouse proximal tubules. Taken together, these findings clearly demonstrate that identical pathways for aminoglycoside uptake exist in both species and that megalin is likely to play an equally crucial role in renal uptake and nephrotoxicity of aminoglycosides in rats and patients.

Accumulation of aminoglycosides in lysosomes with subsequent rupture of the vesicles is considered the main mechanism causing nephrotoxicity in rats and humans (7). Because aminoglycosides are directed to the very same organelles in mouse proximal tubules, murine resistance to aminoglycoside-induced nephrotoxicity likely involves alternative strategies of the mouse to deal with the consequences of intracellular aminoglycoside accumulation. Consistent with this hypothesis, specific protective mechanisms in the mouse to inactivate internalized aminoglycosides or to repair damaged renal tissues are being discussed (8). Elucidation of these mechanisms may reveal possible strategies also to deal with aminoglycoside accumula-

2 J. Hilpert, C. Jacobsen, and T. E. Willnow, unpublished observations.
tion in patients. These investigations are, however, beyond the scope of this study.

In conclusion, acute renal failure caused by aminoglycosides is still an important clinical entity that hampers the unrestricted use of these antibiotics. In industrialized nations, elaborate dosing and monitoring procedures have been implemented to minimize toxic side effects of aminoglycoside treatment. However, these measures result in a dramatic increase in therapy costs. While a standard gentamicin therapy amounts to only $20 for drugs, supplies, and labor costs, therapy requires an additional $17 for monitoring and $180 for treatment of nephrotoxic side effects (36, 37). In developing countries where no monitoring procedures are implemented and aminoglycosides are often available freely over the counter, the dramatic increase in therapy costs. While a standard gentamicin therapy amounts to only $20 for drugs, supplies, and labor costs, therapy requires an additional $17 for monitoring and $180 for treatment of nephrotoxic side effects (36, 37).

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