Role of megalin in renal handling of aminoglycosides

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Received 10 July 2000; accepted in final form 20 April 2001

Nagai, Junya, Hiroaki Tanaka, Naoki Nakanishi, Teruo Murakami, and Mikihisa Takano. Role of megalin in renal handling of aminoglycosides. Am J Physiol Renal Physiol 281: F337–F344, 2001.—The role of megalin in tissue distribution of aminoglycosides was examined in normal rats and maleate-treated rats that shed megalin from the renal brush-border membrane. In normal rats, amikacin administered intravenously accumulated most abundantly in the renal cortex, followed by the renal medulla. No amikacin was detected in other tissues. Tissue distributions of amikacin were well correlated with megalin levels in each tissue. Bolus administration of gentamicin increased urinary excretion of megalin ligands (vitamin D binding protein and calcium), suggesting the competition between gentamicin and these megalin ligands in renal tubules. Ligand blotting showed that binding of $^{45}$Ca$^{2+}$ to megalin was inhibited by aminoglycosides. Both megalin levels and amikacin accumulation in renal cortex were decreased by maleate injection. Then, amikacin accumulation recovered proportionate to megalin levels. These findings suggest that megalin is involved in the renal cortical accumulation of aminoglycosides in vivo. In addition, the interaction between aminoglycosides and calcium in the kidney may be due to the competition among these compounds to bind to megalin.

Nephrotoxicity is a major clinical complication of aminoglycoside antibiotics, which are widely used for the treatment of Gram-negative infectious disease (27, 35). Aminoglycosides are not metabolized in the body, and most of the injected dose is excreted into the urine, whereas a fraction accumulates in the renal proximal tubular cells, where the concentration of aminoglycosides is several times higher than in plasma. The concentrated accumulation of aminoglycosides in the proximal tubular cells is associated with the nephrotoxic effects (14). The transport of aminoglycosides in the kidney has been studied by various techniques in vivo (12, 16, 17, 30) and in vitro (5, 13, 18, 23, 38). These results indicate that aminoglycosides interact with the brush-border membrane in the renal proximal tubular cells and are subsequently taken up by an adsorptive endocytosis. In addition, acidic phospholipids, like phosphatidylinositol, are thought to be the binding sites of aminoglycosides in the brush-border membrane (10, 36, 37). On the other hand, because acidic phospholipids are commonly distributed in the plasma membranes in various tissues, other factors mediating endocytosis of aminoglycosides, specifically in the renal proximal tubular cells, have been assumed to be involved. However, the membrane receptor involved in the binding and endocytosis of aminoglycosides in the kidney has not been identified until recently.

Megalin, initially identified as a Heymann nephritis autoantigen (19, 34), is a large glycoprotein (~600 kDa), functioning as an endocytic receptor. Now also considered a low-density lipoprotein receptor-related protein-2 (LRP-2), megalin is a member of the low-density lipoprotein receptor gene family and is expressed in the renal proximal tubule epithelium, labyrinth epithelium of the ear, retinal epithelium, type II pneumocytes, and yolk sac (40). The cytoplasmic tail of megalin contains the NPXY sequence, which is necessary for coated pit-mediated internalization (3, 34). The ligands binding to megalin represent a variety of classes including Ca$^{2+}$ (4), lipoprotein lipase (21), plasminogen activator-plasminogen activator inhibitor type-1 complexes (26), and receptor-associated protein (RAP) (20). Recently, Nykjaer et al. (28) found that complexes of 25-(OH) vitamin D$_3$ and vitamin D binding protein (DBP) filtered through the glomerulus are taken up by megalin into the proximal tubular cells, where 25-(OH) vitamin D$_3$ is further metabolized to the physiologically active 1a,25-(OH)$_2$ vitamin D$_3$. In addition, megalin would also be involved in the binding and endocytosis of polybasic drugs such as aminoglycosides, aprotinin, and polymyxin B in the renal brush-border membrane (25). The rank order of the apparent affinity of various aminoglycosides for purified megalin agrees with that of their nephrotoxic potentials. However, the role of megalin in the renal handling of these ligands in vivo needs further clarification.
ROLE OF MEGALIN IN AMINOGLYCOSIDE DISTRIBUTION

In the present study, we analyzed the expression of megalin and the distribution of aminoglycosides in tissues of normal and maleate-treated rats. Our results suggest that megalin is involved in the accumulation of aminoglycosides in the renal cortex.

MATERIALS AND METHODS

Materials. The following reagents were used: amikacin sulfate salt (Sigma, St. Louis, MO), maleic acid disodium salt, gentamicin sulfate, 2,4,6-trinitrobenzenesulfonic acid sodium salt, polymixin B sulfate (Nacalai Tesque, Kyoto, Japan), and inulin (Kanto Chemical, Tokyo, Japan). 45Ca2+ (0.72 GBq/mg calcium) was obtained from Amersham International (Buckinghamshire, UK). All chemicals used were of the highest purity available.

Preparation of antiserum against megalin. Rabbit anti-megalin antibody was raised against the synthetic peptide corresponding to the 17-amino acid peptide for the cytoplasmic domain of rat megalin (MEVGKQPVFENPMYAA), with an additional cysteine at the NH2 terminal (1, 34). The synthetic peptide was produced by the peptide synthesizer (PSSM-8, Shimadzu). The peptide was linked to a carrier protein, keyhole limpet hemocyanin (Sigma), with a coupling agent, m-maleimidobenzoic acid N-hydroxysuccinimide ester (Sigma). The cross-linked peptide was mixed with an equal volume of Freund's complete adjuvant (Wako Pure Chemicals, Osaka, Japan), and the emulsion was injected intradermally into New Zealand White female rabbits (2.0–2.5 kg) on day 0. Subsequent immunization was performed with incomplete Freund's adjuvant (Wako Pure Chemicals) at four intervals between days 28 and 64. Serum samples were tested for reactivity with the antigen peptide by dot-blot analysis, using a horseradish peroxidase-conjugated goat antibody to rabbit IgG (ICN Pharmaceuticals, Aurora, OH) as a secondary antibody. At day 36 after the start of immunization, antibodies reactive with the antigen peptide were detected by dot-blot analysis.

Western blot analysis and ligand blotting. Excised tissues were homogenized in 1% Triton X-100 in 20 mM Tris, 150 mM NaCl and centrifuged at 14,000 g for 15 min in an Avanti 30 compact centrifuge with rotor F0630 (Beckman Instruments). The supernatant was solubilized in a loading buffer; 2% SDS, 62.5 mM Tris–HCl, 7% glycerol with or without 5% 2-mercaptoethanol (reducing or nonreducing condition, respectively). Collected urine was dialyzed by use of cellulose membrane and was concentrated by freeze-drying. These samples were subjected to SDS-PAGE with polyacrylamide gels of 6% for megalin detection or 10% for DBP detection. The proteins were transferred for 60 min to polyvinylidene difluoride membrane (Immun-Blot, Bio-Rad) at 4°C. In Western blot analysis, the membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris–HCl, pH 7.5) with 0.05% Tween 20 (TBS-T) overnight at 4°C. The membranes were washed three times for 10 min each in TBS-T and incubated with the rabbit antiserum to the cytoplasmic tail of rat megalin (1: 5,000 dilution) or anti-human DBP rabbit antiserum (1:100 dilution) purchased from DAKO Japan (Kyoto, Japan). The membranes were washed three times in TBS-T and incubated with horse-radish peroxidase-conjugated anti-rabbit IgG antibody (1: 2,000 dilution, ICN Pharmaceuticals), again washed three times in TBS-T, and visualized with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). Ligand blotting with 45Ca2+ was carried out as described by Maruyama et al. (24). Briefly, the membranes were washed with a buffer containing 60 mM KCl, 5 mM MgCl2 and 10 mM imidazole-HCl (pH 6.8), and the buffer was changed three times in 1 h. Subsequently, the membranes were incubated for 10 min in the same buffer containing 45Ca2+ (37 MBq/l) in the absence or presence of various cationic compounds. After being rinsed with 50% ethanol for 5 min, the membranes were dried at room temperature for 3 h. Autoradiograms of the 45Ca2+-labeled proteins were obtained by exposure of the dried membranes to Amersham Hyperfilm for 24–72 h.

Animal experiments. Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, School of Medicine, Hiroshima University. To induce the shedding of megalin from the renal brush-border membrane, male Wistar rats (260–320 g) received sodium maleate (400 mg/kg, pH 7.4) by intraperitoneal injection. Rats injected with the same volume of saline were used as control. Subsequently, the rats were anesthetized with pentobarbital sodium (30 mg/kg), and the femoral artery and vein were cannulated with polyethylene tubing for blood sampling and drug administration, respectively. The urinary bladder was cannulated to collect the urine sample. At a stated time after the maleate injection, amikacin (3.5 mg/kg) and inulin (20 mg/kg) were administered as a bolus via the femoral vein. At 120 min after the administration of amikacin, each tissue was excised. The tissues were weighed and then homogenized with 4 vol of ice-cold 3% perchloric acid. After centrifugation at 3,000 rpm for 15 min, amikacin concentration in the supernatant was assayed as described below.

Analytic methods. Quantitative determination of amikacin was performed by using high-performance liquid chromatography (PU-980, Jasco, Tokyo, Japan) equipped with an ultraviolet spectrophotometric detector (UV-970, Jasco; wavelength 229 nm) after derivatization of the drug by reaction with 2,4,6-trinitrobenzenesulfonic acid (11). The conditions were as follows: column 4.6 × 100 mm, TSK gel ODS-80TM (Tosoh, Tokyo, Japan); mobile phase 0.02 M phosphate buffer (pH 7.5)-acetonitrile-methanol (vol/vol) = 10:11:5; flow rate 1.0 ml/min; and room temperature. Inulin concentrations in the urine were analyzed by spectrophotometric assay as stated previously (22). Urinary calcium was measured with commercial kits available from Wako Pure Chemicals.

Statistical analysis was performed by Student’s t-test. A difference of P < 0.05 was considered statistically significant.

RESULTS

Expression of megalin and accumulation of amikacin in various tissues in normal rats. First, we characterized the rabbit antiserum raised against the synthetic peptide for the cytoplasmic domain of rat megalin. As shown in Fig. 1c, the antiserum detected a high-molecular-weight protein (>400 kDa) in rat renal cortex protein subjected to gel electrophoresis. A band of the same size was observed by blotting with 45Ca2+ (Fig. 1e), which disappeared when reduced with 2-mercaptoethanol (data not shown). The band was not detected when the preimmune serum was used (Fig. 1b). In addition, the band detected by the antiserum disappeared in the presence of the antigen peptide (Fig. 1d). Thus the anti-cytoplasmic peptide antiserum we obtained was shown to specifically recognize megalin. With this anti-megalin antiserum, the tissue distribution of megalin was examined in normal rats by Western blot analysis and immunohistochemical analysis.

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As shown in Fig. 2, the expression of megalin was highest in the renal cortex. The megalin band was also detected in the renal medulla but was not observed in the renal papilla, brain, lung, or liver. Furthermore, the immunohistochemical study showed that megalin was located in the apical membrane of the proximal tubules in rat renal cortex, whereas no staining was observed in the liver (data not shown). Next, the tissue distribution of amikacin was examined 120 min after an intravenous bolus injection of the drug (Fig. 3). Amikacin was more abundant in the renal cortex than other tissues examined. Amikacin was found to a lesser extent in the renal medulla but was not detected in the renal papilla, brain, or liver. Thus the tissue distribution of amikacin was well correlated with the expression level of megalin.

Interaction between aminoglycosides and megalin ligands. The effect of intravenous bolus administration of gentamicin on urinary excretion of such endogenous megalin ligands as DBP and calcium was examined. The administration of gentamicin (3.5 mg/kg) increased the urinary excretion of DBP (Fig. 4). Urinary calcium was also significantly increased by the gentamicin administration (control, 46.1 ± 17.8 μg/120 min, n = 5; gentamicin-administration, 119.8 ± 23.8 μg/120 min, n = 3, means ± SE, P < 0.05). To analyze the involvement of megalin in calcium-aminoglycoside interaction, we examined the effect of aminoglycosides on the binding of 45Ca2+ to megalin (Fig. 5). Unlabeled calcium (2 mM) almost completely inhibited the binding of 45Ca2+ to megalin. 45Ca2+ binding to megalin was inhibited by the presence of aminoglycosides (gentamicin, amikacin). In addition, the binding of 45Ca2+ was inhibited by polymixin B, a basic peptide antibiotic, which was reported to be a ligand of megalin by Moestrup et al. (25).

Effect of maleate administration on megalin level and amikacin accumulation in renal cortex. Bergeron et al. (2) have reported that maleate in rats induces shedding of megalin from the brush-border membrane in the renal proximal tubule cells and increases urinary excretion of megalin. Therefore, maleate-treated rats may be useful as model animals with which to examine the in vivo function of megalin in the renal proximal tubules. To confirm the decrease in megalin in the renal proximal tubules by the administration of maleate in the present study, megalin levels in the renal cortex and in the urine from maleate-treated rats were examined by Western blot analysis and by ligand blotting with 45Ca2+. Similar results were obtained by using both methods: a decrease in megalin level in the renal cortex and the appearance of megalin in the urine of maleate-treated rats (Fig. 6, A and B). Then, renal accumulation of intravenously administered amikacin was examined in normal rats and rats treated with maleate. The results showed that the amount of amikacin accumulated in the renal cortex of maleate-treated rats was significantly lower than that of normal rats (Fig. 7A). The decrease in the amikacin accumulation in the renal cortex would not be due to the change in the amount of amikacin filtered through the glomerulus, because the urinary excretion of amikacin for 120 min (Fig. 7B), as well as that of inulin (data not shown), was not changed by the maleate treatment. Furthermore, the relationship between amikacin and megalin levels in the renal cortex was examined at various intervals after male-
Aminoglycosides are low-protein binding drugs (<10%), freely filtered through the glomerulus in the kidney. Subsequently, aminoglycosides are selectively accumulated and remain, with a long half-life, in the renal cortex (35). Many studies have shown that the uptake of aminoglycosides in the renal proximal tubules is due to an endocytic system, such as a receptor-mediated endocytosis. However, little information is available on the endocytic receptor. Recently, Moestrup et al. (25) reported that polybasic drugs including aminoglycosides bind to purified megalin, which is an endocytic receptor abundantly expressed in the renal proximal tubules. In addition, they found that RAP inhibits the uptake of \[^{3}H\]gentamicin microperfused into single proximal tubules in rat. These observations suggest the possibility that megalin is involved in the accumulation of aminoglycosides in the renal cortex. However, it has not been demonstrated that megalin plays an important role in the tissue distribution of aminoglycosides in vivo. In the present study, we examined the in vivo role of megalin in the tissue distribution of aminoglycosides using normal and maleate-treated rats.

The present results showed that amikacin administered intravenously was accumulated most abundantly in the renal cortex (Fig. 3), where the expression of megalin was highest among various tissues examined (Fig. 2). On the other hand, amikacin was not detected in the renal papilla, brain, lung, and liver (Fig. 3). Zheng et al. (40) detected megalin in brain ependyma and lung pneumocytes type II in an immunohistochemical study, but we did not detect megalin by Western blot analysis using whole tissue homogenates of the brain and lung. Megalin expressed in these specialized regions of the brain and lung might be involved in the endocytosis of megalin ligands in cerebrospinal fluid and respiratory airways, where intravenously administered amikacin is unlikely to be distributed. Therefore, we did not detect amikacin in the brain and lung because of the insensitivity of our analytic method, low expression of megalin in these tissues, or the limited distribution of intravenously administered amikacin where megalin is expressed.
Interestingly, aminoglycosides such as gentamicin, netilmicin, and amikacin are reported to inhibit in vitro binding of the $^{125}$I-urokinase-plasminogen activator inhibitor-1 complex not only to megalin but also to LRP, a member of the low-density lipoprotein receptor gene family (25), suggesting the interaction between aminoglycosides and LRP. The expression pattern for LRP differs strikingly from that of megalin. In contrast to megalin, LRP is abundantly expressed in the liver but not in the renal proximal tubules (40). However, as shown in this study, amikacin was not accumulated in the liver in vivo (Fig. 3), suggesting that LRP does not play an important role in the tissue uptake of aminoglycosides. The reason for this apparent discrepancy between in vitro and in vivo observations is presently unknown. In addition to megalin, other factors may be involved in the selective accumulation of aminoglycosides in the kidney.

Maleate is often used to induce the experimental Fanconi syndrome (2, 32, 33). In the present study, megalin levels in normal and maleate-treated rats were estimated by two methods: ligand blotting with $^{45}$Ca$^{2+}$, which binds to the extracellular domain of megalin, and Western blotting with a rabbit antiserum raised against the synthetic peptide for the cytoplasmic domain of megalin. These two methods revealed a decrease in megalin level in the renal cortex of maleate-treated rats. In addition, megalin was detected in the urine of maleate-treated, but not of normal, rats (Fig. 6). These results indicate that megalin excreted in the urine of maleate-treated rats would retain the cytoplasmic domain (without cleavage in the ectodomain). This is consistent with the previous report that a polyclonal antibody against the cytoplasmic tail of megalin showed a mosaic pattern of megalin staining in the renal tubules in maleate-treated rats (2). However, it is unclear how megalin retaining the cytoplasmic domain sheds from the brush-border membrane by maleate administration.

The administration of maleate reduced the renal accumulation of amikacin, corresponding to the decrease in megalin level in the renal cortex (Fig. 7). Furthermore, we found that amikacin accumulation in the renal cortex of maleate-treated rats recovered over time in accordance with the recovery of megalin level in the renal cortex, suggesting that the renal cortical accumulation of amikacin is dependent on the megalin level in the renal cortex (Fig. 8). There are several previous reports showing that maleate administration increases the decrease in the reabsorption of aprotinin and lysozyme in the renal proximal tubules (9, 39). Recently, both aprotinin and lysozyme are shown to be
ligands of megalin (25, 29). These observations as well as the present results suggest that megalin is involved in the in vivo reabsorption of these cationic compounds. However, the involvement of a nonspecific effect of maleate on endocytosis in the proximal tubules cannot be ruled out.

If megalin plays an important role in the renal uptake of aminoglycosides as described above, the administration of aminoglycosides may induce the increase in urinary excretion of endogenous megalin ligands due to the competitive inhibition of the binding to megalin. To test this possibility, the effect of gentamicin injection was examined. As was expected, the intravenous injection of gentamicin increased the urinary excretion of DBP (Fig. 4) and calcium, both of which are ligands of megalin (4, 28). Consistent with the increase in urinary calcium by the administration of gentamicin in vivo, the binding of $^{45}$Ca$^{2+}$ to megalin blotted on the filter membrane was inhibited by gentamicin and amikacin (Fig. 5). In addition, polymixin B, which has a relatively high affinity for megalin (25), also decreased the calcium binding to megalin. Some reports have focused on the interaction between aminoglycosides and calcium in the kidney. Humes et al. (15) reported that calcium inhibits the binding of aminoglycosides to the renal plasma membranes and that oral calcium loading protects against aminoglycoside nephrotoxicity. Quaram et al. (31) also found that dietary calcium loading attenuates the severity of gentamicin nephrotoxicity in rats. In addition, it has been shown that the hypercalciuria induced by low doses of gentamicin occurs without accompanying nephrotoxicity (7, 8). Moreover, injection of a single, standard clinical dose of gentamicin was shown to produce an immediate and transient increase in urinary excretion of calcium in healthy human subjects (6). Taken together, these findings indicate that megalin is involved in various interactions observed between aminoglycosides and calcium, such as calciuresis induced by aminoglycosides, in the kidney. In addition, inhibition of the renal uptake of DBP by gentamicin may also affect calcium homeosta-

Fig. 7. Accumulation of AMK in renal cortex (A) and AMK excreted in urine (B) in normal and maleate-treated rats. At 45 min after the intraperitoneal injection of maleate (400 mg/kg) or saline (normal), AMK (3.5 mg/kg) was injected intravenously. Urine was collected for 120 min after the administration of AMK, and, at the end of urine collection, the renal cortex was isolated. AMK contents in the renal cortex and in the urine were examined by HPLC. Data are means ± SE of results from 5 rats. Significantly different from normal group, *P < 0.05.

Fig. 8. Time profile of AMK accumulation (A) and megalin level (B) in renal cortex after maleate injection. At 45 min, 2 h 45 min, 4 h 45 min, or 10 h 45 min after the intraperitoneal injection of maleate (400 mg/kg), AMK was administered intravenously. At 120 min after the administration of AMK, the renal cortex was prepared, and the AMK amount and the megalin level were measured by HPLC and Western blot analysis, respectively. Optical density was determined as described in Fig. 4. Open circle, normal; solid circle, maleate-treated. Data are means ± SE of results from 3–5 rats.
sion, because DBP is essential for the transport of 25-(OH) vitamin D$_3$ into the proximal tubular cells, where 25-(OH) vitamin D$_3$ is changed to 1a,25-(OH)$_2$ vitamin D$_3$, a steroid hormone important in the regulation of systemic calcium and bone metabolism.

In conclusion, the present study shows that megalin would be responsible for the aminoglycoside accumulation in the renal cortex under in vivo condition. In addition, it is suggested that the interaction between aminoglycosides and calcium in the kidney is partly due to the competition of these compounds for binding to megalin.

We thank Dr. Hiroki Kuniyasu (First Dept. of Pathology, Faculty of Medicine, Hiroshima University) for his assistance with the immunolocalization study. We also thank the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University, for the use of their facilities.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture in Japan and in part by the Sankyo Foundation of Life Science.

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