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Effect of gentamicin on pharmacokinetics of lysozyme in rats: interaction between megalin substrates in the kidney

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Abstract

To investigate the pharmacokinetic interaction between substrates of megalin, a 600-kDa endocytic receptor abundantly expressed in the renal proximal tubules, we examined the effect of gentamicin infusion on the pharmacokinetics of fluorescein isothiocyanate (FITC)-lysozyme in rats. Infusion of gentamicin did not affect the plasma concentration–time profile of FITC-lysozyme. On the other hand, gentamicin significantly decreased the accumulation of FITC-lysozyme in the renal cortex and medulla, whereas the accumulation in the renal papilla, liver, brain and lung was not changed. Urinary excretion of FITC-lysozyme was increased by gentamicin, whereas there was no change in the biliary excretion of FITC-lysozyme or its degradation products. Gentamicin infusion had little influence on the ATP content in the renal cortex and urinary excretion of glucose, indicating that nephrotoxicity is not induced by short-term infusion of gentamicin. These findings suggest that lysozyme and gentamicin interact with each other in their reabsorption processes in the renal proximal tubules, probably by competing for their binding to megalin expressed in the apical membrane of the renal proximal tubules.

Introduction

Megalin, a member of the low-density lipoprotein (LDL) receptor gene family, is highly expressed on the luminal surface of the renal proximal tubules, but not in the hepatocytes where other members of the LDL receptor family are abundantly expressed (Saito et al 1994; Christensen et al 1998; Willnow 1999). The ligands bound to megalin represent a variety of classes including apolipoproteins, protease/protease inhibitor complexes, vitamin binding proteins, receptor-associated protein and calcium (Christensen & Willnow 1999). Recently, megalin was shown to serve as a renal clearance receptor for low molecular weight proteins filtered through the glomerulus (Leheste et al 1999). In addition, Moestrup et al (1995) reported that aminoglycosides such as gentamicin and amikacin compete for the binding of ^{125}I-urokinase/plasminogen activator inhibitor type-1 complexes to purified rabbit megalin. Further, they showed that the urinary excretion of ^{125}I-aprotinin, a megalin substrate, microinfused into a single proximal tubule was markedly increased in the presence of gentamicin. We also observed that the urinary excretion of endogenous megalin substrates such as vitamin D binding protein and calcium was increased by the intravenous administration of gentamicin to normal rats, suggesting the inhibitory effect of gentamicin on the reabsorption of endogenous megalin substrates in the renal proximal tubular cells (Nagai et al 2001).

Lysozyme (approx. 14 kDa; pI = 11), an endogenous low molecular weight protein, is easily filtered through the glomerulus. The lysozyme concentration in the glomerular filtrate is about 80% of that in the plasma (Maack 1975). The filtered lysozyme is taken up by the epithelial cells of the renal proximal tubules by adsorptive endocytosis (Cojocel et al 1981; Sumpio & Maack 1982; Schöttke et al 1984). The accumulation of lysozyme in the kidney can be entirely accounted for by the subsequent uptake from the luminal side because uptake of lysozyme from the peritubular side is negligible (Maack

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1975; Hysing & Tolleshaug 1986). However, the binding site of lysozyme in the renal proximal tubules was not identified for a long time. Recently, Orlando et al (1998) showed that megalin directly binds lysozyme by using reverse ligand procedures. Leheste et al (1999) observed that urinary excretion of low molecular weight plasma proteins, including lysozyme, is markedly increased in megalin-deficient mice. Thus, megalin appears to have a crucial role in the reabsorption of lysozyme as well as other proteins and drugs from the glomerular filtrate (Nykjaer et al 1999; Schmitz et al 2002).

The present study was designed to investigate the pharmacokinetic interaction between megalin substrates, lysozyme and gentamicin, under in-vivo conditions.

Materials and Methods

Materials

Fluorescein isothiocyanate (FITC) was obtained from Sigma Chemical Co. (St Louis, MO, USA). Lysozyme chloride from egg white and gentamicin sulfate were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used for the experiments were of the highest purity available.

Preparation of FITC-lysozyme

Labelling of lysozyme with FITC was performed according to the method described by Kok et al (1998). Briefly, 2 mg FITC and 200 mg lysozyme were dissolved in 0.1 M borate buffer (pH 9.0). After incubation for 60 min at room temperature, the pH of the mixture was adjusted to 7.5 with 0.1 M boric acid. The solution was then dialysed by cellulose membrane for 48 h at 4°C and concentrated by freeze-drying. When the lyophilized proteins were subjected to SDS-polyacrylamide gel electrophoresis, a single band was observed by staining with Coomassie brilliant blue, and its band size was the same as that with authentic lysozyme, indicating that there was little degradation of lysozyme during the FITC-labelling (data not shown).

Pharmacokinetic studies

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, Graduate School of Biomedical Sciences, Hiroshima University. Male Wistar rats (230–290 g) were anaesthetized by intraperitoneal injection of pentobarbital (30 mg kg^{-1}) and the femoral artery and vein were cannulated with polyethylene tubing for blood sampling and drug administration, respectively. The urinary bladder and bile duct were also cannulated for collection of urine and bile, respectively. The rats were placed on a heating pad to maintain body temperature at 37°C. FITC-lysozyme (4 mg kg^{-1}, 200 μL) was injected into a femoral vein. Co-administration of gentamicin was performed by constant-rate intravenous infusion of gentamicin (30 mg h^{-1} kg^{-1}) at a rate of 2 mL h^{-1}, after the loading dose (11.5 mg kg^{-1}, 200 μL) was introduced by intravenous bolus injection 30 min before FITC-lysozyme administration. The dosage required to give a 100 μM plateau plasma gentamicin concentration was determined based on pharmacokinetic parameters calculated in a preliminary study by the bolus injection of [^{3}H]gentamicin (data not shown). Rats injected with the same volume of saline without gentamicin were used as controls. Blood samples were withdrawn through the femoral artery 1, 5, 10, 20, 30, 45, 60 and 120 min after the administration of FITC-lysozyme. Plasma was separated immediately by centrifugation. Urine and bile samples were collected periodically every 30 min for up to 120 min. At 120 min after the injection of FITC-lysozyme, each tissue was excised. The tissues were weighed and homogenized with 4 vols of phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, pH 7.4). After centrifugation at 3000 rev min^{-1} for 15 min, fluorescence in the supernatant was measured.

Analytical methods

Fluorescence in plasma samples was analysed after an appropriate dilution with PBS, as a measure for intact FITC-lysozyme concentration in plasma because little degradation product was found in the plasma (Haas et al 1993). The urine and bile samples were analysed for total fluorescence. Non-protein-bound fluorescence in these samples was also analysed by the method with trichloroacetic acid (TCA) precipitation. In brief, the sample (100 μL) of urine or bile was added to 100 μL 10% TCA. After centrifugation at 3000 rev min^{-1} for 10 min, the supernatant (100 μL) was neutralized with 30.7 μL 1 M NaOH, and diluted with 869.3 μL PBS. The fluorescence in the solution was assumed to be non-protein-bound fluorescein (degradation product of FITC-lysozyme), and the protein-bound fluorescein (intact FITC-lysozyme) was calculated by subtracting the non-precipitable fluorescein concentration from the total fluorescein concentration. Fluorescein was measured by using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. The ATP content in the renal cortex was measured as described previously (Kunihara et al 1998). Glucose in urine was measured with commercial kits available from Wako Pure Chemicals (Osaka, Japan).

Data analysis

The plasma concentration–time curve of FITC-lysozyme in each rat was fitted to the equation $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ for the plasma concentration C_p at time t, with the aid of KaleidaGraph software (Synergy Software, PA, USA). The total clearance (CL_{tot}) was calculated from the equation CL_{tot} = dose $(A/\alpha + B/\beta)^{-1}$. The apparent volume of the central compartment (V_c) was calculated as V_c = dose

$(A+B)^{-1}$. Statistical analysis was performed using the Student's *t*-test. A difference of $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows the plasma concentration–time profile of FITC-lysozyme after an intravenous bolus injection to rats with or without gentamicin infusion. The plasma concentration of FITC-lysozyme declined biexponentially with time, and profiles were similar between groups with and without gentamicin infusion. The estimated pharmacokinetic parameters of FITC-lysozyme are given in Table 1. Gentamicin infusion did not affect the pharmacokinetic parameters of FITC-lysozyme (Table 1).

The tissue distribution of FITC-lysozyme 120 min after injection was also measured. As shown in Figure 2, accumulation of total fluorescein after intravenous administration of FITC-lysozyme was highest in the renal cortex among the various tissues examined, and moderate in the renal medulla. Gentamicin infusion significantly decreased the accumulation of FITC-lysozyme in the renal cortex and medulla, but not in the renal papilla, liver, brain and lung.

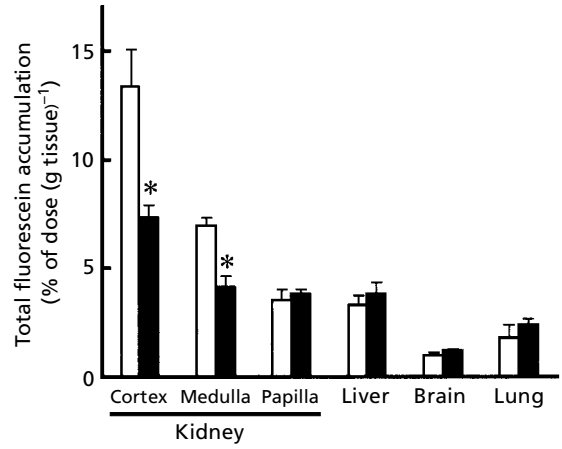

Figure 2 Tissue distribution of total fluorescein 120 min after the intravenous administration of fluorescein isothiocyanate lysozyme to saline- (open column) and gentamicin- (closed column) infused rats. $*P < 0.05$, significantly different compared with the value for the saline-infused group. Values are expressed as mean±s.e. of results from five rats.

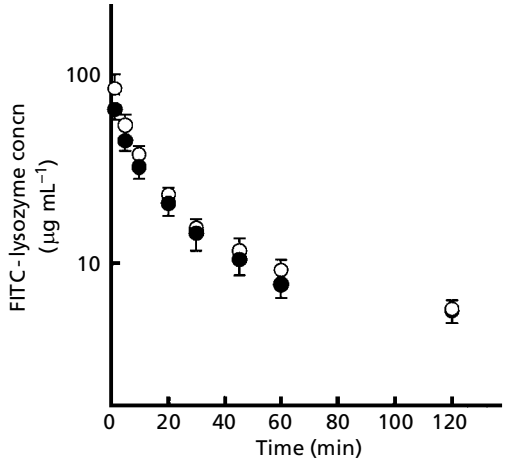

Figure 1 Plasma concentration–time profile of fluorescein isothiocyanate (FITC)-lysozyme administered intravenously to saline- (○) and gentamicin- (●) infused rats. Values are expressed as mean±s.e. of results from five rats.

Table 1 Pharmacokinetic parameters of fluorescein isothiocyanate-labelled lysozyme administered intravenously (4 mg kg^{-1}) to saline- or gentamicin-infused rats.

Parameter	Saline-infused	Gentamicin-infused
A (μg mL^{-1})	84.4±13.3	68.2±7.9
B (μg mL^{-1})	27.6±4.1	22.7±7.7
α (min^{-1})	0.152±0.018	0.157±0.043
β (min^{-1})	0.017±0.003	0.014±0.003
CL_{tot} (mL min^{-1} kg^{-1})	1.86±0.27	2.06±0.21
V_c (mL kg^{-1})	38.6±5.3	49.7±9.5

Gentamicin was administered intravenously as a bolus injection (11.5 mg kg^{-1}) followed by constant-rate infusion (30 mg h^{-1} kg^{-1}). Values are expressed as mean±s.e. of results from five rats.

We then examined the effect of gentamicin infusion on urinary excretion of lysozyme (Figure 3). Urinary excretion of total fluorescein was increased by gentamicin infusion (Figure 3A). Because almost all of the total fluorescein was the protein-bound form (Figure 3A and B), FITC-lysozyme would be excreted into the urine without being degraded. The gentamicin-induced increase in the total fluorescein in the urine (10.6% of dose, n = 5) was comparable with the decrease in the total fluorescein in the renal cortex and medulla (9.3% of dose, n = 5). In contrast to the urine, protein-bound fluorescein and its degradation products were detected in the bile. Neither total nor protein-bound fluorescein in bile was changed by gentamicin infusion (Figure 4A and B).

We also examined whether the short-term infusion of gentamicin used in this study induces nephrotoxicity. The result showed that the infusion of gentamicin had no influence on urinary excretion of glucose, which is almost completely reabsorbed by the sodium-coupled glucose transporter in the renal proximal tubular cells. In addition, the renal cortical ATP content, which is essential to the receptor-mediated endocytosis in the renal proximal tubules, was not affected by the infusion of gentamicin (Table 2).

Discussion

We previously found that the intravenous bolus administration of gentamicin increased the excretion of endogenous megalin substrates such as vitamin D-binding protein and

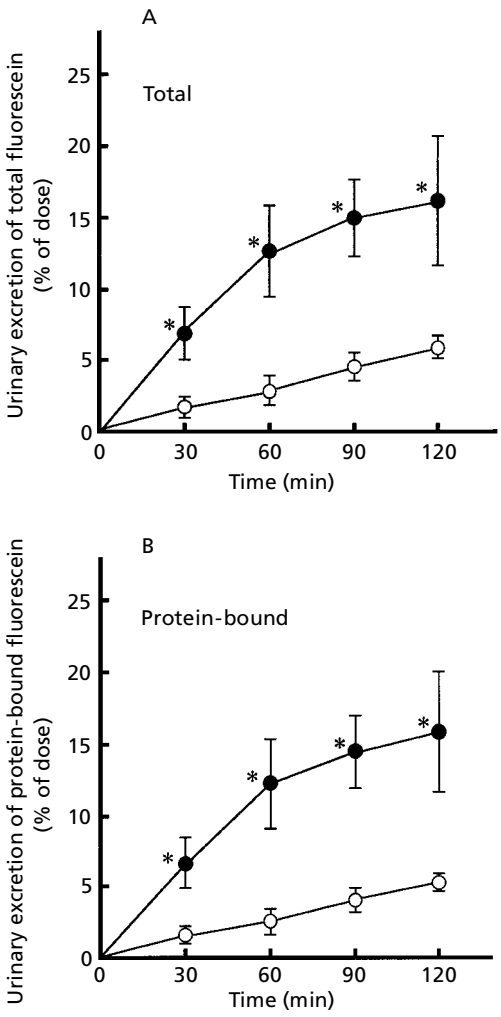

Figure 3 Cumulative urinary excretion of total fluorescein (A) and protein-bound fluorescein (B) in saline- (○) and gentamicin- (●) infused rats. Values are expressed as mean±s.e. of results from four or five rats. *$P < 0.05$, significantly different compared with the value for the saline-infused group.

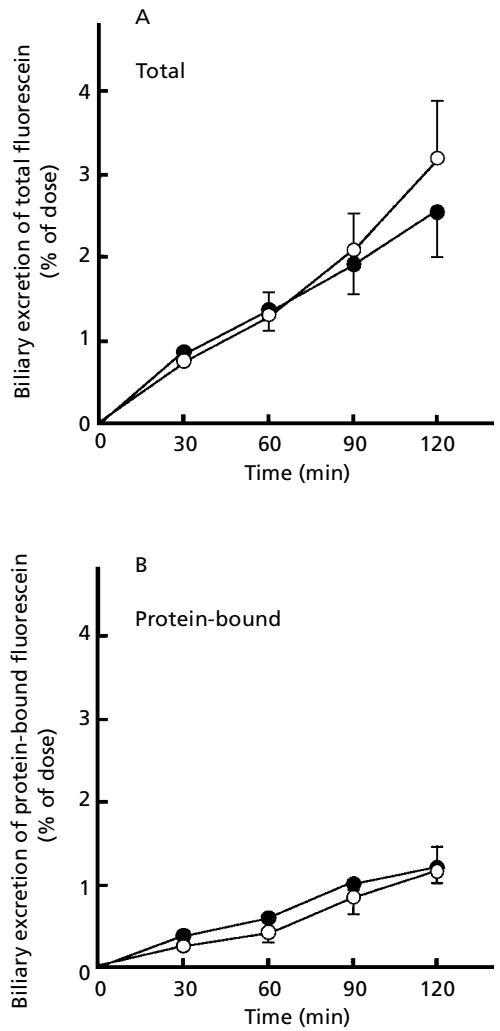

Figure 4 Cumulative biliary excretion of total fluorescein (A) and protein-bound fluorescein (B) in saline- (○) and gentamicin- (●) infused rats. Values are expressed as mean±s.e. of results from three or four rats.

calcium (Nagai et al 2001). These observations indicated that gentamicin and endogenous megalin substrates compete for the renal accumulation by megalin-mediated endocytosis. In order to further examine the interaction between megalin substrates, the effect of gentamicin on the pharmacokinetics of FITC-labelled lysozyme was examined in the present study. Lysozyme (approx. 14 kDa) is readily filtered through the glomerulus and efficiently reabsorbed by the epithelial cells of the proximal tubules. However, little information was available on the endocytic receptor involved in the renal accumulation of lysozyme.

Recently, megalin was shown to directly bind lysozyme by using reverse ligand blotting procedures (Orlando et al 1998). In addition, it was reported that lysozyme interacts with megalin with a Kd value of 0.32 μM, and the binding of lysozyme is inhibited by receptor-associated protein, a substrate of megalin (Leheste et al 1999). Furthermore, sequence analysis of urinary protein from megalin knockout mice identified various low molecular weight proteins, including lysozyme (Leheste et al 1999). In addition, the

Table 2 Urinary excretion of glucose and ATP content in renal cortex in saline- or gentamicin-infused rats.

	Saline-infused	Gentamicin-infused
Urinary glucose (mg/120 min)	0.27±0.07	0.20±0.02
ATP content in renal cortex (μmol (g tissue)$^{-1}$)	1.62±0.10	1.46±0.11

Gentamicin was administered intravenously as a bolus injection (11.5 mg kg^{-1}) followed by constant-rate infusion (30 mg h^{-1} kg^{-1}). Values are expressed as mean±s.e. of results from three rats.

tissue distribution of FITC-lysozyme observed in the present study was well correlated with the megalin level in each tissue, as we observed previously (Nagai et al 2001). Thus, it is likely that megalin plays a crucial role in the renal distribution of endogenous as well as administered lysozyme.

Similar to lysozyme, aminoglycosides such as gentamicin and amikacin accumulate highly and selectively in the renal proximal tubular cells. To date, it has been suggested that aminoglycosides are taken up by an adsorptive endocytosis in the renal proximal tubules (Sastrasinh et al 1982; Takano et al 1994; Molitoris 1997). Recently, several studies have shown that the concentrated accumulation of aminoglycosides in the kidney is owing to megalin-mediated endocytosis (Moestrup et al 1995; Nagai et al 2001). Also, Schmitz et al (2002) showed that renal accumulation of gentamicin was almost negligible in megalin-deficient mice (about 5% of that in wild-type mice), indicating that megalin-mediated endocytosis is the major pathway responsible for renal aminoglycoside accumulation. Therefore, in the present study, we examined whether gentamicin inhibits the reabsorption of lysozyme in the kidney. Co-infusion of gentamicin had no effect on the plasma concentration–time profile of FITC-lysozyme administered intravenously, while FITC-lysozyme accumulation in the renal cortex and medulla was decreased. The accumulation in the other tissues examined was not changed. These results show that gentamicin inhibits the uptake of FITC-lysozyme into the tissues in which megalin is abundantly expressed. In addition, gentamicin increased the urinary excretion of FITC-lysozyme in association with the decrease in the accumulation in the renal cortex and medulla. The infusion of gentamicin had little influence on the urinary excretion of glucose and ATP content in the renal cortex, indicating that the gentamicin-induced effects observed in this study were not owing to the nephrotoxicity of the drug. Taken together, the infusion of gentamicin would competitively inhibit the uptake of lysozyme by megalin-mediated endocytosis in the apical membrane of the renal proximal tubules. However, it is not clear at present whether megalin-mediated endocytosis is the only pathway for renal accumulation of lysozyme, and whether gentamicin interacts with other possible pathways for lysozyme accumulation, because gentamicin is known to interact with various cellular components such as negatively charged phospholipids. Also, because this was the whole-animal study, further studies are needed to show more directly the effect of gentamicin on megalin-mediated endocytosis of lysozyme.

Non-protein-bound fluorescein was observed in the bile of rats administered FITC-lysozyme, indicating that some FITC-lysozyme taken up by the liver was degraded and its degradation products were excreted into the bile. Protein-bound fluorescein was also detected in the bile. This finding indicates that part of the lysozyme taken up by the liver is transcytosed across the hepatocytes into the bile. However, the accumulation in the liver as well as the excretion into the bile was not affected by the co-infusion of gentamicin. Low density lipoprotein-related protein-1 (LRP-1), which is also a member of the LDL receptor family, is expressed in several cells facing the circulation, including hepatocytes (Zheng et al 1994). In the present study, the expected plasma concentration of gentamicin was approximately 100 μM. Moestrup et al (1995) showed that 100 μM gentamicin sufficiently inhibits the binding of ^{125}I-urokinase/plasminogen activator inhibitor type-1 to LRP-1. Therefore, it is unlikely that the uptake of lysozyme in the liver is owing to LRP-1. Further studies are needed to clarify the receptor(s) responsible for the endocytosis and the transcytosis of lysozyme in the liver.

In conclusion, we have shown that megalin substrates such as lysozyme and gentamicin compete for megalin-mediated endocytosis from the apical side of the renal proximal tubular cells. Thus, the renal accumulation of a megalin substrate can be reduced by the co-administration of another megalin substrate that is filtered freely through the glomerulus. These findings could be useful to, for example, establish a new drug delivery system with which the renal accumulation and following nephrotoxicity of aminoglycosides can be reduced.

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