Studies on the Nephrotoxicity of Aminoglycoside Antibiotics and Protection from These Effects (7): Effect of Latamoxef on Binding of Tobramycin to Brush Border Membranes Isolated from Rat Kidney Cortex

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Abstract—We investigated the effect of latamoxef (LMOX) on the binding of tobramycin (TOB) to brush border membranes (BBMs) isolated from rat kidney cortex by calcium precipitation. The simultaneous treatment with TOB (0.2 mM) and LMOX (10 and 20 mM) to the BBMs fraction (about 250 µg protein) significantly inhibited the binding of TOB to BBMs. The addition of the reaction mixture of TOB (0.2 mM) and LMOX (4, 10 and 20 mM) which was preincubated for 3 hr at 37°C, to the BBMs fraction resulted in less binding of TOB to the membranes than that observed in the case of simultaneous treatment with both drugs. Although [14C]-labeled LMOX was taken up by BBMs temperature- and time-dependently, the pretreatment with LMOX showed no obvious differences in inhibition of the TOB binding to BBMs, as compared with the result from simultaneous treatment with both drugs. Additionally, the binding of TOB to the LMOX-treated BBMs that were resuspended in fresh medium after the pretreatment with LMOX for 10 min at 37°C was similar to that of TOB to the non-treated BBMs. These results indicate that LMOX inhibits the binding of TOB to BBMs not by binding to BBMs but by interacting with TOB.

We have already reported that latamoxef (LMOX), which prevents tobramycin (TOB)-induced nephrotoxicity, reduced intrarenal TOB concentration (1). It has been suggested that aminoglycoside antibiotics (AGs) are accumulated into lysosomes by pinocytosis after the binding to renal brush border membranes (BBMs) (2). Thus, in order to clarify the mechanism of the suppressive effect of LMOX on the renal concentration of TOB, we have hypothesized that LMOX may reduce the intrarenal TOB concentration through less binding of TOB to renal BBMs by interaction with TOB. We previously demonstrated the direct interaction of TOB with LMOX in vitro (3). Therefore, in the present study we investigated whether LMOX inhibits the binding of TOB to BBMs isolated from rat kidney cortex to further demonstrate our hypothesis.

Materials and Methods

Animals: Male Sprague-Dawley strain rats weighing about 230 g (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used in all experiments. These animals were housed in an air-conditioned room at 24±1°C.

Preparation of BBMs: BBMs were isolated from rat kidney cortex by the calcium precipitation methods described by Evers et al. (4) and Inui et al. (5). Briefly, the renal cortices of the kidneys from 10 rats were dissected, minced, suspended in 2 mM HEPES/Tris buffer (pH 7.1) containing 10 mM mannitol, and homogenized with a Waring blender (Nissei, Japan) at 18,000 rpm for 2 min. CaCl₂ was added to the homogenate to achieve a final concentration of 10 mM, followed by continuous stirring for 15 min. The suspension was centrifuged at 500×g for 12 min, and the
supernatant was recentrifuged at 15,000×g for 12 min. The resultant pellet was resuspended in 2 mM HEPES/Tris buffer (pH 7.1) containing 10 mM mannitol. CaCl₂ (final concentration: 10 mM) was added to the suspension, followed by centrifugation at 750, 3,000 and 48,000×g for 12, 12 and 20 min, respectively. The final pellet was suspended in 20 mM HEPES/Tris buffer (pH 7.4) containing 100 mM mannitol.

Measurements of enzyme activities: Activities of γ-glutamyl transpeptidase (γ-GTP) and alkaline phosphatase (ALP), marker enzymes of BBMs, were measured by the methods of Tamaoki et al. (6) and Bessey et al. (7), respectively. The activity of Na⁺-K⁺ ATPase for basolateral membranes was assayed by slightly modifying the methods described by Jacobson et al. (8) and Lo et al. (9). Activities of N-acetyl-β-D-glucosaminidase, one of the lysosomal enzymes, and succinate dehydrogenase, a mitochondrial marker enzyme were measured by the methods of Hasebe (10) and Green et al. (11), respectively. Lactate dehydrogenase (LDH) activity was determined using a LDH Linia Neo 3A kit (Shinotest, Japan) to assess the degree of contamination of cytosolic fractions. The membrane proteins were measured by the BIO-RAD Protein Assay with bovine serum albumin as the standard.

Electron microscopy of isolated BBMs: The ultrathin sections of the isolated BBMs were prepared by a slight modification of the methods described by Evers et al. (4) and Josepovitz et al. (12). In brief, aliquots of BBMs were fixed in suspension with 0.5% glutaraldehyde buffered with 0.2 M cacodylate (pH 7.4). After centrifugation, the resultant pellet was treated with 2% OsO₄, dehydrated in graded alcohols and embedded in epoxy plastic (Epon 812). The ultrathin sections were stained with 4% uranyl acetate and observed by electron microscopy (LEM-2000, Akashi, Japan). For negative staining, aliquots of BBMs were stained with 2.5% ammonium molybdate on a grid with collodion membrane by the method of Brenner and Horne (13) and observed by the LEM-2000.

Binding assay of TOB with BBMs: The binding of TOB to BBMs was assayed by slightly modifying the method of Ishikawa et al. (14). In a general assay, 200 µl of membrane suspension (200–250 µg protein) was incubated at 37°C for 10 min with 200 µl of 0.2 mM TOB in 20 mM HEPES/Tris buffer (pH 7.4) containing 100 mM mannitol, followed by centrifugation at 45,000×g for 30 min at 4°C. After removal of the supernatant, 200 µl of H₂O and 200 µl 5% trichloroacetic acid were added, centrifuged at 1,600×g for 20 min, and the resultant supernatant was used for measuring TOB concentration. The TOB concentration was determined by a substrate-labeled fluorescent immunoassay (Ames TDA tobramycin assay kit, Miles-Sankyo Co., Ltd., Tokyo, Japan) (15). According to the methods of the binding assay described above, we designed the following experiments: 1) the effect of simultaneous treatment and pretreatment with LMOX on TOB binding to BBMs, 2) the effect of the reaction mixture of TOB and LMOX, which was preincubated for 3 hr at 37°C to make the complex of both drugs (3), on TOB binding to BBMs, and 3) the binding of TOB to the LMOX-treated and non-treated BBMs. TOB (final concentration: 0.2 mM) and LMOX (final concentration: 2, 4, 10 or 20 mM) were dissolved in 20 mM HEPES/tris buffer (pH 7.4) containing 100 mM mannitol. Before examining the effect of LMOX on TOB binding to BBMs, we investigated the effect of the osmolarity of the medium for suspending BBMs on TOB binding to BBMs and the binding of TOB to BBMs was analyzed by the Scatchard plot method.

Uptake of LMOX by BBMs: The uptake of LMOX by BBMs was determined by modifying the method of Inui et al. (16). Twenty microliters of the membranes (about 200 µg protein) suspended in 20 mM HEPES/Tris buffer (pH 7.4) containing 100 mM mannitol was incubated at 4 or 37°C for 10, 30 or 60 min with the substrate medium (20 µl) including 50 µM of [¹⁴C]-labeled LMOX (36 µCi/mM). The reaction was stopped by adding 1 ml of ice-cold 20 mM HEPES/Tris buffer (pH 7.4). [¹⁴C]-LMOX bound to BBMs was separated from free [¹⁴C]-LMOX by rapid filtration using a 0.45 µm nitrocellulose Millipore filter, and the incubation tube was washed three times with 2 ml of ice-cold 20 mM HEPES/Tris buffer (pH 7.4). The radioactivity on the filter was counted in 10 ml of
Sintisol EX-H (Dojin, Japan) using a Packard scintillation counter. Additionally, the non-specific binding of \([^{14}C]\)-LMOX to the Millipore filter was determined under the condition in which BBMs were not contained in the reaction mixture.

**Statistical analysis:** The data in the figures and tables are expressed as the mean±S.E. or S.D. Statistical significance of differences was evaluated by the Student’s t-test and the Mann-Whitney U-test. Differences were considered significant if the P value was <0.05.

**Results**

1. **Purity of the isolated BBMs:** Table 1 summarizes the specific activities of the various marker enzymes in the crude homogenate and BBMs fractions to elucidate the purity of the isolated BBMs. The relative specific activities of \(\gamma\)-GTP, ALP and Na\(^{+}\)-K\(^{+}\)ATPase in the isolated BBMs fraction were 14.6, 11.5 and 0.60, respectively. Only slight contamination with other fractions such as mitochondria and cytosol was found, as assessed by the respective marker enzymes. This indicates that the BBMs were highly purified by the method of Ca precipitation.

2. **Electron micrographs of the isolated BBMs:** As shown in Fig. 1, the isolated BBMs showed spherical vesicles of different size, small elongated structures, and a right-side-out orientation of the membrane vesicles, as reported by Evers et al. (4). Additionally, the BBMs fraction included a very small amount of membrane fragments.

3. **Binding assay of TOB with BBMs:** In a preliminary study, inasmuch as the TOB binding to BBMs showed a steady state after incubation for 10 min at 37°C, all subsequent binding assays were carried out at 37°C for 10 min.

   The amounts of TOB bound to BBMs (about 50 nmol/mg protein) was found to be unaffected by alterations in the buffer osmolarity (0–800 mM of mannitol in 20 mM HEPES/Tris, pH 7.4) (data not shown). The Scatchard plot of the binding of TOB to BBMs showed that the dissociation constant between TOB and the binding site on BBMs was 34.52 \(\mu\)M and that the maximal binding capacity was 82.54 nmol/mg of protein. The linearity of the Scatchard plot indicated that a single class of binding sites for TOB existed on BBMs (Fig. 2).

   Figure 3 illustrates the effect of simultaneous treatment with LMOX on TOB binding to BBMs. The addition of LMOX (2 and 4 mM) to the BBMs fraction showed no significant inhibition of the TOB binding to BBMs, whereas 10 and 20 mM of LMOX significantly inhibited the TOB binding by 16 and 23%, respectively.

   Figure 4 summarizes the effect of the reaction mixture of TOB and LMOX on TOB binding to BBMs. Each reaction mixture of TOB (0.2 mM) and LMOX (4, 10 and 20 mM)

| Marker enzyme                      | Specific activity                      | Relative specific activity |
|------------------------------------|----------------------------------------|----------------------------|
|                                    | Homogenate                             | BBM fraction              |                             |
| \(\gamma\)-Glutamyl transpeptidase | \(\mu\)mol/hr/mg protein              | 3.57±0.16                 | 53.62±3.43                 | 14.6                        |
| Alkaline phosphatase               | \(\mu\)mol/hr/mg protein              | 0.04±0.002                | 0.46±0.03                  | 11.5                        |
| Na\(^{+}\)-K\(^{+}\)ATPase         | \(\mu\)mol/hr/mg protein              | 0.42±0.07                 | 0.25±0.04                  | 0.60                        |
| N-Acetyl-\(\beta\)-D-glucosaminidase| \(\mu\)mol/hr/mg protein              | 11.59±0.37                | 2.21±0.12                  | 0.19                        |
| Succinate dehydrogenase            | \(\mu\)mol/hr/mg protein              | 1.26±0.02                 | 0.04±0.02                  | 0.03                        |
| Lactate dehydrogenase              | U/hr/mg protein                        | 250±30                    | 40±5                       | 0.02                        |

The values are expressed as the mean±S.E. of five separate experiments.
suppressed the TOB binding to BBMs by 25, 52 and 74%, respectively, as compared with the results from simultaneous treatment with both drugs.

Figure 5 summarizes the effect of pretreatment with LMOX on TOB binding to BBMs. No significant differences in the TOB binding to BBMs were observed between pretreat-
Fig. 3. Effect of LMOX on TOB binding to BBMs. LMOX (2, 4, 10 and 20 mM) and TOB (0.2 mM) were added simultaneously to the BBMs fraction and incubated for 10 min at 37°C. To correct for the effect of sodium, in the addition of LMOX, on the TOB binding to BBMs, TOB binding in the treatment with TOB alone was monitored in the presence of NaCl solution containing the same sodium content as that in each concentration of LMOX. The values are expressed as the mean±S.D. *P<0.05, **P<0.01, compared with the values by TOB in the presence of NaCl (*: Mann-Whitney U-test *: Student’s t-test).

Fig. 4. Effect of the reaction mixture of TOB and LMOX on TOB binding to BBMs. TOB and LMOX were mixed and preincubated for 3 hr at 37°C to form the complex of both drugs. The preincubated reaction mixture was added to the BBMs fraction and incubated for 10 min at 37°C. The values are expressed as the mean±S.D. *P<0.05, **P<0.01, compared with the values by TOB and LMOX (simultaneous treatment). Significant differences were evaluated by Student’s t-test.
Fig. 5. Effect of pretreatment with LMOX on TOB binding to BBMs. Pretreatment with LMOX: BBMs were preincubated with LMOX for 10 min at 37°C, followed by the incubation with TOB for 10 min at 37°C. Simultaneous treatment with LMOX: LMOX and TOB were added simultaneously to the BBMs fraction and incubated for 10 min at 37°C. The values are expressed as the mean±S.D.

Fig. 6. Binding of TOB to the LMOX-treated and non-treated BBMs. BBMs were preincubated for 10 min at 37°C in the medium (100 mM mannitol, 20 mM HEPES/Tris, pH 7.4) with or without LMOX, centrifuged at 45,000 x g for 30 min, and then the membrane pellets were resuspended in the fresh medium (100 mM mannitol, 20 mM HEPES/Tris, pH 7.4). The resuspended BBMs were incubated for 10 min at 37°C with 0.2 mM TOB. The values are expressed as the mean±S.D.
Inhibitory Effect of LMOX on TOB Binding to BBMs

As shown in Fig. 6, the binding of TOB to the LMOX-treated BBMs was almost similar to that of TOB to the non-treated BBMs.

4. Uptake of LMOX by BBMs: As shown in Table 2, the incubation of [14C]-LMOX with BBMs at 4°C showed a plateau of the uptake of LMOX by BBMs 30 min after incubation. When [14C]-LMOX was incubated with BBMs at 37°C, the amounts of [14C]-LMOX taken up by the membranes were greater than that observed in incubation at 4°C, and no steady state of the uptake of LMOX was observed until 60 min of incubation.

Discussion

The aim of the present study was to clarify whether LMOX inhibits the binding of TOB to BBMs isolated from the rat kidney cortex. Before examining the effect of LMOX on TOB binding to BBMs, we investigated the influence of osmolarity of the medium suspending BBMs on TOB binding to BBMs and the binding kinetics of TOB to the membranes. The alteration of osmolarity of the medium, which led to changes in intravesicular space of BBMs, showed no effect on the binding of TOB to BBMs. This result indicates that TOB binds to BBMs, but are not transported into the vesicular space of BBMs. This finding was in agreement with the results reported by Williams et al. (17). Additionally, the Scatchard plot analysis showed a single class of binding sites for TOB on BBMs (Fig. 2) as demonstrated by Sastrasinh et al. (18).

In the binding assay of TOB with BBMs, we found that LMOX added simultaneously with TOB to the BBMs fraction inhibited the binding of TOB to BBMs (Fig. 3). As the molecular interaction of TOB and LMOX is associated with the suppression of intrarenal TOB concentration (R. Kojima et al., unpublished data), we have postulated that interaction between both drugs may be involved in the inhibition by LMOX of TOB binding to BBMs. Therefore, we determined the effect on TOB binding to BBMs of the reaction mixture of TOB and LMOX which was preincubated for 3 hr at 37°C to form the complex of both drugs. As shown in Fig. 4, the treatment with the reaction mixture showed greater suppression of the TOB binding to BBMs than that observed in the simultaneous addition of TOB and LMOX. This result suggested that the molecular interaction of TOB and LMOX contributed to the inhibition of the binding of TOB to BBMs.

However, the possibility could not be excluded that the binding of LMOX to BBMs and the transport of LMOX into the vesicular space of BBMs might inhibit the binding of TOB to the membranes, because some cephalosporin antibiotics were taken up by rat renal BBMs (19, 20). Thus, we examined whether LMOX is taken up by BBMs using the rapid filtration technique. The assay of uptake of [14C]-labeled LMOX by BBMs showed that LMOX was taken up by BBMs temperature- and time-dependently (Table 2). In this uptake assay, although it is not clear whether the uptake of LMOX by BBMs represents binding to the membranes or transport into the vesicular space of the membranes, there are two possibilities regarding the mechanism of the suppressive action of LMOX on TOB binding to BBMs: 1) LMOX bound to sites close to the binding site of TOB on BBMs might interfere with the binding of TOB by covering the binding site of TOB with LMOX molecules and 2) LMOX transported into the vesicular space of BBMs might inhibit the binding of TOB to the membranes from inside of the vesicles.

### Table 2. Uptake of [14C]-labeled LMOX by BBMs

| Incubation time (min) | 10 | 30 | 60 |
|-----------------------|----|----|----|
| **4°C**               | 13.60±7.87 | 38.86±9.27 | 42.22±11.02 |
| **37°C**              | 38.62±11.42 | 67.51±8.04 | 82.41±20.76 |

The values are expressed as the mean±S.E. of three separate experiments.
However, pretreatment with LMOX, which allowed LMOX to be taken up by BBMs, showed no significant reductions in TOB binding to BBMs compared to simultaneous treatment with both drugs (Fig. 5). Additionally, no obvious differences in TOB binding to BBMs were observed between LMOX-treated and non-treated BBMs (Fig. 6). Thus, these results indicate that the uptake of LMOX by BBMs is not involved in inhibiting the binding of TOB to BBMs. The possibility would not be likely that LMOX might suppress TOB binding to BBMs by competitively inhibiting the binding of TOB to binding sites on BBMs, because the binding site of AGs has been shown to be composed of acidic phospholipids including phosphatidylinosities (18, 21), and LMOX can not gain access to and bind to the anionic site of phospholipids because of the repulsive force arising from the anionic charges of two carboxyl groups in the LMOX molecule. Thus, it is concluded from the present study that LMOX inhibits the binding of TOB to BBMs by interacting with TOB.

We speculate that the inhibitory action of LMOX on TOB binding to BBMs may be due to the decrease in the positive charge and the conformational change of TOB by the interaction of both drugs, which would reduce the electrostatic (22), hydrophobic (23), and hydrogen (24) bondings between TOB and acidic phospholipids of BBMs.

The present study provides strong support for our hypothesis that the suppressive effect of LMOX on intrarenal TOB level (1) may result from less binding of TOB to BBMs due to the interaction of TOB with LMOX.

Recently, Williams et al. (17) reported that polyaspartic acid (PAA) inhibited the binding of gentamicin (GM) to BBMs vesicles in vitro and protected rat kidneys from the GM-induced nephrotoxicity in vivo. Of particular interest is the fact that treatment with PAA was ineffective in reducing the intrarenal GM concentration. The authors suggested that the binding of AGs to BBMs, but not accumulation of AGs in renal tubular cells, played an important role in the pathogenesis of AGs-induced nephrotoxicity. Moreover, Williams et al. (25) have suggested that the degree of binding affinity of AGs to BBMs is closely correlated to the nephrotoxic action of AGs. In addition, Au et al. (26, 27) and Ramsammy et al. (28) have reported that GM causes alterations in the fluidity and permeability of the liposome membranes containing phosphatidylinositols-4,5-bisphosphate. They have also suggested the significance of the binding of AGs to the membranes in the changes of membrane properties induced by AGs. Thus, our observation that LMOX inhibits the binding of TOB to BBMs also may suggest one mechanism for the protective action of LMOX against TOB-induced nephrotoxicity.

In conclusion, the results of the present study indicate that LMOX inhibits the binding of TOB to BBMs by interacting with TOB, and they support our hypothesis regarding the mechanism of the suppressive action of LMOX on intrarenal TOB concentration.

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