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学位記番号	博乙第269号
学位授与の日付	平成15年9月30日
学位授与の要件	論文博士（学位規則第4条第2項）
学位授与の題目	Effects of Bacterial Toxins, Endotoxin and Shiga-like Toxin II on Drug Transport System（薬物輸送機構に及ぼすエンドトキシンと志賀様毒素Ⅱの影響）
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学位論文要旨

SUMMARY

The effects of bacterial toxins, endotoxin derived from *Klebsiella pneumoniae* and Shiga-like toxin II (SLT-II) isolated from *Escherichia coli* O157:H7, on drug transport system were investigated in rats or mice. We found that endotoxin decreases P-glycoprotein-mediated drug biliary and renal excretion by decreasing the expression of *mdr1a*, which is likely due to overproduction of plasma TNF-α levels and that SLT-II induces damage to renal tubule, but not glomerulus and impairs renal excretion of drug by decreasing GFR and causing decreased renal plasma flow, whereas it does not alter the protein levels of drug transporting P-glycoprotein and Mrp2 in the kidney. On the other hand, the present study also found that endotoxin has no effect on the brain capillary integrity and P-glycoprotein-mediated drug transport across the blood-brain barrier (BBB), whereas SLT-II impairs the BBB function and drug transport across the BBB in spite of overexpression of P-glycoprotein in the brain.

Bacterial endotoxin, which is a major component of the outer membrane of Gram-negative bacteria, is believed to play an important role in the pathologenecity of Gram-negative sepsis, shock, and the development of multiple organ failure, and is mainly responsible for the high mortality by Gram-negative bacterial infections. Endotoxin derived from various bacterial families shares a common architecture. Endotoxin administered systemically is rapidly distributed into various tissues, such as the liver, lungs, spleen and kidneys, and is mainly eliminated by the liver. Antibiotics are often used in the treatment of patients suffering Gram-negative bacterial infections and these may enhance the liberation of endotoxin from bacteria to the body. It is well known that endotoxin released from bacteria induces a variety of pathophysiological and immunological changes in the body including circulatory shock, disseminated intravascular coagulation, and damages to numerous organs such as

the central nervous system, liver, kidney, heart, and lungs. These endotoxin-mediated activities may contribute to the development of tissue injury and consequently lead to shock and death.

It is generally difficult to distinguish the biological effects mediated by endotoxin from those of other contaminations derived from intact bacteria. The animal model appears to be useful for the prediction of various changes occurring in the human body during Gram-negative bacterial infections, because purified endotoxin can be prepared and injected into the animals either intravenously or intraperitoneally avoiding the possibly active compounds of the various contaminations derived from living bacteria. The choice of animal model must be carefully considered, however, because humans are known to be much more sensitive to endotoxin than animals, and different species and strain of experimental animals have large variations in their susceptibility to the lethal toxicity induced by endotoxin. For example, mice and rats are relatively resistant, but rabbits generally exhibit a relatively higher susceptibility to endotoxin. Moreover, there are a number of factors influencing the effects of endotoxin in the body, such as the dose, routes and schedules of endotoxin injection. In general, endotoxin, which was derived from *E. coli* O55 and O111, is widely used in animal experiments since *E. coli* is the most frequent Gram-negative bacterial pathogen in patients with sepsis, although there are strain- and species-related differences in potency among Gram-negative bacteria.

Escherichia coli O157:H7 infection induces colonization of the bowel and production of powerful Shiga-like toxins (SLTs), which are thought to enter the circulation system and to cause injury to target endothelial cells in various organs, such as the renal glomeruli and the gastrointestinal tract. The SLTs can be divided into two major types: SLT type I (SLT-I) and type II (SLT-II). SLT-II is known to induce nonspecific diarrhea, hemorrhagic colitis, and severe hemolytic-uremic syndrome (HUS). In particular, HUS is the most serious complication of *E. coli* O157:H7 infection and contributes to renal dysfunction and mortality. However, which drug therapy and how it should be used in the treatment of this infection has not yet been clinically clarified. Relevant animal models for *E. coli* O157:H7 infection are needed to study the physiological and pathological states of *E. coli* O157:H7 infectious disease in humans because of the difficulties associated with conducting clinical trials with humans. A wide variety of animal species, such as rabbits, dogs, and mice, have been used as models for human *E. coli* O157:H7 infections. However, there is only one study that rats were used as an animal model of HUS and hemorrhagic colitis by intravenous injection of SLT-I derived from *E. coli* O157. We, therefore, succeeded in the development of *E. coli* O157:H7 infectious rat and mouse models by intravenous injection of SLT-II.

Drug pharmacokinetics (absorption, distribution, metabolism and excretion) is altered in certain disease states such as Gram-negative and *E. coli* O157:H7 infections, due to functional changes occurring in the body, especially in the kidney and liver. The kidney is the most important organ for excretion of drugs and their metabolites from the body. Excretion of drugs and their metabolites into the urine involves three processes including glomerular filtration, active tubular secretion, and passive tubular reabsorption. As well as kidneys, the liver, which consists of hepatic paranchymal cells, vascular endothelial cells and Kupffer cells, also plays an important role in the specific elimination and detoxification of endogenous and exogenous substances. Some organic anionic and cationic drugs are eliminated from blood to bile by three processes including uptake into the

sinusoidal membrane, intracellular transport, and excretion into the bile canalicular membrane. Furthermore, the brain possesses unique and efficient protective systems controlling the passage of drugs and chemicals from the blood into the cerebral tissue, due to the presence of tight junctions between these endothelial cells.

The drug transport systems at the liver, kidney and blood-brain barrier (BBB) are important in regulating the concentration of drugs in their tissues. Among numerous drug transporters, the most important drug transporting protein, P-glycoprotein, which is a 170 kDa transmembrane glycoprotein that utilizes energy from ATP hydrolysis, is expressed in various tissues such as liver, kidneys, intestine and brain, and this protein in these tissues has a protective function of excluding endogenous and exogenous substances from the body. However, the effects of endotoxin and SLT-II on drug transporters have not yet been clarified, since it is thought that some disease states could exert a large influence on the expression and function of drug transporters. Then, we focused on the effects of endotoxin and SLT-II on P-glycoprotein-mediated drug transport system.

In a series of our studies on the effect of endotoxin, which is derived from *K. pneumoniae* Kasuya strain, and SLT-II on drug disposition, we have found that endotoxin reduces biliary and renal excretion of various organic anionic drugs as a result of changes in the biliary and renal tubular secretory systems [*Drug Metab. Dispos.*, **21**:611 (1993) and **22**:8 (1994), *Antimicro. Agents Chemother.*, **37**:1781 (1993) and **39**:2258 (1995), and *J. Pharm. Pharmacol.*, **48**:744 (1996)] and that it reduces hepatic cytochrome P450-dependent drug-metabolizing enzymes by overproducing of nitric oxide (NO) in plasma, although the precise mechanisms responsible for these changes are now unclear [*J. Pharm. Pharmacol.*, **50**:871 (1998) and *Antimicro. Agents Chemother.*, **43**:2697 (1999)]. Most recently, we found that SLT-II, which was prepared from NGY12, a mutant strain of *E. coli* O157:H7, does not induce liver injury, while it decreases the protein levels of both CYP3A2 and CYP2C11 [*Antimicrob. Agents Chemother.*, **47**:1636 (2003)]. The effect of *K. pneumonia* endotoxin and SLT-II on drug transport systems in the liver, kidney and brain, however, has not yet been investigated.

We designed a series of experiments to investigate the effects of *K. pneumoniae* endotoxin and SLT-II on drug transport systems. First, we investigated whether *K. pneumoniae* endotoxin could modify P-glycoprotein-mediated biliary and renal transport systems in rats. Rhodamine-123 was chosen as the model drug, since this compound is primarily excreted into the bile and urine by P-glycoprotein. We also measured the expression of P-glycoprotein mRNA (*mdr1a*) in the liver and kidney at different time intervals after intraperitoneal injection of endotoxin (1 mg/kg, i.v.), by reverse transcriptase-polymerase chain reaction (RT-PCR). The effect of the typical substrates for P-glycoprotein, cyclosporin A, colchicine and erythromycin, on the biliary excretion of P-glycoprotein substrate, rhodamine-123 at steady state concentration was investigated in rats. The P-glycoprotein inhibitors cyclosporin A and erythromycin dramatically inhibited the biliary clearance of rhodamine-123, whereas the organic cationic drug cimetidine did not inhibit, suggesting that rhodamine-123 is mainly transported by P-glycoprotein. Endotoxin significantly decreased the steady-state biliary, renal and tubular secretory clearances of rhodamine-123 and the glomerular filtration rate 6 h after injection, whereas these parameters returned to control levels by 24 h. These results suggest that endotoxin-induced decreases in P-glycoprotein-mediated biliary excretion and renal handling of rhodamine-123

were probably due to impairment of P-glycoprotein-mediated transport ability. The contribution of tumor necrosis factor-α (TNF-α) to the endotoxin-induced decrease in the biliary excretion of rhodamine-123 was investigated. Pretreatment of pentoxifylline (50 mg/kg) significantly inhibited endotoxin-induced increase in TNF-α levels in plasma, which ameliorated the endotoxin-induced reduction of the biliary excretion of rhodamine-123. It is likely that endotoxin-induced impairment of the transport of rhodamine-123 is caused, in part, by overproduction of TNF-α. The effect of endotoxin on the expression of P-glycoprotein mRNA in liver and kidney of rats was investigated by using RT-PCR. The expression of *mdr1a* mRNA in both liver and kidney decreased 6 h after endotoxin injection and returned to control levels after 24 h, whereas the expression of *mdr1b* mRNA in liver increased at both times and that in kidney decreased at 24 h. These findings suggest that *K. pneumoniae* endotoxin dramatically decreases P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 probably by decreasing the expression of *mdr1a*, which is due to increased plasma TNF-α levels [*Antimicrob. Agents Chemother.*, **45**:3462 (2001)].

Second, the effect of SLT-II (2 μg/animal) on the renal transport system and the expression and function of Mrp2 and P-glycoprotein in the kidney of rats was investigated. A quinolone antimicrobial agent, levofloxacin (LVX), was chosen as a model drug of quinolone antimicrobial agents, as it is mainly excreted into the urine through active tubular secretion by drug transporters and used for the treatment of *E. coli* O157 infection. In histopathological examination, the acute tubular injury was observed in SLT-II-treated rats, but the glomeruli were not injured. SLT-II significantly delayed disappearance of LVX from plasma 24 h after injection, suggesting that SLT-II decreases the renal excretion of LVX. The effect of SLT-II on the renal handling of LVX was investigated. SLT-II significantly increased the steady-state plasma concentration of LVX to 1.5-fold that of control rats and induced significant decreases in the GFR and CL_R of LVX. SLT-II slightly, but significantly, increased the unbound fraction and decreased renal plasma flow with no change in the extraction ratio of *p*-aminohippurate, suggesting that the decreased GFR may be caused by decreased RPF. SLT-II significantly increased concentrations of TNF-α and nitrite and nitrate in plasma. The TNF-α inhibitor pentoxifylline (50 mg/kg) partly, but significantly, inhibited SLT-II-induced decreases in the GFR and CL_R of LVX. By contrast, *S*-methylisothiourea, a selective inhibitor of inducible nitric oxide synthase (10 mg/kg), did not. Western blot analysis revealed that SLT-II did not alter the level of the multidrug resistance-associated protein 2 (Mrp2) and P-glycoprotein, in kidney 24 h after injection, assuming the lack of involvement of Mrp2 as well as P-glycoprotein in SLT-II-induced acute renal tubular injury and renal handling of LVX, observed 24 h after SLT-II injection. The present study suggests that SLT-II impairs the renal handling of LVX by decreasing GFR and causing decreased renal plasma flow [*Antimicrob. Agents Chemother.*, **46**:1522 (2002)].

Third, the effect of *K. pneumoniae* endotoxin on *in vivo* blood-brain barrier (BBB) transport of doxorubicin and P-glycoprotein function was investigated. Doxorubicin was chosen as a model drug, as it is a typical P-glycoprotein substrate, and fluorescein isothiocyanate labeled dextran 4 (FD-4) was used as an integrity marker of BBB. Doxorubicin (30 mg/kg) was administered into the tail vein or FD-4 was infused (20 μg/min) into the right jugular vein of mice injected intravenously with endotoxin (10 mg/kg) 6 or 24 h earlier. Blood and brain samples were collected 4 h after injection of doxorubicin or 1 h after infusion of FD-4. We examined using

Western blotting the influence of endotoxin on the expression of P-glycoprotein in brains obtained 6, 12, and 24 h after injection. Endotoxin did not change the plasma and brain concentrations and brain-to-plasma concentration ratio (K_p) of FD-4. No histopathological changes in brain capillaries were observed. These results suggest that endotoxin does not cause damage to brain capillaries. Plasma and brain concentrations of doxorubicin in mice treated 6 h earlier with endotoxin were significantly higher than those in control and mice treated 24 h earlier. However, endotoxin did not significantly change the K_p value of doxorubicin. The protein level of P-glycoprotein was significantly, but slightly down-regulated 6 h after endotoxin treatment. However, the levels remained almost unchanged after 12 and 24 h. The present results suggest that *K. pneumoniae* endotoxin has no effect on the brain capillary integrity and doxorubicin transport across the BBB in mice. It is likely that P-glycoprotein function might be sufficient to transport doxorubicin in spite of decreased levels of P-glycoprotein in the brain [*Eur. J. Pharmacol.*, **445**:115 (2002)].

Fourth, the effect of SLT-II on the brain distribution of a P-glycoprotein substrate, doxorubicin, and on P-glycoprotein expression and function in mice was investigated. Doxorubicin (30 mg/kg) was administered intravenously or FD-4 was infused (20 μg/min) in mice, which had received intravenous injection of SLT-II (0.2 μg/animal) 6 or 24 h earlier. Blood and brain samples were collected 4 h after injection of doxorubicin or 60 min after infusion of FD-4. SLT-II significantly elevated the K_p of FD-4 24 h after injection, but did not alter 6 h after. No significant differences in the plasma and brain concentrations and K_p value of FD-4 were observed between control and mice treated 6 h earlier with SLT-II. To the contrary, SLT-II significantly increased the plasma and brain concentrations and K_p value of doxorubicin in mice 24 h after injection. Cyclosporin A (200 mg/kg) significantly increased the plasma and brain concentrations and K_p value of doxorubicin in the control mice. However, cyclosporin A did not alter the K_p value of doxorubicin in mice treated 24 h earlier with SLT-II. The TNF-α inhibitor pentoxifylline (100 mg/kg) protected SLT-II-induced increases in the brain concentrations of both drugs and K_p value of FD-4, suggesting that TNF-α, at least in part, causes damage to the brain capillaries. Western blotting analysis revealed that SLT-II increased the protein level of P-glycoprotein in the brain of mice 6 h after injection and the increased level remained unchanged for 24 h. SLT-II did not change ATP content in the brain of mice. These results suggest that the increased P-glycoprotein level cannot explain SLT-II-induced increase in the accumulation of doxorubicin in the brain of mice treated with SLT-II. The present findings indicate that SLT-II impairs the BBB function and doxorubicin transport across the BBB, while overexpressed P-glycoprotein [*Brain Res.*, **956**:246 (2002)].

Conclusion, the present results are the first to report the effects of endotoxin and SLT-II on the drug transport system. Our findings firstly revealed that *K. pneumoniae* endotoxin decreases P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 probably due to decrease in the expression of P-glycoprotein mRNA, and that TNF-α may be associated with these pathophysiological alterations. SLT-II also decreases the renal excretion of LVX by decreasing GFR and induces renal tubular necrosis. It has no effect on the protein levels of Mrp2 and P-glycoprotein in the kidney, which differs from endotoxin. The protein levels of Mrp2 and P-glycoprotein could not explain SLT-II-induced decrease in the renal excretion of

LVX. This phenomenon is probably due to accumulation of endogenous toxic substances and/or putative substrates for Mrp2 and P-glycoprotein in plasma and kidney, or the possibility that TNF-α and NO do not play major roles in SLT-II-induced renal tubular damage.

Concerning the effect of endotoxin and SLT-II on the P-glycoprotein-medicated BBB drug transport system, we demonstrated that *K. pneumoniae* endotoxin does not impair BBB integrity and doxorubicin transport across the BBB in mice. This study is the first to describe the effect of endotoxin on the protein level of P-glycoprotein in the brain. Differing from endotoxin, SLT-II impairs the integrity of the brain capillary endothelial cells and doxorubicin transport across the BBB and up-regulates P-glycoprotein in the brain. SLT-II suppresses P-glycoprotein-mediated efflux of doxorubicin from the brain, which could not be explained by SLT-II-induced increase in the protein level of P-glycoprotein. The mechanism may be involved in the accumulation of endogenous P-glycoprotein substrates in plasma and brain.

Our findings may provide some useful guides for clinical treatment for those patients with *K. pneumoniae* or *Escherichia coli* O157:H7 infections. The dosage adjustment should be needed to avoid the side effects of these drugs

学位論文審査結果の要旨

グラム陰性菌感染症によるエンドトキシン血症および腸管出血性大腸菌 O157:H7 感染症病態における生体膜輸送機構の変化は未だ明らかにされていない。本論文では、Klebsiella pneumoniae LEN-1 (03: K1⁻) のエンドトキシンおよびE.coli O157:H7 strain NGY12 から分離した滋賀様毒素Ⅱ (SLT-Ⅱ) の薬物の生体膜輸送機構に及ぼす影響をラットあるいはマウスを用いて検討し、以下のような知見を得た。

(1) エンドトキシンは肝臓および腎臓に障害を与えないが、P糖蛋白質 mRNA の発現を低下させることによってP糖蛋白質介在性の胆汁排泄能および腎排泄能を低下させるが、この低下には炎症性サイトカインイン TNF-α が一部関与していること。

(2) エンドトキシンは血液脳関門の形態およびP糖蛋白質発現量に変化を与えることなく、P糖蛋白質介在性薬物の血液脳関門輸送に影響を与えないこと。

(3) SLT-Ⅱは糸球体には病理組織学的変化を及ぼさないが、尿細管障害を誘発し、糸球体濾過速度および尿細管分泌能を低下させることによって薬物の腎排泄速度を遅延させるが、腎臓のP糖蛋白質の発現量には変化を与えないこと。

(4) SLT-Ⅱは脳毛細血管内皮細胞間隙を開口し、薬物の単純拡散に基づく脳移行を高めること。

以上の結果は、臨床分離菌由来の内毒素エンドトキシンと外毒素 SLT-Ⅱを用いた感染症病態モデルにおける薬物の生体膜輸送および体内動態の変化とトランスポーターの発現レベルと機能変化の関与について明らかにしたものである。本研究の成果は肺炎桿菌感染によるエンドトキシン血症および腸管出血性大腸菌 O157:H7 感染症患者に対する適正な治療法を確立するための重要な情報を提供するものであり、本論文は博士（薬学）論文に値すると判定した。