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# Down-regulation of cytochrome P450 proteins and its activities by Shiga-like toxin II from *Escherichia coli* O157:H7☆

Kiyoyuki Kitaichi[a], Hironao Nakayama[a], Jun Ueyama[a], Masayuki Nadai[b], Kenji Baba[c], Kenji Takagi[a], Kenzo Takagi[a], Michio Ohta[d], Takaaki Hasegawa[a,*]

[a]*Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20, Daikominami, Higashi-ku, Nagoya 461-8673, Japan*
[b]*Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-0077, Japan*
[c]*Third Department of Internal Medicine, Aichi Medical School, School of Medicine, 21 Nagakute-cho, Aichi-gun, Aichi 480-1195, Japan*
[d]*Department of Microbiology, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan*

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**Abstract**

*Escherichia coli* O157:H7 infection frequently induces clinical complications such as hemolytic uremic syndromes and intestinal dysfunctions. These changes could alter the disposition of drugs, consequently changing their efficacy. However, the possible changes of drug-metabolizing activities by *E. coli* O157:H7 infection have not been addressed. Thus, we have investigated the effect of Shiga-like toxin type II (SLT-II), derived from *E. coli* O157:H7, on the hepatic cytochrome P450 (CYP) content and its activity in rats. SLT-II (2 μg per animal, i.v.) time-dependently decreased total CYP content and the contents of CYP2C11 and CYP3A2 in hepatic microsomal preparations up to 24 hr following injection. Consistently, SLT-II time-dependently decreased CYP activity *in vivo*, as represented by systemic clearance of antipyrine. An inhibitor of inducible nitric oxide synthase, *S*-methylisothiourea, restored the decreased systemic clearance of antipyrine by SLT-II, suggesting the involvement of the overproduction of nitric oxide by SLT-II. Moreover, dexamethasone restored the decreased systemic clearance of antipyrine by SLT-II. In the hepatic microsomal preparation, dexamethasone restored the SLT-II-induced decrease of CYP3A2 whereas *S*-methylisothiourea did not affect both CYP subtypes. Taken together, these results suggest that SLT-II might alter hepatic drug-metabolizing function during *E. coli* O157 infection and that more than one cytokines induced by SLT-II, including nitric oxide, might make a critical contribution to the decrease of CYP content and its activity.


*Keywords:* SLT-II; Cytochrome P450; CYP3A; Nitric oxide; Inflammatory cytokines; Rats

## 1. Introduction

*Escherichia coli* O157:H7 (*E. coli* O157:H7) infection produces SLTs, subsequently causing clinical symptom including hemolytic uremic syndromes (HUS) [1]. SLTs possess one A-subunit and five B-subunits [2]. The B-subunits of SLTs binds to a cell surface receptor named $Gb_3$/CD77 [1]. Then, SLTs-$Gb_3$/CD77 complexes are endocytosed and are transported retrogradely through the Golgi apparatus to the endoplasmic reticulum. Finally, their A-subunits are translocated to the cytosol upon which the effect of this complex becomes apparent; i.e. apoptosis/cytotoxicity [1,3,4]. However, the sites of tissue damage in human by *E. coli* O157:H7 infection are not co-related with the site of Gb3/CD77 expression [5]. For example, the renal damage in HUS is limited primarily to the glomeruli [6,7], although Gb3/CD77 expression is much lower here than in renal tubular epithelial cells in human [8]. These results suggest the involvement of indirect intoxication by SLTs in the clinical symptoms of HUS.

In consideration of several reasons, thus far, there are few options to treat *E. coli* O157:H7 infection with drugs. It is naturally considered that antibiotics should be the first

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* Corresponding author. Tel.: +81-52-719-3008x1558; fax: +81-52-719-3009.
*E-mail address:* hasegawa@met.nagoya-u.ac.jp (T. Hasegawa).

*Abbreviations:* CYP, cytochrome P450; SLT, Shiga-like toxin; NO, nitric oxide; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; NOS, nitric oxide synthase; SMT, *S*-methylisothiourea; L-NAME, *N*ω-nitro-L-arginine methyl ester; DEX, dexamethasone.

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choice for the bacterial infections. However, the clinical use of antibiotics against *E. coli* O157:H7 is limited or contraindicated since several cases have been reported in which antibiotics have increased the risk of HUS by increasing the release of SLTs from injured bacteria [9,10]. Moreover, the use of antimotility drugs against bloody diarrhea is contraindicated due to the slow elimination of SLTs from the bowel [11]. Furthermore, drugs, excreted into urine are unlikely to be recommended for the treatment of *E. coli* O157:H7 infection due to HUS in the infection, although there are no reports available regarding undue toxicity of drugs. Indeed, the renal excretion of levofloxacin was significantly reduced in SLT-II-treated rats [12]. Thus, depending on the status of this disease, drugs against disseminated intravascular coagulation, blood cell transfusion against blood loss, and therapeutic treatment against HUS such as haemoidialysis, peritoneal dialysis, and therapeutic plasma exchange are routinely used.

In order to select the appropriate drugs against *E. coli* O157 infection, it is deemed to be important to obtain information about possible altered drug-pharmacokinetics, including hepatic drug metabolizing activities. If the *E. coli* O157 infection causes hepatic dysfunction like endotoxemia [13], hepatic-metabolizing drugs could cause severe side-effects due to relatively higher concentrations or levels of them in the blood circulation than in normal subjects. To a lesser extent, SLT type II (SLT-II) likely causes the hepatic dysfunction since we recently found that SLT-II impairs hepatobiliary transport of doxorubicin [14].

Recently, several researchers, including our group, have demonstrated that SLTs significantly increase cytokines levels *in vitro* and *in vivo* [12,15–20]. For example, SLT type I (SLT-I) stimulated the *in vitro* release of TNF-α in a monocytic cell line THP-1 as well as from human peripheral blood monocytes [16]. Moreover, it has been reported that SLT-II increased the plasma levels of TNF-α and NO in rats [12]. Another bacterial toxin, endotoxin (LPS), also stimulates the release of cytokines. Our group and others have previously reported that LPS altered the pharmacokinetic behaviors of various drugs, including antibiotics [13,21–24]. These changes occur in both urinary-excreting and hepatic-metabolizing drugs. For example, LPS decreased the renal clearance of urinary-excreted antibiotics, amikacin, tobramycin and cefazolin [21,23,24]. Alternatively, LPS decreased the systemic clearance of a hepatic-metabolizing drug, antipyrine [22,25], which is metabolized by cytochrome P450 1A2 (CYP1A2), CYP2B6, CYP2C8, CYP2C9, CYP2C18 and CYP3A4 in humans [26] and their orthologues in rats [25,27]. These results suggest that LPS-stimulated release of cytokines might play a crucial role in the pharmacokinetics of drugs.

There are numerous reports that various inflammatory mediators such as TNF-α, interferon-γ, IL-6 and NO regulate the contents and activities of hepatic cytochrome P450 (CYP) [28,29]. In case of NO, two possible mechanisms have been proposed to decrease CYP activities. One is that NO binds to the heme moiety of cytochrome P450 resulting in its inactivation [30–33], and another is that NO decreases the contents of CYP subtypes by decreasing their mRNA expressions [34]. Indeed, decreased *in vivo* CYP activity and increased plasma NO level by *Klebsiella pneumoniae* LPS were restored by SMT [35], an inducible nitric oxide synthase (iNOS) inhibitor [22]. Alternatively, TNF-α decreased the contents of CYP subtypes, CYP3A2 and CYP2C11 in rats [29]. Thus, it would be expected that SLTs could reduce CYP contents and its activities by stimulating the release of cytokines.

Hence, in order to evaluate the effect of SLT-II on the CYP contents and their activities; total CYP contents, the contents of each CYP subtype, and *in vivo* CYP activities, as estimated by the systemic clearance of antipyrine [22,25], were initially investigated. Secondly, considering the fact that SLT-II stimulates the release of cytokines [12] and that excess cytokines (NO and TNF-α) decreased CYP contents, the effect of SLT-II on CYP subtypes was investigated. Among CYPs, CYP3A2 and CYP2C11, were selected for investigation since they are major CYPs in rats [27], are metabolized by antipyrine [26], and are sensitive to several cytokines including TNF-α [29]. Finally, in order to investigate the involvement of SLT-II-induced cytokines, we employed NOS inhibitors and DEX to regulate the effect of SLT-II.

## 2. Materials and methods

The procedures involving animals and their care conformed to the international (‘‘Principles of laboratory animal care’’ (NIH publication No. 85-23, revised 1985) and domestic (‘‘Guiding Principles for the Care and Use of Laboratory Animals’’ provided by Nagoya University, Japan) guidelines.

### 2.1. Animals

Eight- to nine-week-old male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were used in all experiments. The animals were maintained in a temperature- and humidity-regulated room (22–24° and 55 ± 5%, respectively) with food and water ad libitum under controlled lighting (lights on: 08:00–20:00 hr) for at least 3 days before the experiment and surgery.

### 2.2. Chemicals

Antipyrine, phenacetin, SMT, L-NAME) and DEX were purchased from Sigma Chemical Company. All other chemicals used were obtained commercially and were used without further purification. Antipyrine, SMT and L-NAME were dissolved in isotonic saline and DEX in isotonic saline containing 5% ethanol.

### 2.3. Preparation of SLT-II

SLT-II was prepared from a clinically isolated *E. coli* O157:H7 strain NGY12 according to the methods reported previously [12,36]. The concentration of SLT-II in the crude preparation was determined to be 20 μg/mL by using a reverse passive latex agglutination kit (VTEC-RPLA, DENKA SEIKEN Co.).

### 2.4. Surgery

One day before starting the experiments, rats were anesthetized with sodium pentobarbital (25 mg/kg body weight) and the right jugular vein was cannulated with polyethylene tubes for drug administration and blood sampling. The dose of pentobarbital used (25 mg/kg) was relatively lower than that to induce hepatic CYP (over 75 mg/kg for several days) as shown in a previous report [37]. However, to avoid confusion, we administered pentobarbital (25 mg/kg) to all animals used in this study.

### 2.5. Pharmacokinetic experiments

#### 2.5.1. Experimental schedule

In the time-course experiments, rats received a bolus intravenous injection of antipyrine (20 mg/kg of body weight) at 6, 12, and 24 hr after an intravenous injection of SLT-II (2 μg per animal). In the experiments using drugs, SMT (5 mg/kg, i.p.) was administered 2 hr after the intravenous injection of SLT-II (2 μg per animal), L-NAME (10 mg/kg, i.p.) was administered 30 min after SLT-II, and DEX was administered (8 mg/kg, i.p.) 30 min before SLT-II. The effect of drugs was assessed 24 hr after SLT-II injection by injecting antipyrine. The doses of SMT and L-NAME were selected to inhibit LPS-induced NO overproduction [22,35,38], respectively. The dose of DEX was selected to inhibit the overproduction of both TNF-α [39] and NO [38] by LPS. After administered antipyrine (20 mg/kg), blood samples (approximately 0.25 mL each) were collected at designated intervals of 30, 60, 90, 120, 180, 240, and 300 min after antipyrine administration. Plasma samples were immediately obtained by centrifugation at 6000 *g* for 10 min, and were stored at −40° until analyzed.

#### 2.5.2. Drug analysis

Concentrations of antipyrine in the plasma were measured by high-performance liquid chromatography (HPLC) with a slight modification of a previously described method [40]. The HPLC apparatus was an LC-VP system (Shimadzu) consisting of an LC-10AD$_{VP}$ liquid pump, an SPD-10A$_{VP}$ UV-Vis spectrophotometric detector, and an SIL-10AD$_{VP}$ autoinjector. A Cosmosil 5C$_{18}$ column (4.6 mm × 150 mm; Nacalai Tesque) was used with a column oven (OTC-10AC$_{VP}$) heated to 40°. The UV detector was set at 254 nm. The mobile phase was 30% methanol in distilled water (v/v), and the flow rate was 1.5 mL/min. Phenacetin was used as an internal standard. Standard curves for measuring antipyrine in the plasma proved to be linear for concentrations ranging from 0.5 to 50 μg/mL with a correlation coefficient of 0.999. The intra- and interassay coefficients of variation for the HPLC assay were less than 6% at concentrations of 5 and 20 μg/mL. The detection limit of antipyrine was 0.2 μg/mL.

#### 2.5.3. Data analysis

Plasma concentration-time data for antipyrine in each rat were analyzed individually by noncompartmental methods. The area under the plasma concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated by the trapezoidal rule method up to the last measured plasma concentration and were extrapolated to infinity by adding the following: the value of the last measured plasma concentration divided by the terminal elimination rate constant, which was calculated by determining the slope of the least-squares regression line from the terminal portion of the log concentration–time data. Systemic clearance ($CL_{SYS}$) was calculated by dividing the dose by the AUC. The steady-state volume of distribution ($V_{SS}$) was calculated as $V_{SS} = CL_{SYS} \times MRT$, where MRT represents the mean residence time, which was calculated as MRT = AUMC/AUC. All computer analyses were performed with the nonlinear least-squares regression program WinNonlin (ver 2.1, Pharsight Co.).

### 2.6. Biochemical determinations

#### 2.6.1. Experimental procedures

Time-course effect of SLT-II and the effects of drugs on biochemical and histochemical parameters were performed independently to pharmacokinetic studies (see Table 1). As described above, rats were anesthetized with sodium pentobarbital (25 mg/kg, i.p.) and the right jugular vein was cannulated with polyethylene tubes 1 day before starting the experiments. On the day of the experiments, rats were intravenously administered SLT-II (2 μg per animal) and/or drugs (SMT or DEX) for the series of experiments.

#### 2.6.2. Measurement of plasma $NO_x$ and TNF-α levels

Two and 12 hr after SLT-II injection, blood samples were collected to determine plasma TNF-α and $NO_x$ ($NO_2^-$ and $NO_3^-$) levels. Collected blood samples were centrifugated (6000 *g* for 10 min) and were kept at −80° until analyzed. Blood samples at 2 hr after SLT-II injection were used for measuring plasma TNF-α levels and at 12 hr after SLT-II injection for measuring plasma $NO_x$ levels since plasma TNF-α and $NO_x$ levels were reached to maximum 2 and 12 hr after SLT-II injection, respectively [12]. Plasma $NO_x$ ($NO_2^-$ and $NO_3^-$) and plasma TNF-α levels were measured by using available commercial kits ($NO_x$: Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical; TNF-α: Rat ELISA TNFα, Endogen Inc.) using a microplate reader (Molecular Devices Ltd.).

Table 1
Time-course effect of SLT-II on the pharmacokinetic parameters of antipyrine in rats[a]

| Parameters | AUC (mg/L hr) | $CL_{SYS}$(L/hr/kg) | $t_{1/2}$ (hr) | $V_{SS}$ (L/kg) |
|---|---|---|---|---|
| Control | 29.9 ± 1.1 | 0.67 ± 0.03 | 1.02 ± 0.20 | 0.98 ± 0.11 |
| 6 hr after SLT-II | 45.9 ± 2.5 | 0.46 ± 0.05[b] | 1.47 ± 0.15 | 0.97 ± 0.12 |
| 12 hr after SLT-II | 51.3 ± 3.7[b] | 0.41 ± 0.10[b] | 1.69 ± 0.31[c] | 0.98 ± 0.14 |
| 24 hr after SLT-II | 68.4 ± 5.5[b] | 0.30 ± 0.07[b] | 2.30 ± 0.62[b] | 0.96 ± 0.09 |

[a] Data represent the mean ± SEM of six to seven animals. As described in Material and methods section, 2, 6, 12 and 24 hr after SLT-II (2 μg per animal, i.v.), antipyrine (20 mg/kg, i.v.) was injected, blood samples were collected at designate time-points, and pharmacokinetic analysis was performed. Saline was administered in control group.
[b] $P < 0.05$.
[c] $P < 0.01$ vs. control.

#### 2.6.3. Hepatic microsome preparation

Rats were anesthetized with sodium pentobarbital (25 mg/kg, i.p.) 6, 12 and 24 hr after SLT-II injection. Then, each liver was excised after the perfusion of 150 mL ice-cold saline to remove most of blood, and was kept at −80°. The preparation of microsomes were described elsewhere [41]. Briefly, liver (approx. 1 g) was homogenized at 4° with a Teflon homogenizer using 1.15% KCl. The homogenate was centrifuged (12,000 *g*, 25 min, 4°). The supernatant was further centrifuged (100,000 *g*, 90 min, 4°) to obtain the microsomal fraction. The obtained pellet was resuspended in 1.15% KCl. The protein concentration of the microsomal fraction was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories) using bovine serum albumin (Sigma Chemical Co.) as a standard. The fraction was kept at −80° until analysis.

#### 2.6.4. Determination of total hepatic microsomal CYP content

Hepatic microsomal CYP content was measured by the different spectrum for their carbon oxide-reduced form by the method of Omura and Sato [41]. Briefly, microsomal preparations were adjusted to 1 mg/mL with 0.1 M phosphate buffer (pH 7.2) containing 20% glycerin, and were placed in both sample and reference cells. After recording the baseline, the sample cell was carefully bubbled with carbon oxide for 30 s to formulate carbon oxide–CYP complex. Then, a few milligrams of solid $Na_2S_2O_4$ was added to reduce carbon oxide–CYP complex. Solid $Na_2S_2O_4$ was also added to the reference cell. The difference in absorbance between sample and reference cell was scanned again from 400 to 500 nm.

CYP content was calculated by the following formula:

$$\text{CYP content } (n \text{ mole mg protein}^{-1}) = \frac{OD_{450} - OD_{490}}{91} \times 1000$$

#### 2.6.5. Western blot analysis for CYP2C11 and CYP3A2

The protein (1 μg) was separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 4% nonfat dry milk, detected by rabbit monoclonal antibody to rat CYP3A2 (Amersham Bioscience Co.) and goat monoclonal antibody to rat CYP2C11 (Sigma Chemical Co.). The immune complexes were visualized using horseradish peroxidase-labeled secondary antibody with ECL Western blot detection reagents (Amersham Bioscience Co.). To quantify the relative levels of CYPs in each gel, the intensity of the stained bands was measured by NIH image.

#### 2.6.6. Statistical analysis

Results were expressed as means ± standard errors for the indicated number of experiments. Statistical comparisons among the groups were assessed by ANOVA with StatView (ver. 4.54, Abacus Concepts Inc.). When *F* ratios were significant ($P < 0.05$), Scheffe post hoc tests between two groups were done, and *P* values less than 0.05 were considered statistically significant post hoc differences.

## 3. Results

### 3.1. The time-course effect of SLT-II on the contents of total CYP, CYP2C11 and CYP3A2

SLT-II time-dependently decreased total CYP contents (Fig. 1A), assessed by carbon oxide-exposing method [41], showing a significant effect 12 and 24 hr after SLT-II injection. Similar to total CYP contents, SLT-II time-dependently decreased CYP subtypes; CYP2C11 significantly decreased 12 and 24 hr after SLT-II injection (Fig. 1B) and CYP3A2 significantly decreased 24 hr after SLT-II injection (Fig. 1C).

### 3.2. The time-course effect of SLT-II on the disappearance of antipyrine from plasma

Time-course effect of SLT-II on the concentration–time data of antipyrine was illustrated in Fig. 2 as mean semilogarithmic plasma concentration–time curves for antipyrine. SLT-II time-dependently increased the level of antipyrine in the plasma, i.e. markedly delayed the disappearance of antipyrine from plasma. Further pharmacokinetic analysis

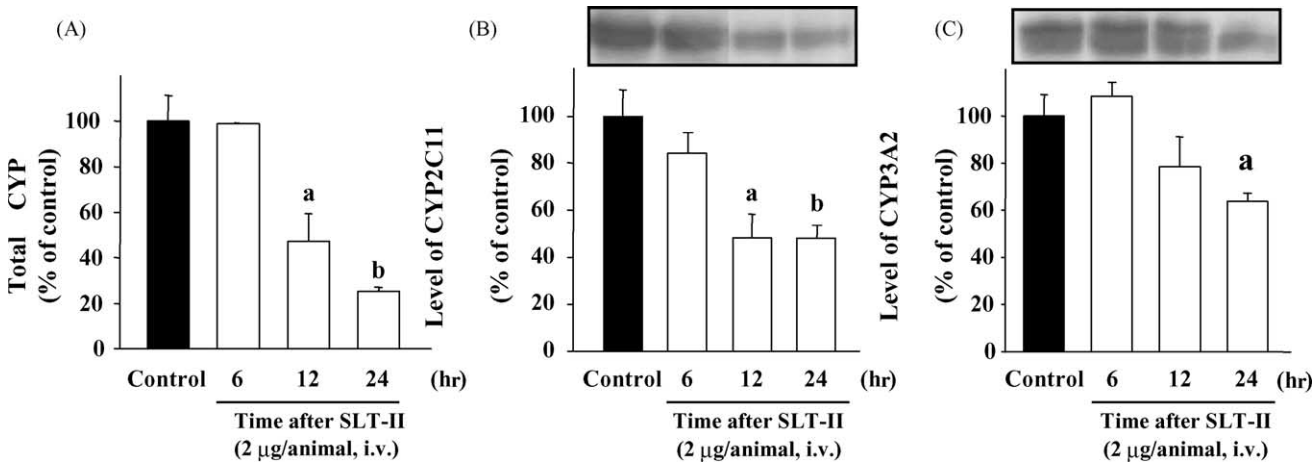


Fig. 1. The time-course effect of SLT-II (2 μg per animal) on total CYP contents (A) and the contents of CYP2C11 (B) and CYP3A2 (C). The methods in detail were described in Section 2. Data represent mean ± SEM of three animals. **a** $P < 0.05$ and **b** $P < 0.01$ vs. control.

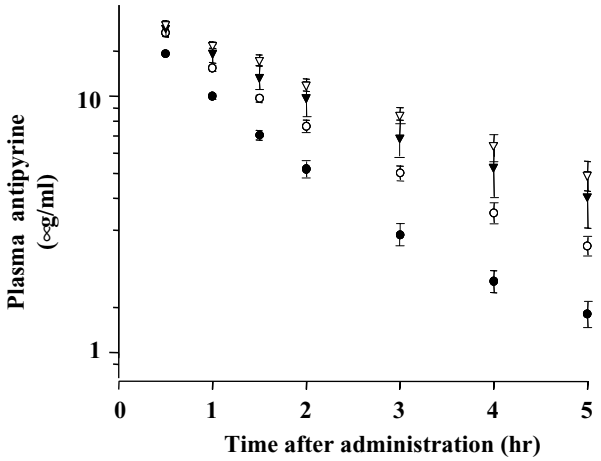


Fig. 2. Mean semilogarithmic plots of plasma concentration–time data for antipyrine in rats treated with/without SLT-II (2 μg per animal). Each symbol represents the mean ± SEM (N = 6–7). Symbols: (●), control; (○), 6 hr after SLT-II; (▼), 12 hr after SLT-II; (▽), 24 hr after SLT-II.

revealed that SLT-II significantly decreased systemic clearance ($CL_{SYS}$) of antipyrine [$F(3, 22) = 33.823$, $P < 0.0001$], increased plasma half-life of antipyrine ($t_{1/2}$) [$F(3, 22) = 13.583$, $P < 0.0001$] as well as AUC of antipyrine [$F(3, 22) = 13.583$, $P < 0.0001$]. In contrast, SLT-II did not affect volume of distribution of antipyrine at steady-state ($Vd_{SS}$) [$F(3, 22) = 0.037$, $P > 0.05$].

### 3.3. The effect of SMT and DEX on elevated plasma $NO_x$ and TNF-α levels by SLT-II

Plasma levels of $NO_x$ and TNF-α reached their maximum 2 and 12 hr after SLT-II injection, respectively, as reported previously [12]. The elevation of plasma $NO_x$ by SLT-II was significantly restored towards basal levels by both SMT (5 mg/kg) and DEX (8 mg/kg) (Fig. 3A). Moreover, DEX also significantly restored towards basal levels the elevated TNF-α levels by SLT-II (Fig. 3B). It should be noted that the effect of L-NAME (10 mg/kg) was not

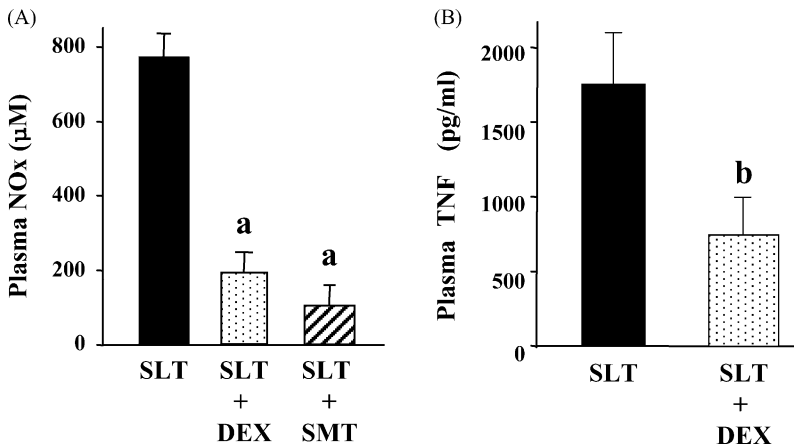


Fig. 3. The effect of SMT and DEX on SLT-II-induced increases in plasma $NO_x$ (A) and TNF-α (B). Each column represents the mean ± SEM (N = 5–6). **a** $P < 0.05$ and **b** $P < 0.01$ vs. the corresponding SLT-II-treated group (Scheffe's post hoc test).

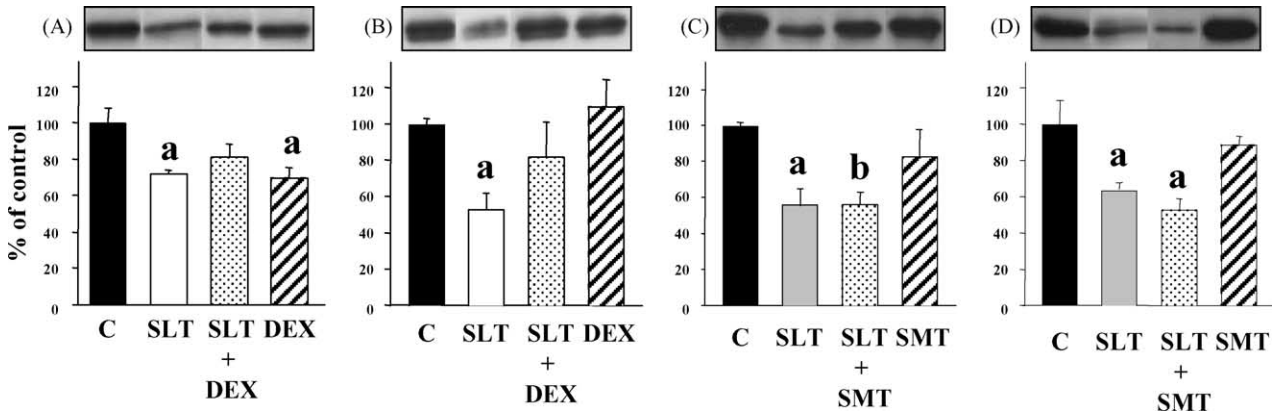


Fig. 4. The effect of DEX (A and B) and SMT (C and D) on decreased expression of CYP2C11 (A and C) and CYP3A2 (B and D) by SLT-II. Each column represents the mean ± SEM (N = 5–6). **a** $P < 0.05$ and **b** $P < 0.01$ vs. the corresponding SLT-II-treated group (Scheffe's post hoc test). C: control; SLT: SLT-II.

evaluated since animals treated with SLT-II and L-NAME were dead within 2–6 hr (data not shown).

### 3.4. The effect of SMT and DEX on the decrease of CYP subtypes by SLT-II

The effect of SMT and DEX on the expression of hepatic CYP was assessed 24 hr after SLT-II injection. The difference in CYP2C11 level between control and SLT-II treatments was reduced to a non-significant level by DEX, although DEX itself decreased CYP2C11 expression (Fig. 4A). The SLT-II-induced decrease in CYP3A2 was restored by DEX without affecting CYP3A2 by DEX alone (Fig. 4B). SMT did not restore the decrease of both subtypes by SLT-II (Fig. 4C and D).

### 3.5. The effect of SMT and DEX on the decreased disappearance of antipyrine by SLT-II

Both SMT and DEX significantly restored the decreased $CL_{SYS}$ of antipyrine by SLT-II (Fig. 5). DEX, but not SMT, significantly decreased $CL_{SYS}$ of antipyrine (Fig. 5).

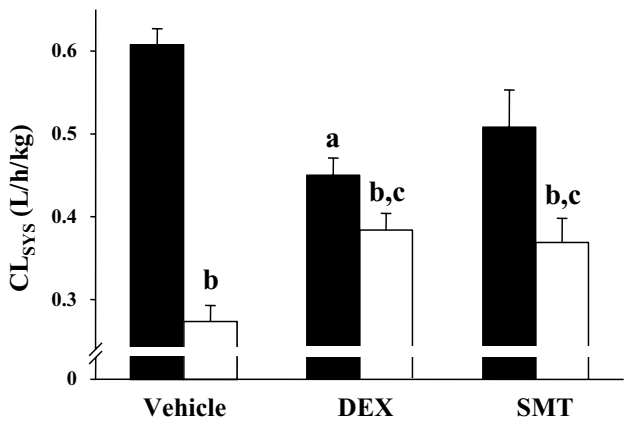


Fig. 5. The effect of DEX and SMT on the systemic clearance of antipyrine ($CL_{SYS}$) in rats treated with/without SLT-II (2 μg per animal). Each column represents the mean ± SEM (N = 6–7). **a** $P < 0.05$ and **b** $P < 0.01$ vs. control; **c** $P < 0.05$ vs. SLT-II (Scheffe's post hoc test).

## 4. Discussion

SLT-II is an important contributor to producing the clinical syndrome of *E. coli* O157 infection in humans including hemolytic uremic syndromes (HUS) and bloody diarrhea [2]. Considering the occurrence of HUS, in the treatment of *E. coli* O157 infection drugs that are not excreted by kidney would be recommended. However, there is little or no information available concerning whether hepatic-metabolizing drugs are appropriate to use in the case of *E. coli* O157 infection. Hence, the purpose of the present study was to investigate the hepatic CYP activity in rats exposed to SLT-II. It has been reported that SLT-II stimulates the release of NO and TNF-α, and that these cytokines differentially affect the contents of CYP subtypes and their activities [29]. Thus, we initially investigated the time-course effect of SLT-II on total CYP content, CYP subtypes, and *in vivo* hepatic CYP activity as revealed by the systemic clearance of antipyrine [22,25]. Then, we investigated the effect of the stimulated release of $NO_x$ and TNF-α by SLT-II on SLT-II-induced changes in CYP contents and its activities.

The present study found that both total CYP contents and protein levels of major CYP subtypes in rats, CYP2C11 and CYP3A2 [27], were time-dependently decreased by SLT-II. Consistent with this, *in vivo* hepatic CYP activity, as expressed by $CL_{SYS}$ of antipyrine [18,21], was also decreased in a time-dependent fashion. It is most likely that the effect of SLT-II on CYP activity is not due to its hepatotoxic effects since no histological or biochemical changes were found using this experimental regimen [14]. Moreover, decreased elimination of antipyrine might not be due to hepatic dysfunction since antipyrine is mainly metabolized by liver without being affected by hepatic blood flow [25,26]. Taken together, these results suggest that SLT-II might decrease the contents and activities of CYP without causing serious hepatic damage in this animal model.

How does SLT-II affect the hepatic CYP contents and its activities? One possibility is that SLT-II acts via certain

receptors on hepatocytes. A putative receptor for SLT-II in eukaryotic cells is now considered to be globotriasoyl ceramide ($Gb_3$/CD77) [1]. However, there are no reports available showing that $Gb_3$/CD77 are present on Kupffer cells or hepatocytes [42,43]. Thus, it is likely that SLT-II could have an indirect effect to change the hepatic CYP.

SLT-II stimulates the release of various cytokines, such as TNF-α, IL-1, IL-6, CXC chemokines including IL-8, and NO from the variety of cells [15–19]. Thus, it is possible that SLT-II indirectly altered hepatic CYP contents and activities by these cytokines. In fact, injection of IL-1 [44–47], NO donors [48,49], or TNF-α [44,50] decreases certain CYP activities and/or their mRNA expressions, although it is difficult to distinguish which mediator(s) play(s) important roles in SLT-II-induced decreases in hepatic CYP content/activity. Thus, we investigated the involvement of these cytokines in SLT-II-induced changes of CYP contents and activity by using several cytokine inhibitors.

NO is one of the important inflammatory mediators to regulate the contents and activities of CYP [22,29–34]. NO is synthesized by both constantly-expressed endothelial NOS (eNOS) and iNOS. NO is subsequently found in plasma in its oxidized forms, $NO_2$/$NO_3$ ($NO_x$) [51]. In the present study, we applied a selective iNOS inhibitor, SMT, and an eNOS/iNOS dual inhibitor, L-NAME. As a result, SMT restored the elevated plasma $NO_x$ by SLT-II. No alteration of eNOS by SLT-II has already been reported [52]. Thus, these results suggest the importance of iNOS in elevated plasma $NO_x$ by SLT-II. Notably, an eNOS/iNOS dual inhibitor, L-NAME, killed all animals within 6 hr of SLT-II injection (data not shown), consistent with a previous report indicating that L-NAME increased lethality in mice treated with SLTs, although the selective iNOS inhibitor aminoguanidine did not [53]. Thus, it is likely that eNOS inhibition deteriorates SLT-II-induced syndrome. Further studies will be required to address this issue.

A previous study revealed that bacterial toxin causes the decreased clearance of antipyrine together with NO overproduction, in agreement with the present findings [22]. Thus far, there are two ways that NO is known to be able to regulate CYP; i.e. (i) NO can reduce CYP mRNA expression, subsequently decreasing CYP protein [31,33,48] and (ii) NO binds to the heme moiety of CYP resulting in its inactivation [30–32]. In our experimental conditions, we reason that the overproduction of NO by SLT-II dominantly affects CYP activity rather than CYP contents. Consistent with this, SMT restored the decreased antipyrine clearance by SLT-II along with a significant reduction of SLT-II-induced increases in plasma $NO_x$ levels; however, SMT did not improve the decreased expression of CYP subtypes. This assumption is likely to be true since our recent findings that the injection of an NO donor, FK-409 [54], failed to decrease the contents of CYP2C11 and CYP3A2 (unpublished observation) although it decreases the systemic clearance of antipyrine [22].

The current experiments also showed that DEX restored the SLT-II-induced decreased antipyrine clearance, together with reducing the SLT-II-induced overproduction of NO. These results suggest that DEX might partially act by regulating NO level. However, DEX differentially modulated CYP subtypes. That is, DEX restored the SLT-II-induced decrease in the protein levels of CYP3A2. It is likely to be excluded out the possibility that DEX induced CYP3A2 although it is well known that DEX is an inducer of CYP3A [24]; since (1) DEX itself did not induce CYP3A2 in the present experiment (Fig. 5) and (2) a previous report demonstrated that higher doses and longer durations of DEX treatment (50 mg/kg for 4 consecutive days [55]) are needed to induce the CYP3A, which are different from ours (8 mg/kg). Several studies revealed that the candidate cytokines for the regulation of CYP3A protein level are IL-1, IL-6, TNF-α [29]. DEX has an ability to inhibit the cytokine release induced by other bacterial toxins [38,39]. In the present study, DEX prevented the overproduction of TNF-α as well as NO by SLT-II. Taken together, several cytokines including TNF-α and NO might be involved in SLT-II-induced decrease in CYP subtype contents. Another report revealed that relatively higher dose of DEX (over 50 mg/kg) than used in our study (8 mg/kg) significantly induces an alternate human CYP3A4 orthologue, named CYP3A1, having similar substrate specificities [56,57]. Thus, it would be of interest to investigate in detail how the effect of DEX against SLT-II.

In conclusion, the present study is the first to show that SLT-II decreases the contents and activities of CYP. More specific determination of changing activities of each CYP subtype would be useful to find out which hepatic-metabolizing drug(s) are dangerous during *E. coli* O157:H7 infection. Moreover, SLT-II-induced cytokines, including NO, might to be involved in the regulation of CYP. The results reported here, at least, could give us a caution when we use the hepatic-metabolizing drugs against *E. coli* O157:H7 infection. In *E. coli* O157:H7 infectious animal model, SLT-II also alters the renal and hepatobiliary transport [12,14] and the brain penetration of several drugs [36]. Thus, we believe it to be important to investigate further the mechanism whereby SLT-II changes the penetration/elimination/metabolizing processes of drugs. This should help establish appropriate dosing regimens for therapeutic drugs in patients with *E. coli* O157:H7 infection.

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