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Role of tumor necrosis factor-α in down-regulation of hepatic cytochrome *P*450 and P-glycoprotein by endotoxin

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Abstract

We investigated the role of tumor necrosis factor-α (TNF-α) in the down-regulation of hepatic P-glycoprotein and cytochrome *P*450 (CYP) by endotoxin, using TNF-α gene-deficient (TNF-$\alpha^{-/-}$) mice. In the case of P-glycoprotein, endotoxin (10 mg/kg) significantly decreased the expression of hepatic P-glycoprotein in wild-type mice 6 h, but not 24 h, after intraperitoneal injection, with no significant differences in the constitutional expression of P-glycoprotein between wild-type mice and TNF-$\alpha^{-/-}$ mice. However, endotoxin had no effect on the expression of P-glycoprotein in TNF-$\alpha^{-/-}$ mice either 6 or 24 h after injection. When doxorubicin was administered intravenously to TNF-$\alpha^{-/-}$ mice treated 6 h earlier with and without endotoxin, no significant differences in the plasma concentrations of doxorubicin 3 h after injection were observed between endotoxin-treated and untreated TNF-$\alpha^{-/-}$ mice. These results suggest that TNF-α plays a pivotal role in the down-regulation of P-glycoprotein by endotoxin. In the case of CYP, the constitutive expression of hepatic CYP3A2 and CYP2C11 had a tendency to decline in TNF-$\alpha^{-/-}$ mice compared with that in wild-type mice. Endotoxin significantly decreased the expression of hepatic CYP3A2 and CYP2C11 in wild-type mice 24 h after injection, and that decreased expression was significantly greater in TNF-$\alpha^{-/-}$ mice than wild-type mice. When antipyrine was administered intravenously to wild-type mice and TNF-$\alpha^{-/-}$ mice treated 24 h earlier with endotoxin, the plasma concentrations of antipyrine in TNF-$\alpha^{-/-}$ mice 3 h after injection were significantly higher than those in wild-type mice. These findings suggest that TNF-α plays a key role in endotoxin-induced down-regulation of hepatic P-glycoprotein, as well as plays a protective role in the regulation of hepatic CYP3A2 and CYP2C11 against endotoxin-induced acute inflammatory response. In TNF-$\alpha^{-/-}$ mice, other cytokines appear to function as compensation for the lack of endogenous TNF-α.

Keywords: Tumor necrosis factor-α (TNF-α); Cytochrome *P*450 (CYP); P-glycoprotein; Endotoxin; TNF-α knockout mice

1. Introduction

It is well known that bacterial infections impair hepatic drug metabolism in humans, and that endotoxin (lipopolysaccharide), a major component of the cell wall of Gram-negative bacteria, plays a key role in this phenomenon. Endotoxin is known to secrete various inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-2, and interleukin-6. These inflammatory mediators have been shown to regulate the content and activities of hepatic cytochrome *P*450 (CYP) in humans and animals (Chen et al., 1992; Manuel, 1996; Morgan, 1997; Shedlofsky et al., 1994). Among them, NO is significantly released after exposure to endotoxin, subsequent to the expression of inducible NO synthase (Bredt and Snyder, 1994; Khatsenko et al., 1993; Moncada et al., 1991). We and other investigators have demonstrated that NO is one

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of the important inflammatory mediators regulating the contents and activities of CYP (Kitaichi et al., 1999, 2004; Gergel et al., 1997; Khatsenko and Kikkawa, 1997; Khatsenko et al., 1993; Minamiyama et al., 1997; Morgan, 1997; Sewer and Morgan, 1997; Takemura et al., 1999; Ueyama et al., 2004; Wink et al., 1993). On the other hand, it has also been reported that the endotoxin-induced down-regulation of CYP3A2, CYP2C11, and CYP2E1 is NO-independent (Sewer and Morgan, 1997, 1998). The mechanism responsible for the NO-mediated down-regulation of CYP isoforms is still unclear.

In addition to NO, TNF-α is also thought to be of major importance in the down-regulation of CYP isoforms in endotoxemia. It has been reported that TNF-α decreases the contents of the CYP isoforms CYP3A2 and CYP2C11 in rats (Monshouwer et al., 1996; Sewer and Morgan, 1997). In contrast, Warren et al. (1999), in animal experiments using TNF-α receptor (p55/p75)-deficient mice, reported that TNF-α does not play a key role in the endotoxin-induced down-regulation of CYP isoforms, including CYP1A and CYP3A.

On the other hand, inflammatory cytokines, including TNF-α, interleukin-1, interleukin-2, and interleukin-6, might play an important role in endotoxin-induced changes in certain drug transporter-mediated hepatobiliary excretion systems (Hirsch-Ernst et al., 1998; Simpson et al., 1997). We previously reported that *Klebsiella pneumoniae* endotoxin significantly reduces hepatobiliary excretion of the β-lactam antibiotic, cefoperazone, which is a substrate for multidrug resistance-associated protein 2 (Mrp2) (Haghgoo et al., 1995; Nadai et al., 1998), suggesting that some inflammatory mediators released by endotoxin contribute to the impairment of the hepatobiliary excretion of drugs by reducing the expression and/or function of Mrp2 in the canalicular membrane of hepatocytes. Endotoxin is known to induce cholestasis and hyperbilirubinemia by down-regulating Mrp2, an efflux pump for bile acids and bilirubin, due to the secretion of some cytokines, including TNF-α and interleukin-1 (Green et al., 1996; Nakamura et al., 1999; Trauner et al., 1997). In contrast, there is evidence that TNF-α induces the up-regulation of transporter genes or MRP1 protein in human colon carcinoma cells and mdr1 in rat hepatoma cells (Chapekar et al., 1991; Stein et al., 1997). However, the precise role of TNF-α in the down-regulation or up-regulation of hepatic Mrp2 by endotoxins remains unclear.

Like Mrp2, the ATP-binding cassette transport protein, P-glycoprotein, is expressed in many eliminating organs such as the liver and kidney (Cordon-Cardo et al., 1989; Thiebaut et al., 1987), and acts as the efflux transport protein for endogenous and exogenous toxic substances (Schinkel et al., 1996; Thiebaut et al., 1987). Thus, these two drug transport proteins, P-glycoprotein and Mrp2, might exert a protective function of excluding various lipophilic substrates from the liver. There is evidence that TNF-α reduces the expression of P-glycoprotein (Sukhai et al., 2000). In our previous studies, it was suggested that endotoxin dramatically decreases the P-glycoprotein-mediated hepatobiliary excretion of rhodamine 123 by reducing the expression of mdr1a due to increased plasma TNF-α levels (Ando et al., 2001). From the above findings, the expression of both hepatic P-glycoprotein and Mrp2 appears to be regulated by inflammatory cytokines, including TNF-α. Interestingly, there is evidence that the numerous substrates of P-glycoprotein, CYP3A, and Mrp2 largely overlap, and that these proteins are located at hepatocytes and have similar functions of removing various drugs from the body (Mayer et al., 1995; Oude Elferink et al., 1995; Wacher et al., 1995). On the basis of these observations, it is possible that endotoxin might simultaneously down-regulate hepatic P-glycoprotein, CYP3A, and Mrp2. However, to our knowledge, there is no information confirming whether endotoxin simultaneously regulates the expression of CYP3A, P-glycoprotein, and Mrp2. Taken together, it is, at present, difficult to clarify the role of endogenous TNF-α in the regulation of hepatic CYP isoforms and drug transporters.

In the present study, we focused on the effect of TNF-α on the expression of the drug transporter P-glycoprotein and the major CYP isoforms, CYP3A2 and CYP2C11. It is considered that mice with targeted deletions of the TNF-α gene (TNF-$\alpha^{-/-}$ mice) are useful as an animal model for evaluating the role of endogenous TNF-α in the down-regulation of CYP isoforms by endotoxin. The aim of the present study was to clarify the role of TNF-α in the endotoxin-induced down-regulation of P-glycoprotein and these CYP isoforms, using mice with a targeted disruption of the gene encoding TNF-α (Taniguchi et al., 1997), which can block the effects of TNF-α.

2. Materials and methods

2.1. Chemicals

Endotoxin was obtained from *K. pneumoniae* LEN-1 (O3:K1^{-}), which was identical to that used in previous studies (Ando et al., 2001; Kitaichi et al., 1999; Nadai et al., 1998; Zhao et al., 2002). Doxorubicin hydrochloride and daunorubicin hydrochloride were purchased from Sigma (St. Louis, MO, USA). Doxorubicin hydrochloride, in the form of a commercial preparation for injection, was purchased from Kyowa Hakko Kogyo (Adriamycin; Tokyo, Japan). Antipyrine, testosterone, 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 2α-hydroxytestosterone were purchased from Sigma. All other chemicals were commercially available and were of analytical grade. Endotoxin and antipyrine were dissolved in isotonic saline.

2.2. Animals

Nine- to 10n-week-old male C57BL/6^{-} TNF-$\alpha^{-/-}$ and C57BL/6 mice were used in the present experiment.

C57BL/6^{-} TNF-$\alpha^{-/-}$ mice were obtained from embryonic stem (ES) cells of the TT2 line by backcrossing C57BL/6 by more than eight generations (Taniguchi et al., 1997). The wild-type mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed under controlled environmental conditions (temperature of 22–24 °C and humidity of 55±5%) with a commercial food diet and water freely available to the animals. All animal experiments were carried out in accordance with the guidelines of the Nagoya University School of Medicine for the care and use of laboratory animals.

2.3. Histopathological examinations

Mice under light anesthesia with diethyl ether were killed by exsanguination 24 h after injection of endotoxin or saline. For light microscopy, the liver was immediately removed and small pieces of liver tissues were fixed in 20% formaldehyde in 0.1 M phosphate-buffered saline (PBS). Fixed tissue specimens were embedded in paraffin wax, and paraffin sections were then treated with hematoxylin and eosin stain. Veterinary pathologists performed the histopathological examinations according to a method reported previously (Zhao et al., 2002).

2.4. Elimination of antipyrine and doxorubicin

Under light anesthesia with sodium pentobarbital, the right jugular vein of each mouse was cannulated with a polyethylene tube for the injection of antipyrine or doxorubicin. Doxorubicin (30 mg/kg) was administered intravenously in TNF-$\alpha^{-/-}$ mice 6 h after injection of endotoxin (10 mg/kg) or saline. Antipyrine (20 mg/kg) was administered intravenously in wild-type and TNF-$\alpha^{-/-}$ mice 24 h after injection of endotoxin or saline. Three hours after injection of doxorubicin or antipyrine, blood samples were collected by exsanguinations from the abdominal aortas of mice under light anesthesia with diethyl ether. Plasma samples were obtained from the blood samples by centrifugation at 4000×g for 10 min at 4 °C, and were stored at −40 °C until analyzed.

2.5. Hepatic microsome preparation for Western blot

Mice were anesthesized with sodium pentobarbital (25 mg/kg) 24 h after injection of endotoxin or saline. Then, each liver was excised after perfusion of 10 ml of iced-cold saline to remove most of the blood. The microsomes were prepared according to the method of Omura and Sato (1964). Briefly, the liver (approximately 0.5 g) was homogenized at 4 °C with a tight homogenizer (10 strokes up and down) using 1.15% KCl. The homogenate was centrifuged at 12,000×g for 25 min at 4 °C. The supernatant was further centrifuged at 80,000×g for 90 min at 4 °C to obtain the microsomal fraction. The obtained pellet was washed with 1.15% KCl and then resuspended in 1.15% KCl. The protein concentration of the microsomal fraction was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin (Sigma) as a standard. The fraction was kept at −80 °C until analysis.

2.6. Testosterone hydroxylase activity

The liver (approximately 0.5 g) obtained by the methods described above was homogenized at 4 °C with Teflon homogenizer (10 strokes up and down) using a buffer solution consisting of 250 mM sucrose, 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, and 10 mM KCl. The homogenate was centrifuged at 9000×g for 25 min at 4 °C. The supernatant was further centrifuged at 105,000×g for 60 min at 4 °C to obtain the microsomal fraction. The obtained pellet was dissolved in the buffer solution, and again centrifuged at 105,000×g for 60 min at 4 °C.

A volume of 480 μl of 100 mM phosphate-buffered incubation medium (pH 7.4) containing 3.3 mM $MgCl_2$, 1.5 mM β-$NADP^{+}$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase (G6PD), and 50 μl of the obtained microsomal protein (about 1 mg/ml protein) was placed in a sample tube, and preincubated for 1.5 min at 37 °C. The reaction was initiated by addition of 20 μl of 5 mM testosterone as a substrate. Incubations were performed for 15 min at 37 °C and were terminated by adding 1 ml of ice-cold ethyl acetate and 20 μl of 250 mM 11α-hydroxyprogesterone as internal standard. The samples were vortex-mixed for 30 s and centrifuged at 11,000×g for 10 min. The organic phase was evaporated under a nitrogen stream, and the residue was dissolved in 50% methanol and subjected to high-performance liquid chromatography (HPLC) analysis.

2.7. Western blot analysis for CYP3A2 and CYP2C11 in microsomal fraction

The protein (1 μg) was separated by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked in PBS solution containing 0.1% Tween 20 and 4% nonfat dry milk, and detected by rabbit polyclonal antibody to rat CYP3A2 (Daiichi Pure Chemicals, Tokyo, Japan) and goat polyclonal antibody to rat CYP2C11 (Daiichi Pure Chemicals). Immune complexes were visualized using horseradish peroxidase-labeled secondary antibody, antirabbit IgG (Amersham Biosciences, Piscataway, NJ, USA) for CYP3A2, and antigoat IgG (Sigma) for CYP2C11 with ECL Western blot detection reagents (Amersham Biosciences).

To quantify the relative levels of each protein in each gel, the intensity of the stained bands was measured by the NIH image program (Bethesda, MD, USA). The levels were expressed as 100% of those in mice treated with saline in place of endotoxin.

2.8. Western blot analysis for hepatic P-glycoprotein

The liver was obtained from mice 24 h after injection of endotoxin or saline. Each liver was excised after a perfusion of 10 ml of iced-cold saline to remove most of the blood. Each liver (approximately 0.2 g) was suspended in 10-fold volumes of 10 mM Tris–HCl buffer (pH 8.0) containing 1.5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (Sigma). The suspension was homogenized with a tight homogenizer (20 strokes up and down) and centrifuged at 3000×*g* for 10 min at 4 °C. The supernatant was centrifuged at 30,000×*g* for 60 min at 4 °C. The pellet was dissolved in 100 μl of 10 mM Tris–HCl buffer (pH 8.0) containing 0.5% Nonidet P40 (Daiichi Pure Chemicals). The protein (40 μg) was separated by electrophoresis on 8% polyacrylamide gels containing 0.1% SDS and transferred to a PVDF membrane (Millipore). The membrane was blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk, and detected by C219 mouse monoclonal antibody to P-glycoprotein (DakoCytomation, Glostrup, Denmark).

The intensity of the stained bands was measured as described above. The levels were expressed as 100% of those in mice treated with saline in place of endotoxin.

2.9. Measurement of plasma nitrate/nitrite (NO_x) levels

Blood samples were collected to determine plasma NO_x (nitrate/nitrite) 24 h after the injection of endotoxin. The plasma samples were ultrafiltered (molecular cutoff of 10,000) at 6000×*g* for 60 min at 4 °C. The concentration of NO_x in the ultrafiltrate was measured by a commercially available kit (Nitrate/Nitrite Colorimetric Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) using a microplate reader (Molecular Devices, Crawley, UK). Nitrite recovery in this assay was over 95%.

2.10. Drug analysis

HPLC analyses were performed using a Shimadzu LC-10A system (Kyoto, Japan) consisting of an LC-10A liquid pump and an auto injector SIL-10Advp, and equipped with a UV–VIS detector (SPD-10 AV) set at wavelength of 254 nm for antipyrine and 247 nm for testosterone metabolites, and a fluorescence detector (RF-10AXL) (excitation, 480 nm; emission, 560 nm) for doxorubicin. The assay conditions were as follows: column, a Cosmocil $5C_{18}$ (Nacalai Tesque, Kyoto, Japan) for antipyrine and doxorubicin, and a Cosmocil 5CN-MS (Tosho, Tokyo, Japan) for testosterone metabolites; mobile phase, 30% methanol in water (vol/vol) for antipyrine, 0.5% phosphoric acid–methanol (40:60, vol/vol) solution for doxorubicin, and water/methanol/acetonitrile (76:22:2, vol/vol) for testosterone metabolites; temperature, 40 °C for antipyrine and testosterone metabolites, and 50 °C for doxorubicin; flow rate, 1.0 ml/min for antipyrine and testosterone metabolites, and 1.2 ml/min for doxorubicin.

2.11. Statistical analysis

Results are expressed as mean±S.E.M. Statistical differences between means were assessed by Student's *t* test or one-way analysis of variance (ANOVA). When *F* ratios were significant ($P<0.05$), Scheffe's post-hoc tests between the groups were done, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of endotoxin on histopathological findings in wild-type and TNF-$\alpha^{-/-}$ mice

Light micrographs obtained by the histopathological examinations revealed that there was no difference in the light microscopy of liver tissues between wild-type mice and TNF-$\alpha^{-/-}$ mice, indicating that the livers of both possess almost the same morphological characteristics. Endotoxin induced only a mild infiltration with no evidence of a massive necrotic or apoptotic area in either the wild-type or TNF-$\alpha^{-/-}$ mice.

3.2. Effect of endotoxin on expression of hepatic P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice

The time-dependent effects of endotoxin on the expression of P-glycoprotein in wild-type mice and TNF-$\alpha^{-/-}$ mice after intraperitoneal injection of endotoxin (10 mg/kg) are illustrated in Fig. 1. The expression of P-glycoprotein in wild-type mice significantly decreased 6 h after the endotoxin injection but returned to the control level by 24 h. The expression of P-glycoprotein did not change in TNF-$\alpha^{-/-}$ mice either at 6 or 24 h after injection with endotoxin. No significant differences in the constitutive expression of P-glycoprotein were observed between wild-type and TNF-$\alpha^{-/-}$ mice. To evaluate in vivo whether the function of P-glycoprotein is maintained

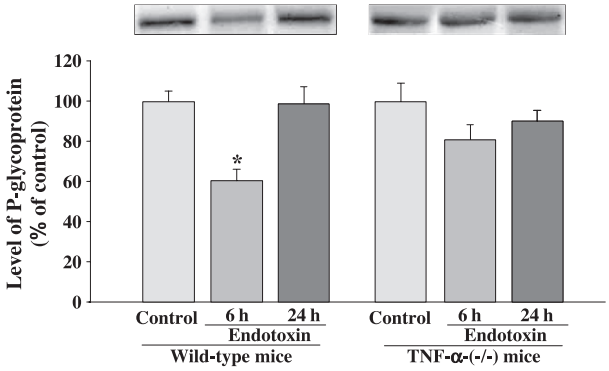

Fig. 1. Time-dependent effects of endotoxin on hepatic expression of P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control ($P<0.05$).

in endotoxin-treated TNF-α$^{-/-}$ mice, we measured the plasma concentrations of doxorubicin 3 h after injection in TNF-α$^{-/-}$ mice treated 6 h earlier with and without endotoxin. No significant differences in the plasma concentrations of doxorubicin were observed between endotoxin-treated and untreated TNF-α$^{-/-}$ mice (0.43±0.02 and 0.34±0.04 μg/ml, respectively), although endotoxin significantly increased the plasma concentrations of doxorubicin in wild-type mice. These results indicate that endogenous TNF-α is involved in the down-regulation of P-glycoprotein.

3.3. Effect of endotoxin on expression of CYP3A2 and CYP2C11 in wild-type and TNF-α$^{-/-}$ mice

The constitutive expression of hepatic CYP3A2 and CYP2C11 in wild-type and TNF-α$^{-/-}$ mice is represented in Fig. 2. The constitutive expression of CYP3A2 and CYP2C11 in TNF-α$^{-/-}$ mice showed a tendency to decline compared to that in wild-type mice, although the differences failed to reach the 5% level of statistical significance. The effects of endotoxin on the expression of hepatic CYP3A2 and CYP2C11 in wild-type and TNF-α$^{-/-}$ mice 24 h after injection of endotoxin are illustrated in Figs. 3 and 4. Endotoxin significantly reduced the expression of CYP3A2 and CYP2C11 in both wild-type and TNF-α$^{-/-}$ mice, although the degree of reduced expression in the latter was greater than in the former.

3.4. Effect of endotoxin on formation from testosterone to its metabolites in wild-type and TNF-α$^{-/-}$ mice

It is reported that the major metabolites of testosterone, 6β-hydroxytestosterone, and both 16α-hydroxytestosterone and 2α-hydroxytestosterone are indicative of CYP3A2 and CYP2C11 enzymes, respectively (Takahashi et al., 1999).

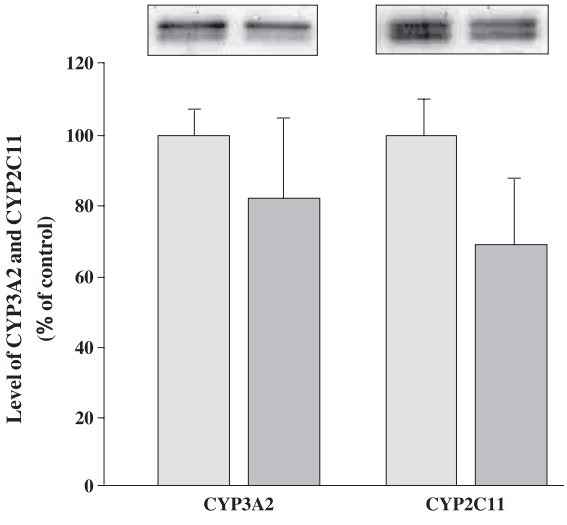

Fig. 2. Constitutive expression of CYP3A2 and CYP2C11 in liver of wild-type and TNF-α$^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6).

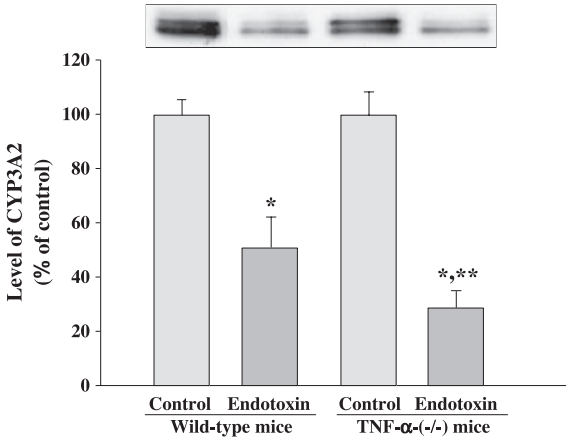

Fig. 3. Effect of endotoxin on hepatic expression of CYP3A2 in wild-type and TNF-α$^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

Therefore, we measured the formation rate of 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 2α-hydroxytestosterone in the microsomal fractions obtained from wild-type and TNF-α$^{-/-}$ mice treated 24 h earlier with or without endotoxin. Endotoxin significantly reduced the formation rate of 6β-hydroxytestosterone in both wild-type and TNF-α$^{-/-}$ mice (1.87±0.18 to 0.57±0.07 and 1.53±0.18 to 0.59±0.08 nmol/mg protein/min, respectively). Furthermore, CYP2C11 activity was assessed by its ability to catalyze testosterone to 16α-hydroxytestosterone and 2α-hydroxytestosterone. The formation rate of 16α-hydroxytestosterone and 2α-hydroxytestosterone in wild-type mice was 0.15±0.06 and 0.08±0.05 nmol/mg protein/min, respectively, while that in TNF-α$^{-/-}$ mice was 0.13±0.02 and 0.02±0.01 nmol/mg protein/min, respectively. On the other hand, endotoxin completely suppressed the activity of CYP2C11 in both wild-type and TNF-α$^{-/-}$ mice.

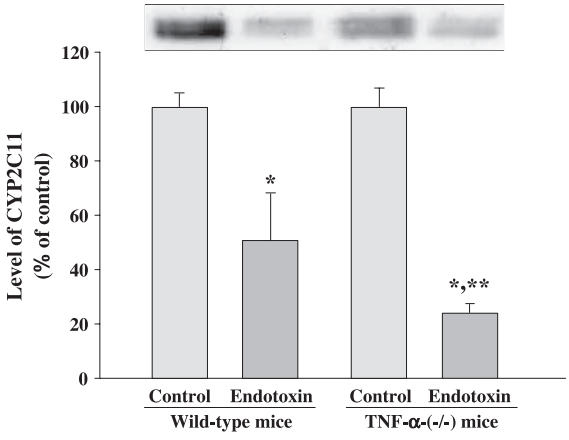

Fig. 4. Effect of endotoxin on hepatic expression of CYP2C11 in wild-type and TNF-α$^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

3.5. Effect of endotoxin on antipyrine elimination in wild-type and TNF-α$^{-/-}$ mice

Antipyrine is widely used as a tool to evaluate the capacity of drug metabolism in various pathological animal models, since it is almost completely metabolized by the hepatic CYP isozymes in rats (Balani et al., 2002; Kitaichi et al., 1999, 2004). We previously reported that the systemic clearance of antipyrine correlates well with the expression of CYP3A2 and CYP2C11 (Ueyama et al., 2004). Therefore, estimating the plasma concentrations of antipyrine at 3 h after injection in wild-type and TNF-α$^{-/-}$ mice treated 24 h earlier with endotoxin or saline, we found that the plasma concentration of antipyrine in the former was 0.23±0.01 μg/ml, and its value had been increased twofold by the injection of endotoxin (0.45±0.08 μ.g/ml). On the other hand, the concentration in TNF-α$^{-/-}$ mice was 0.40±0.02 μg/ml, and its value had been increased threefold by injection of endotoxin (1.26±0.19 μg/ml). Although no marked difference in the plasma concentration of antipyrine was observed between untreated wild-type mice and TNF-α$^{-/-}$ mice, a significant difference was observed between endotoxin-treated wild-type mice and TNF-α$^{-/-}$ mice.

3.6. Effect of endotoxin on plasma levels of nitrate/nitrite (NO_x) in wild-type and TNF-α$^{-/-}$ mice

We previously reported that plasma levels of NO_x in rats 24 h after the injection of endotoxin were significantly higher than those in untreated rats (Kitaichi et al., 1999). We then measured plasma levels of NO_x in wild-type and TNF-α$^{-/-}$ mice 24 h after the injection of endotoxin or saline. As shown in Fig. 5, no significant differences in the constitutive levels of NO_x in plasma were observed between wild-type and TNF-α$^{-/-}$ mice. Endotoxin significantly increased the plasma levels of NO_x by 15-fold and 4-fold in wild-type and TNF-α$^{-/-}$ mice, respectively. The plasma levels of NO_x in endotoxin-treated wild-type mice were 3.5-fold higher than those in endotoxin-treated TNF-α$^{-/-}$ mice.

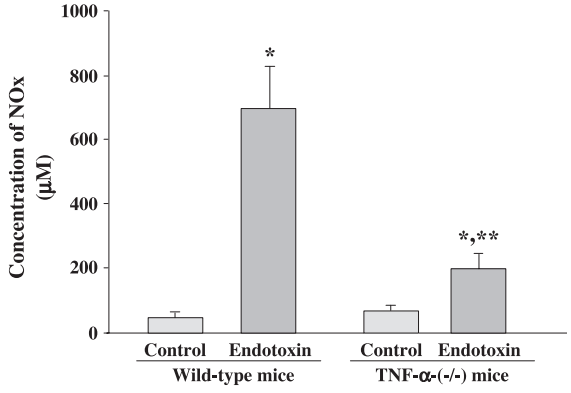

Fig. 5. Effect of endotoxin on plasma concentrations of NO_x in wild-type and TNF-α$^{-/-}$ mice. Values are shown as mean±S.E.M. (n=3). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

4. Discussion

Endotoxin is known to increase the levels of cytokines, including TNF-α, and the elevation of these cytokines might play an important role in endotoxin-induced changes in certain transporter-mediated biliary excretion systems (Hirsch-Ernst et al., 1998; Simpson et al., 1997). We previously reported that the expression of P-glycoprotein mRNA (mdr1a mRNA) in the liver of rats treated 6 h earlier with endotoxin declined, and returned to control levels after 24 h, and that pentoxifylline, which inhibits the overproduction of TNF-α, ameliorated the endotoxin-induced reduction in the P-glycoprotein-mediated hepatobiliary excretion of rhodamine 123, which is transported specifically by P-glycoprotein (Ando et al., 2001). These results suggest that TNF-α plays an important role in regulating the expression and function of P-glycoprotein. In the present study, we focused on TNF-α and selected mice with a targeted disruption of the gene encoding TNF-α (Taniguchi et al., 1997).

First, we examined the role of TNF-α in the endotoxin-induced down-regulation of hepatic P-glycoprotein. A significant down-regulation of hepatic P-glycoprotein was observed in wild-type mice at 6 h, but not at 24 h, after the injection of endotoxin. This down-regulation was in good agreement with that seen in our previous studies using rats (Ando et al., 2001), and is further supported by in vitro studies demonstrating that TNF-α reduces the expression of *mdr1* gene in human colon carcinoma cells (Walther and Stein, 1994). In addition, the present results may be supported by our previous study showing that the net biliary excretion of doxorubicin, a substrate of P-glycoprotein, was significantly reduced by the down-regulation of hepatic P-glycoprotein in rats treated with Shiga-like toxin II from *Escherichia coli* O157:H7, which induces an overproduction of TNF-α (Foster et al., 2000; Hidemura et al., 2003). In contrast, it has been reported that endotoxin has no effect on the expression of hepatic P-glycoprotein in TNF-α$^{-/-}$ mice at either 6 or 24 h after injection. We then measured plasma concentrations of doxorubicin to confirm whether the function of P-glycoprotein in TNF-α$^{-/-}$ mice treated 6 h earlier with endotoxin persists; no significant differences in the plasma concentrations of doxorubicin were observed between endotoxin-treated and untreated TNF-α$^{-/-}$ mice, a finding that was in good agreement with the results of Western blot analysis reported previously (Zhao et al., 2002). It is likely that endotoxin-treated TNF-α$^{-/-}$ mice maintain the normal function of P-glycoprotein. Consequently, these results strongly suggest that endogenous TNF-α plays a crucial role in the down-regulation of hepatic P-glycoprotein by endotoxin. However, whether only TNF-α alone is involved in the down-regulation of P-glycoprotein remains to be established.

It has been reported that TNF-α, which is implicated as an important mediator of the pathophysiological effects of

endotoxin, has been shown to reduce total CYP as well as CYP subfamilies such as CYP1A, CYP3A, and CYP2E (Monshouwer et al., 1996; Sewer and Morgan, 1997). However, the precise roles of these cytokines in the expression of CYP isoforms remain unclear. Among CYP isoforms, CYP3A2 and CYP2C11 were selected for this study, since they are major CYP isoforms in rats (Souček and Gut, 1992), are enzymes metabolizing antipyrine (Engel et al., 1996), and are sensitive to TNF-α (Morgan, 1997). Secondly, we examined the role of TNF-α in the endotoxin-induced down-regulation of these two CYP isoforms. Endotoxin significantly reduced the protein levels of CYP3A2 and CYP2C11 in livers of both wild-type mice and TNF-$\alpha^{-/-}$ mice, with the degree of down-regulation being more marked in the latter than in former. These results suggest the possibility that TNF-α may play a protective role in the down-regulation of hepatic CYP3A2 and CYP2C11 by endotoxin. We assume that the reduced activity of both CYP3A2 and CYP2C11 by endotoxin is, at least, caused by TNF-α-independent mediators besides TNF-α. Most recently, we reported that antipyrine clearance obviously reflects the activity of hepatic CYP3A2 and CYP2C11 (Ueyama et al., 2004). We then measured the plasma concentrations of antipyrine 3 h after intravenous injection in wild-type and TNF-$\alpha^{-/-}$ mice treated with or without endotoxin. The endotoxin-induced delay of CYP-mediated antipyrine metabolism was more pronounced in TNF-$\alpha^{-/-}$ than in wild-type mice, a finding that was in agreement with the results of Western blot analysis.

NO is one of the important inflammatory mediators regulating the contents and activities of CYP isoforms (Gergel et al., 1997; Khatsenko and Kikkawa, 1997; Kitaichi et al., 1999; Minamiyama et al., 1997; Morgan, 1997; Wink et al., 1993). NO is synthesized by both continuously expressed endothelial NO synthase and inducible NO synthase. It is well known that inducible NO plays an important role in the elevation of plasma NO_x by endotoxin. Finally, in the present study, we measured plasma concentrations of NO_x in wild-type and TNF-$\alpha^{-/-}$ mice 24 h after the injection of endotoxin or saline, and found that the plasma NO_x elevated by endotoxin was approximately fourfold higher in wild-type than TNF-$\alpha^{-/-}$ mice, although endotoxin significantly elevated the plasma NO_x in both types. There is evidence that inflammatory cytokines, including TNF-α, are important inducers of NO generation in macrophages and other cells (Kolios et al., 1995; Saito and Nakano, 1996). Considering that the slight elevation in plasma NO_x in TNF-$\alpha^{-/-}$ mice was induced by NO derived from endothelial NO synthase, inducible NO synthase, and other mediators besides TNF-α, it is likely that the contribution of TNF-α-mediated NO to the elevation of plasma NO_x is a significant one. Based on result from the present study, it is unlikely that the down-regulation of CYP3A2 and CYP2C11 by endotoxin is due to the overproduction of NO in plasma. This speculation is supported by a report, which demonstrates that the inactivation of hepatic CYP observed 24 h after the injection of endotoxin is not induced by NO (Takemura et al., 1999). However, results from the present study cannot provide irrefutable evidence that NO is not involved in the endotoxin-induced suppression of the expression of CYP isoforms.

In conclusion, our results show for the first time that TNF-α is a key mediator in the down-regulation of hepatic P-glycoprotein by endotoxin, but not in the down-regulation of hepatic CYP3A2 and CYP2C11. However, the role of TNF-α is still unclear, although it appears to play a protective role in regulating the hepatic expression of CYP3A2 and CYP2C11 in endotoxemic mice.

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References

Ando, H., Nishio, Y., Ito, K., Nakao, A., Wang, L., Zhao, Y.L., Kitaichi, K., Takagi, K., Hasegawa, T., 2001. Effect of endotoxin on P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 in rats. Antimicrob. Agents Chemother. 45, 3462–3467.

Balani, S.K., Zhu, T., Yang, J., Liu, Z., He, B., Lee, F.W., 2002. Effective dosing regimen of 1-aminobenzotriazole for inhibition of antipyrine clearance in rats, dogs, and monkeys. Drug Metab. Dispos. 30, 1059–1062.

Bredt, D.S., Snyder, S.H., 1994. Nitric oxide: a physiologic messenger molecule. Annu. Rev. Biochem. 63, 175–195.

Chapekar, M.S., Huggett, A.C., Cheng, C., 1991. Dexamethasone prevents the growth inhibitory effects of recombinant tumor necrosis factor in a rat hepatoma cell line Reuber-RC-3: an association with the changes in the messenger RNA levels for multidrug resistance gene. Biochem. Biophys. Res. Commun. 181, 1524–1531.

Chen, Y.L., Florentin, I., Batt, A.M., Ferrari, L., Giroud, J.P., Chauvelot-Moachon, L., 1992. Effects of interleukin-6 on cytochrome *P*450-dependent mixed-function oxidases in the rat. Biochem. Pharmacol. 44, 137–148.

Cordon-Cardo, C., O'Brien, J.P., Casals, D., Rittman-Grauer, L., Biedler, J.L., Melamed, M.R., Bertino, J.R., 1989. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood–brain barrier sites. Proc. Natl. Acad. Sci. U. S. A. 86, 695–698.

Engel, G., Hofmann, U., Heidemann, H., Cosme, J., Eichelbaum, M., 1996. Antipyrine as a probe for human oxidative drug metabolism: identification of the cytochrome *P*450 enzymes catalyzing 4-hydroxyantipyrine, 3-hydroxymethylantipyrine, and norantipyrine formation. Clin. Pharmacol. Ther. 59, 613–623.

Foster, G.H., Armstrong, C.S., Sakiri, R., Tesh, V.L., 2000. Shiga toxin-induced tumor necrosis factor alpha expression: requirement for toxin enzymatic activity and monocytes protein kinase C and protein tyrosine kinases. Infect. Immun. 68, 5183–5189.

Gergel, D., Misik, V., Riesz, P., Cederbaum, A.I., 1997. Inhibition of rat and human cytochrome *P*4502E1 catalytic activity and reactive oxygen radical formation by nitric oxide. Arch. Biochem. Biophys. 337, 239–250.

Green, R.M., Beier, D., Gollan, J.L., 1996. Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. Gastroenterology 111, 193–198.

Haghgoo, S., Hasegawa, T., Nadai, M., Wang, L., Nabeshima, T., Kato, N., 1995. Effects of a bacterial lipopolysaccharide on biliary excretion of a β-lactam antibiotic, cefoperazone, in rats. Antimicrob. Agents Chemother. 39, 2258–2261.

Hidemura, K., Zhao, Y.L., Ito, K., Nakao, A., Kanazawa, H., Takagi, K., Ohta, M., Hasegawa, T., 2003. Shiga-like toxin II impairs hepatobiliary transport of doxorubicin in rats by down-regulation of hepatic P glycoprotein and multidrug resistance-associated protein Mrp2. Antimicrob. Agents Chemother. 47, 1636–1642.

Hirsch-Ernst, K.I., Ziemann, C., Foth, H., Kozian, D., Schmitz-Salue, C., Kahl, G.F., 1998. Induction of mdr1b mRNA and P-glycoprotein expression by tumor necrosis factor alpha in primary rat hepatocyte cultures. J. Cell. Physiol. 176, 506–515.

Khatsenko, O., Kikkawa, Y., 1997. Nitric oxide differentially affects constitutive cytochrome *P*450 isoforms in rat liver. J. Pharmacol. Exp. Ther. 280, 1463–1470.

Khatsenko, O.G., Gross, S.S., Rifkind, A.B., Vane, J.R., 1993. Nitric oxide is a mediator of the decrease in cytochrome *P*450-dependent metabolism caused by immunostimulants. Proc. Natl. Acad. Sci. U. S. A. 90, 11147–11151.

Kitaichi, K., Wang, L., Takagi, K., Iwase, M., Shibata, E., Nadai, M., Takagi, K., Hasegawa, T., 1999. Decreased antipyrine clearance following endotoxin administration: in vivo evidence of the role of nitric oxide. Antimicrob. Agents Chemother. 43, 2697–2701.

Kitaichi, K., Nakayama, H., Ueyama, J., Nadai, M., Baba, K., Takagi, K., Takagi, K., Ohta, M., Hasegawa, T., 2004. Down-regulation of cytochrome *P*450 proteins and its activities by Shiga-like toxin II from *Escherichia coli* O157:H7. Biochem. Pharmacol. 67, 1427–1435.

Kolios, G., Brown, Z., Robson, R.L., Robertson, D.A., Westwick, J., 1995. Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. Br. J. Pharmacol. 116, 2866–2872.

Manuel, P., 1996. The CYP3A family. In: Ioannides, C. (Ed.), Cytochrome *P*450: Metabolic and Toxicological Aspects, Chapter 10. Stern, London, UK, pp. 241–270.

Mayer, R., Kartenbeck, J., Buchler, M., Jedlitschky, G., Leier, I., Kuppler, D., 1995. Expression of the *MRP* gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. J. Cell Biol. 131, 137–150.

Minamiyama, Y., Takemura, S., Imaoka, S., Funae, Y., Tanimoto, Y., Inoue, M., 1997. Irreversible inhibition of cytochrome *P*450 by nitric oxide. J. Pharmacol. Exp. Ther. 283, 1479–1485.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Monshouwer, M., McLellan, R.A., Delaporte, E., Witkamp, R.F., van Miert, A.S., Renton, K.W., 1996. Differential effect of pentoxifylline on lipopolysaccharide-induced downregulation of cytochrome *P*450. Biochem. Pharmacol. 52, 1195–1200.

Morgan, E.T., 1997. Regulation of cytochromes *P*450 during inflammation and infection. Drug Metab. Rev. 29, 1129–1188.

Nadai, M., Matsuda, I., Wang, L., Itoh, A., Naruhashi, K., Nabeshima, T., Asai, M., Hasegawa, T., 1998. Granulocyte colony-stimulating factor enhances endotoxin-induced decrease in biliary excretion of the antibiotic cefoperazone in rats. Antimicrob. Agents Chemother. 42, 2178–2183.

Nakamura, J., Nishida, T., Hayashi, K., Kawada, N., Ueshima, S., Sugiyama, Y., Ito, T., Sobue, K., Matsuda, H., 1999. Kupffer cell-mediated down regulation of rat hepatic *CMOAT/MRP2* gene expression. Biochem. Biophys. Res. Commun. 255, 143–149.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem. 239, 2370–2378.

Oude Elferink, R.P., Meijer, D.K., Kuipers, F., Jansen, P.L., Groen, A.K., Groothuis, G.M., 1995. Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. Biochim. Biophys. Acta 1241, 215–268.

Saito, S., Nakano, M., 1996. Nitric oxide production by peritoneal macrophages of *Mycobacterium bovis* BCG-infected or non-infected mice: regulatory role of T lymphocytes and cytokines. J. Leukoc. Biol. 59, 908–915.

Schinkel, A.H., Wagenaar, E., Mol, C.A., van Deemter, L., 1996. P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J. Clin. Invest. 97, 2517–2524.

Sewer, M.B., Morgan, E.T., 1997. Nitric oxide-independent suppression of *P*450 2C11 expression by interleukin-1beta and endotoxin in primary rat hepatocytes. Biochem. Pharmacol. 54, 729–737.

Sewer, M.B., Morgan, E.T., 1998. Down-regulation of the expression of three major rat liver cytochrome *P*450s by endotoxin in vivo occurs independently of nitric oxide production. J. Pharmacol. Exp. Ther. 287, 352–358.

Shedlofsky, S.I., Israel, B.C., McClain, C.J., Hill, D.B., Blouin, R.A., 1994. Endotoxin administration to humans inhibits hepatic cytochrome *P*450-mediated drug metabolism. J. Clin. Invest. 94, 2209–2214.

Simpson, A.E., Tomkins, P.T., Cooper, K.L., 1997. An investigation of the temporal induction of cytokine mRNAs in LPS-challenged thioglycollate-elicited murine peritoneal macrophages using the reverse transcription polymerase chain reaction. Inflamm. Res. 46, 65–71.

Souček, P., Gut, I., 1992. Cytochromes *P*-450 in rats: structures, functions, properties and relevant human forms. Xenobiotica 22, 83–103.

Stein, U., Walther, W., Laurencot, C.M., Scheffer, G.L., Scheper, R.J., Shoemaker, R.H., 1997. Tumor necrosis factor-alpha and expression of the multidrug resistance-associated genes LRP and MRP. J. Natl. Cancer Inst. 89, 807–813.

Sukhai, M., Yong, A., Kalitsky, J., Piquette-Miller, M., 2000. Inflammation and interleukin-6 mediate reductions in the hepatic expression and transcription of the *mdr1a* and *mdr1b* genes. Mol. Cell. Biol. Res. Commun. 4, 248–256.

Takahashi, J., Furuhata, Y., Ikeda, A., Takahashi, M., Iwata, H., Kazusaka, A., Fujita, S., 1999. Characterization of hepatic cytochrome *P*450 isozyme composition in the transgenic rat expressing low level human growth hormone. Xenobiotica 12, 1203–1212.

Takemura, S., Minamiyama, Y., Imaoka, S., Fumie, Y., Hirohashi, K., Inoue, M., Kinoshita, H., 1999. Hepatic cytochrome *P*450 is directly inactivated by nitric oxide, not by inflammatory cytokines, in the early phase of endotoxemia. J. Hepatol. 30, 1035–1044.

Taniguchi, T., Takata, M., Ikeda, A., Momotani, E., Sekikawa, K., 1997. Failure of germinal center formation and impairment of response to endotoxin in tumor necrosis factor alpha-deficient mice. Lab. Invest. 77, 647–658.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1987. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc. Natl. Acad. Sci. U. S. A. 84, 7735–7738.

Trauner, M., Arrese, M., Soroka, C.J., Ananthanarayanan, M., Koeppel, T.A., Schlosser, S.F., Suchy, F.J., Keppler, D., Boyer, J.L., 1997. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. Gastroenterology 113, 255–264.

Ueyama, J., Kitaichi, K., Nadai, M., Iwase, M., Tomyo, N., Kanazawa, H., Suzuki, R., Takagi, K., Takagi, K., Hasegawa, T., 2004. Effect of pioglitazone on endotoxin-induced decreases in hepatic drug-metabolizing enzyme activity and expression of CYP3A2 and CYP2C11. Eur. J. Pharmacol. 498, 257–265.

Wacher, V.J., Wu, C.-Y., Benet, L.Z., 1995. Overlapping substrate specificities and tissue distribution of cytochrome *P*450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. Mol. Carcinog. 13, 129–134.

Walther, W., Stein, U., 1994. Influence of cytokines on *mdr1* expression in human colon carcinoma cell lines: increased cytotoxicity of MDR relevant drugs. J. Cancer Res. Clin. Oncol. 120, 471–478.

Warren, G.W., Poloyac, S.M., Gary, D.S., Mattson, M.P., Blouin, R.A., 1999. Hepatic cytochrome *P*-450 expression in tumor necrosis factor-

alpha receptor (p55/p75) knockout mice after endotoxin administration. J. Pharmacol. Exp. Ther. 288, 945–950.

Wink, D.A., Osawa, Y., Darbyshire, J.F., Jones, C.R., Eshenaur, S.C., Nims, R.W., 1993. Inhibition of cytochrome *P*450 by nitric oxide and a nitric oxide-releasing agent. Arch. Biochem. Biophys. 300, 115–123.

Zhao, Y.L., Du, J., Kanazawa, H., Sugawara, K., Takagi, K., Kitaichi, K., Tatsumi, Y., Takagi, K., Hasegawa, T., 2002. Effect of endotoxin on doxorubicin transport across blood–brain barrier and P-glycoprotein function in mice. Eur. J. Pharmacol. 445, 115–123.