Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine in Rats

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ABSTRACT: **Methamphetamine, a psychostimulant drug, produces both acute psychomotor stimulation and long-lasting behavioral effects including addiction and psychosis. To identify anatomical substrates for the discriminative stimulus effects of methamphetamine in rats, we examined the drug discrimination-associated c-Fos expression in the brains of rats that were trained to discriminate methamphetamine from saline under a two-lever fixed ratio (FR-20) schedule of food reinforcement. c-Fos expression in the brains of rats trained to discriminate methamphetamine from saline was significantly increased in the nucleus accumbens (NAc) and the ventral tegmental area (VTA) as compared with the expression in the control rats that were maintained under the FR-20 schedule, but no alternation was observed in other areas including the cerebral cortex, caudate putamen, substantia nigra, hippocampus, amygdala, and habenulla. Methamphetamine treatment in the trained rats caused a significant increase in c-Fos expression in the VTA, and a decrease in the NAc core, as compared to saline treatment. However, c-Fos expression in the NAc and VTA of rats that received chronic intermittent methamphetamine administration without discrimination training, did not differ from the expression in saline-treatment animals. These results suggest that the VTA and the NAc play an important role in the discriminative stimulus effects of methamphetamine.**

KEYWORDS: **drug dependence; drug discrimination; c-Fos; methamphetamine; nucleus accumbens; ventral tegmental area**

INTRODUCTION

Methamphetamine (MAP), an addictive drug, produces various behavioral effects that are mainly mediated by the dopaminergic (DA) neuronal system.[1] It has been known that the discriminative stimulus effects of psychostimulants in experi-

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mental animals are related to their subjective effects in humans.[2] Therefore, the drug discrimination procedures have been proven to be a valuable means for elucidating the mechanism of action underlying the unique properties of addictive drugs.[1,3]

Quantification of the changes in the expression of the immediate early gene *c-fos* has been proven to be a very useful method by which the distribution of neurons that are activated by physiological or pharmacological stimuli may be mapped.[4] Several studies have shown that acute methamphetamine administration dose-dependently produces c-Fos-like immunoreactivity in wide areas of the brains including the nucleus accumbens and striatum,[5] and that chronic methamphetamine or amphetamine administration abolishes the inducibility of *c-fos* in the striatum.[6,7] In the present study, to identify potential anatomical substrates of the discriminative stimulus effects of methamphetamine in rats, we examined the c-Fos expression in the brain of rats trained to discriminate methamphetamine from saline.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (7 weeks old, Charles River Japan, Yokohama), weighing 230 ± 10 g at the beginning of experiment, were used in this study. They were under controlled laboratory conditions (12-h light/dark cycle with lights on at 9:00 h, 23 ± 0.5°C, 50 ± 0.5% humidity). Their body weights were gradually reduced to approximately 80% of the free-feeding weight by limiting daily access to food. Water was available *ad libitum*. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Methamphetamine Discrimination Procedure

Methamphetamine discrimination procedures were conducted as described in detail by Mori *et al.*[3] Rats were injected 10 min before the session with either saline or methamphetamine (0.5 mg/kg, s.c.). Discrimination training sessions were conducted 5 days per week under a double-alternation schedule (i.e., MMSSMMSS, etc., where M is methamphetamine; S, saline).

c-Fos Immunohistochemistry

A total of 44 of rats were prepared. Four groups were prepared in order to investigate the neural circuitry underlying the discriminative stimulus effects of methamphetamine: (1) naive rats that were subjected to neither food restriction nor drug discrimination training, (2) control rats that were maintained on the FR-20 schedule of food reinforcement without drug discrimination training, and (3) saline- and (4) methamphetamine-injected trained rats that had met the criteria for learning methamphetamine discrimination. Control rats were subjected to the FR-20 schedule of food reinforcement, while saline- and methamphetamine-injected rats were subjected to the test session of methamphetamine discrimination. Accordingly, the three groups of animals except naive rats obtained the same number (20 pellets) of food reinforcement by almost equal numbers of lever pressing. The saline- and methamphetamine-injected rats had the same drug history during the drug discrim-

ination training sessions, but received different drug treatment (methamphetamine vs. saline) on the test day. Rats were killed 2 h after the drug discrimination test for c-Fos immunohistochemistry. An additional four groups of rats were prepared in order to examine the effects of acute and chronic intermittent methamphetamine treatment on c-Fos expression—two groups of rats received chronic intermittent methamphetamine treatment at a dose of 0.5 mg/kg under a double-alternation schedule (i.e., MMSSMMSS etc., where M is methamphetamine; S, saline) without discrimination training. On the final day, half of these rats were challenged by methamphetamine 0.5 mg/kg or saline, respectively, and killed 2 h after the challenge. Another two groups of rats were injected with saline daily to examine the acute effects of methamphetamine. On the final day, half of the rats were injected with methamphetamine 0.5 mg/kg, while the others were treated with saline and killed 2 h after the treatment. Three separate groups of rats were treated with single saline or methamphetamine (0.5 mg/kg or 2 mg/kg, s.c.) to examine the dose-dependent effects of acute methamphetamine treatment on c-Fos expression, and killed 2 h after the treatment.

The c-Fos immunohistochemical procedure was the same as previously described methods.[8] Quantitative analysis of c-Fos immunohistochemistry was conducted by a computer-assisted image analysis system (C. Imaging Systems; Compix Inc., Mars, PA) as described previously.[9]

RESULTS

c-Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine

c-Fos expression were observed in all examined areas of the brain. Significant differences in c-Fos expression among the four groups of rats were observed in four brain areas including the cingulate cortex, the core and shell of NAc, and VTA (TABLE 1). There was a marked difference in c-Fos expression between control and trained groups in the core and shell of the NAc, and the VTA. Moreover, the number of c-Fos–positive cells was significantly smaller in the NAc core of methamphetamine-injected trained rats than in that of saline-injected trained rats, whereas it was increased in the VTA of methamphetamine-injected trained rats compared with saline-injected trained rats. No alteration in c-Fos expression was observed in other brain areas examined.

Effects of Acute and Chronic Intermittent Methamphetamine Treatment on c-Fos Expression

No significant alteration of c-Fos expression was observed in rats treated with chronic intermittent methamphetamine (0.5 mg/kg), whereas the number of c-Fos–positive cells in the NAc core of acute methamphetamine-treated rats was significantly higher than that of saline-treated rats.

Single administration with methamphetamine (0.5 and 2 mg/kg) produced a dose-dependent and significant increase in the number of c-Fos–positive cells in the NAc core, while methamphetamine 2 mg/kg, not 0.5 mg/kg, produced a significant increase in the NAc shell and the VTA.

TABLE 1. c-Fos expression in the brain subregions of rats trained for methamphetamine discrimination

Brain area	Naive ($n = 3$)	Control ($n = 4$)	Saline ($n = 4$)	Methamphetamine ($n = 4$)
Cerebral cortex				
Cingulate	72.3 ± 2.8*	102.4 ± 5.9	110.0 ± 3.4	115.7 ± 6.9
Motor	54.1 ± 3.2	66.7 ± 4.5	68.3 ± 3.4	68.0 ± 1.9
Somotosensory	38.8 ± 3.5	48.7 ± 1.8	51.4 ± 1.2	49.2 ± 2.7
Nucleus accumbens				
Core	36.8 ± 1.2	50.6 ± 4.3	21.9 ± 10.9***	90.3 ± 6.7*,#
Shell	30.6 ± 3.0	33.6 ± 5.8	86.0 ± 7.9***	71.2 ± 4.3**
Ventral tegmental area	16.7 ± 1.3	21.0 ± 4.7	57.4 ± 7.1**	90.4 ± 5.9***,##

Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were subjected to the drug discrimination test after either saline or methamphetamine (0.5 mg/kg) treatment. Control rats were maintained under the FR-20 schedule of food reinforcement without drug discrimination training. Naive rats were subjected to neither food restriction nor the methamphetamine discrimination training. c-Fos expression in each area is indicated as the number of c-Fos–positive cells per mm^2. Each value represents the mean ± SE. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus control. $^{\#}P < .05$, $^{\#\#}P < .01$ versus saline.

DISCUSSION

It is well known that DA plays a major role in the discriminative stimulus effects of methamphetamine.[1] In the discrimination test in rats, DA uptake inhibitors and D1 or D2 receptor agonists substituted for methamphetamine, whereas their antagonists completely blocked the discriminative stimulus effects.[1] In the present study, we demonstrated immunohistochemically that development of the ability to discriminate methamphetamine from saline in rats is associated with increases in c-Fos expression only in the VTA and NAc. This activation is unlikely due to lever-press behavior or food reinforcement because c-Fos expression in these brain areas did not increase in the control group maintained on the FR-20 schedule of food reinforcement (TABLE 1). Further, it is also unlikely that the changes are due to chronic intermittent methamphetamine treatment because such treatment without discrimination training did not increase c-Fos expression in the VTA and NAc.

Dopaminergic projections from the VTA to the NAc are involved in investigatory behavior evoked by novel stimuli and the reinforcement of adaptive investigatory approaches evoked by naturally occurring rewards and by addictive drugs.[10–12] It has also been suggested that the NAc core is more important than the NAc shell in response-reinforcement learning[13] and in behavioral response to motivationally significant stiumuli in general.[14,15] Accordingly, our findings suggest that the development of methamphetamine discrimination is associated with a selective activation of the VTA-NAc, probably the dopaminergic neuronal system. Compared to the shell region, c-Fos expression in the NAc core is more extensive, indicating that the

core region of the NAc is more important for the discriminative stimulus effects of methamphetamine.

Interestingly, both saline and methamphetamine treatment in rats that fulfilled the criteria of the discrimination led to an increase in c-Fos expression in the VTA and the core and shell of the NAc. Therefore, it is suggested that once the animals acquired the ability to discriminate methamphetamine from saline, these brain areas were selectively activated even after saline challenge. The mechanism underlying this activation is unclear, but it will provide an insight into the mechanisms for the discriminative stimulus effects of methamphetamine.

In conclusion, our findings suggest that the VTA and the NAc as possible neuronal substrates play a role in the discriminative stimulus effects of methamphetamine. To prove the hypothesis, further related experiments will be necessary.

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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

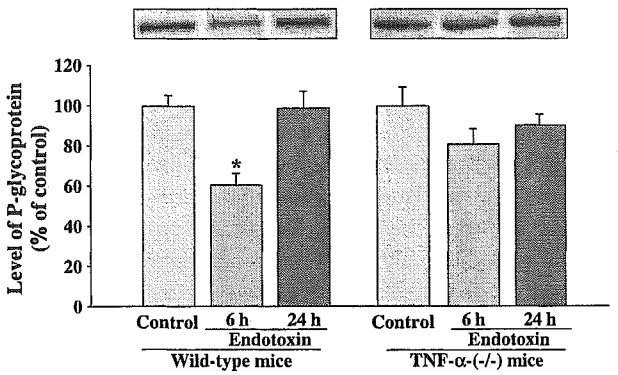

Fig. 1. Time-dependent effects of endotoxin on hepatic expression of P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control ($P<0.05$).

in endotoxin-treated TNF-$\alpha^{-/-}$ mice, we measured the plasma concentrations of doxorubicin 3 h after injection in TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ mice

The constitutive expression of hepatic CYP3A2 and CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice is represented in Fig. 2. The constitutive expression of CYP3A2 and CYP2C11 in TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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).

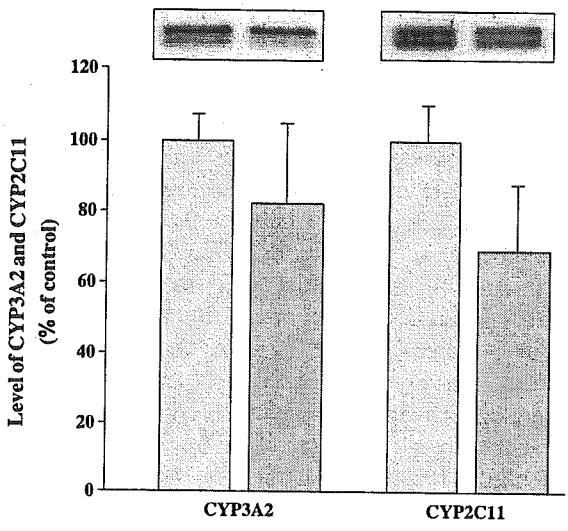

Fig. 2. Constitutive expression of CYP3A2 and CYP2C11 in liver of wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6).

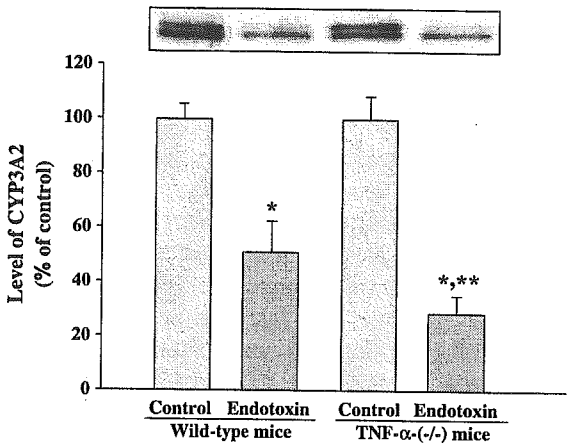

Fig. 3. Effect of endotoxin on hepatic expression of CYP3A2 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type 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-$\alpha^{-/-}$ mice treated 24 h earlier with or without endotoxin. Endotoxin significantly reduced the formation rate of 6β-hydroxytestosterone in both wild-type and TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ mice.

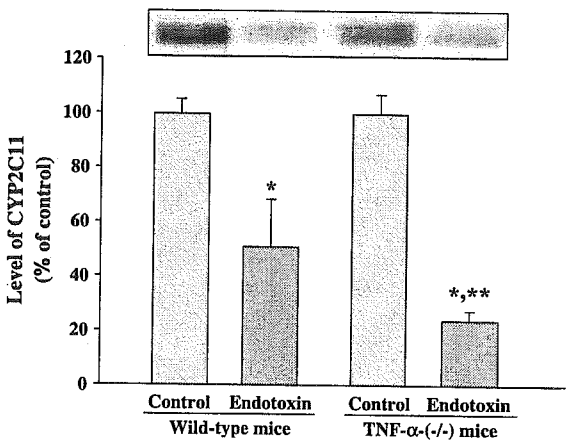

Fig. 4. Effect of endotoxin on hepatic expression of CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type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

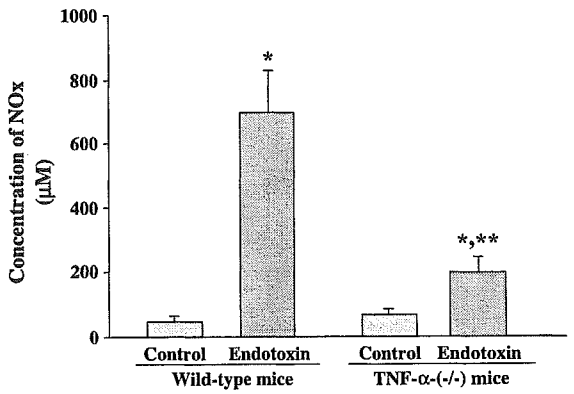

Fig. 5. Effect of endotoxin on plasma concentrations of NO_x in wild-type and TNF-α$^{-/-}$ mice. Values are shown as mean±S.E.M. (n=3). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

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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Inflammation-induced GDNF improves locomotor function after spinal cord injury

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Activation of microglia/macrophages after injury occurs limitedly in the CNS, which finding may explain unsuccessful axonal regeneration. Therefore, the relationship between lipopolysaccharide (LPS)-induced inflammation and recovery of locomotor function of rats after spinal cord injury was examined. High-dose LPS improved locomotor function greater than low-dose LPS, being consistent with the expression of neurotrophic factor (GDNF) in microglia/macrophages. Experiments using GDNF gene mutant mice confirmed that the increase in the GDNF mRNA level, rather than the reduction in the mRNA level of inducible NO synthase, could be correlated with the restoration activity of locomotor function. These results suggest that a higher degree of inflammation leads to a higher degree of repair of CNS injuries through GDNF produced by activated microglia/macrophages. *NeuroReport* 16:99–102 © 2005 Lippincott Williams & Wilkins.

Key words: Glial cell line-derived neurotrophic factor (GDNF); Inducible NO synthase (iNOS); Lipopolysaccharide (LPS); Inflammation; Microglia/macrophages; Spinal cord injury

INTRODUCTION

Axonal regeneration fails to succeed in the CNS, unlike in the peripheral nervous system (PNS). This failure is thought to result from molecules inhibitory toward axonal growth [1], from the lack of neurotrophic factors [2], and/or from inflammatory reactions [3]. Inflammation is a response that occurs after CNS injury, and it causes secondary injury and facilitates neuronal dysfunction. This inflammatory response is characterized by the invasion of leucocytes into the injury site and the activation of resident glial cells, the activities of which are regulated by chemokines [4,5]. Microglia/macrophages infiltrating the injury site are thought to be involved in the secondary injury through the production of nitric oxide (NO), reactive oxygen species, and certain cytokines [6–8]. However, the activation of microglia/macrophages occurs rather more limitedly in the CNS than in the PNS [9,10]; and thus the facilitating effects of these cells on nerve regeneration and/or the inhibitory effects on secondary injury would be less in the CNS. Inflammation is also thought to contribute to the supply of neurotrophic factors: glial cell line-derived neurotrophic factor (GDNF), a neurotrophic factor for motoneurons [11,12], is expressed in activated microglia/macrophages of the injured CNS [2,13,14]. These facts suggest that the quality of activation determines if microglia/macrophages produce molecules harmful or trophic for neurons. Optimal inflammation induced by properly-activated microglia/macrophages may thus favor CNS nerve regeneration.

In the current study, we found that high-dose lipopolysaccharide (LPS) improved locomotor function to a greater degree than low-dose LPS in rats with spinal cord injury. This improvement was correlated with GDNF mRNA level in the activated microglia/macrophages, which was confirmed by experiments using GDNF gene mutant mice. Our data thus suggest that a higher degree of inflammation results in a higher amelioration of spinal cord injury through facilitated production of GDNF.

MATERIALS AND METHODS

Surgery and evaluation of locomotor function: Male Wistar rats (7–8 weeks old) or GDNF gene-deficient mice were cared for according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. Animals were anesthetized with pentobarbital (30 mg/kg), and the left side of the spinal cord was hemitransected at the level of the 9th thoracic vertebra. Then, LPS (1, 10 or 100 μg) in 10 μl phosphate-buffered saline (PBS) for rats or 2 μg LPS in 2 μl PBS for mice was injected into the injury site. Locomotor function of the left hind limb was evaluated according to the BBB locomotor rating scale [15]. For the reverse transcription-polymerase chain reaction (RT-PCR) experiment, the spinal cords were dissected out; and the segments just rostral or caudal to the injury site (5 mm length each) were collected. The generation of GDNF gene-deficient mice was described elsewhere [16].

Reverse transcription-polymerase chain reaction: RT-PCR was performed as described [17]. The specific primers used for GDNF, inducible NO synthase (iNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: up-primer 5′-GAGAGGAATCGGCAGGCTGCAGCTG-3′ and down-primer 5′-CAGATACATCCACATCGTTTAGCGG-3′ for GDNF (product size: 337 bases); up-primer 5′-CAGAGGACCCAGAGACAAGC-3′ and down-primer 5′-ACTGGGTGAACTCCAAGGTG-3′ for iNOS (product size: 488 bases); and up-primer 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and down-primer 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ for GAPDH (product size: 309 bases). The GAPDH gene was used as an internal control.

Immunohistochemical study: Immunohistochemical detection of GDNF and CD11b was performed as described earlier [17].

RESULTS

Effects of LPS on locomotor function: Recovery of locomotor score was significantly facilitated by administration of 10 or 100 μg LPS into the injury site of the rat spinal cord (Fig. 1a). Administration of 10 μg LPS effectively promoted the recovery of locomotor function on and after day 10 (Fig. 1b). These results suggest that the sufficiently activated microglia/macrophages play a stimulatory role in nerve regeneration after spinal cord injury.

Induction of GDNF or iNOS mRNA in the spinal cord: Earlier we observed that GDNF synthesis is upregulated in cultured macrophages by LPS [17]. Therefore, after LPS administration we examined the time-dependent change in mRNA expression of GDNF and of iNOS, a key enzyme for the generation of the neurotoxin NO, in the segment next to the transaction site (Fig. 2a). The expression of GDNF mRNA was significantly enhanced at day 0.5 or 1 and returned to the control level by day 3 after the LPS injection. An increase in the iNOS mRNA was found only at day 0.5 after the injury. Namely, the LPS administration caused an increase in mRNA expression that lasted much longer for GDNF than for iNOS, suggesting that the neurotrophic effects of GDNF would exceed the neurotoxic ones of iNOS. We propose that these conditions would favor recovery of locomotor function.

Cells expressing LPS-induced GDNF-immunoreactivity: Most of the cells bearing CD11b (a specific protein of microglia/macrophages) were positive for GDNF-immunoreactivity (ir) irrespective of LPS stimulation (Fig. 2b). As the difference between vehicle- and LPS-treated expression of GDNF or iNOS mRNA was greatest 12 h after the spinal cord injury, as was shown in Fig. 2a, we evaluated the effect of LPS on the cell number at that time. The cell number was counted in serial sagittal sections prepared from the rostral and caudal areas (500 μm each) adjacent to the injury site. The number of CD11b-positive cells was 290.3±23.8 (vehicle, n=4) and 376.8±24.4 (LPS, n=4), and the difference was significant (Student's t-test, $p<0.05$). The number of GDNF-positive cells was 278.3±20.1 (vehicle, n=4) and 360.0±19.9 (LPS, n=4), and this difference was also significant (Student's t-test, $p<0.05$, n=4). Cells expressing CD11b antigen or GDNF-ir were thus significantly increased in number after LPS administration.

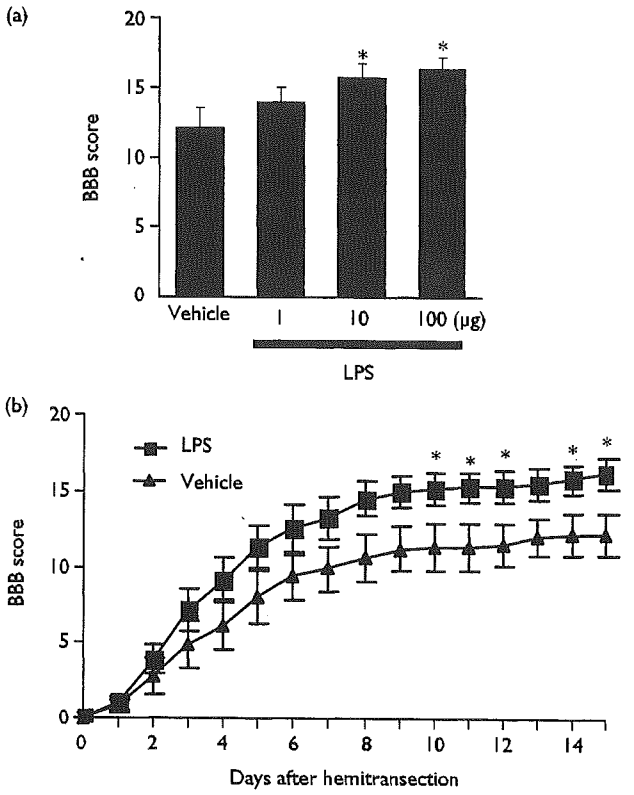

Fig. 1. Effect of LPS on locomotor function of rats with spinal cord injury. The left hemisphere of the spinal cord was transected, and a single injection (10 μl) of 1, 10 or 100 μg LPS or vehicle (PBS) was then administered to the injury site. **(a)** Locomotor function of the hind limb on the injured side was evaluated 14 days after the injury. The values are expressed as the means±s.e. (n=4–9). Significant differences from the value of the vehicle group were determined using ANOVA with Newman-Keuls *post hoc* test. *$p<0.05$. **(b)** Locomotor function of the hind limb was evaluated daily after a single injection of LPS (10 μg; squares) or vehicle (triangles). The values are expressed as means±s.e. (n=6 or 9). Significant differences from the value of the vehicle group on the same days were determined using Student's t-test. *$p<0.05$.

Effects of LPS on locomotor function of GDNF gene-deficient mice: Homozygous mice (−/−) could not be used because they died immediately after birth [16]. LPS facilitated the recovery of locomotor function of the +/+ mice, but showed no effects on that of +/− mice. The +/− mice recovered at a much slower rate than the +/+ ones irrespective of LPS administration (Fig. 3a), suggesting the GDNF expression level to be critical for the recovery rate and the degree of locomotor function.

The GDNF mRNA level in the intact spinal cord of the +/− mice was about half of that of the +/+ animals. Spinal cord injury upregulated GDNF mRNA expression around the injury site, and LPS injection into the injury site facilitated GDNF mRNA expression much more than vehicle administration in both genetic types of mice (Fig. 3ba). On the other hand, although iNOS mRNA expression was also enhanced by the spinal cord injury or LPS administration (Fig. 3bb), similarly as for GDNF mRNA expression, there was no

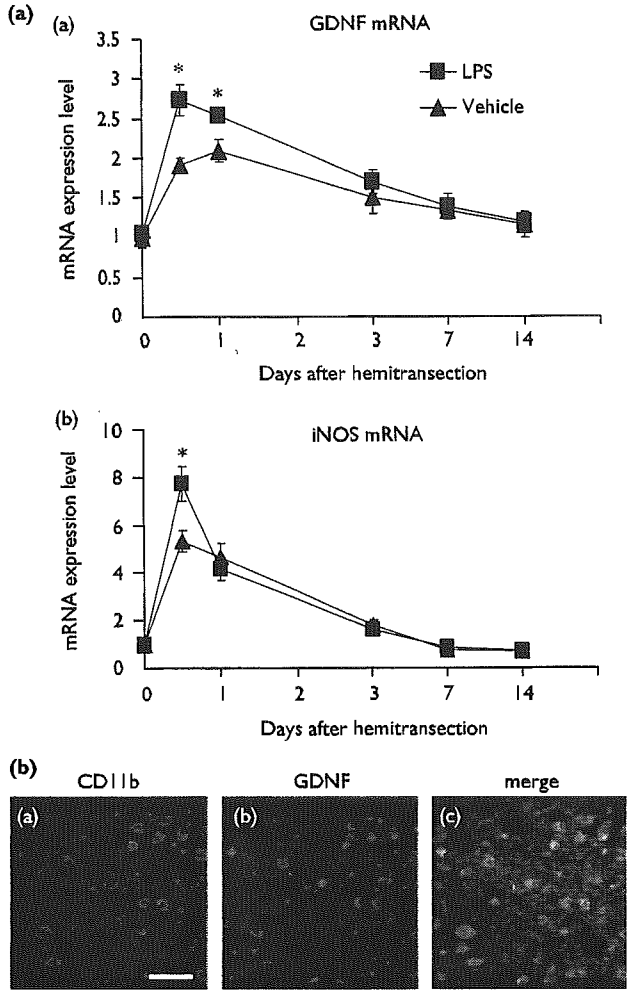

Fig. 2. Expression of GDNF and iNOS mRNAs (**a**) and colocalization of GDNF-ir in the CD11b-positive cells (**b**) in the injured rat spinal cord to which LPS or vehicle was administered. (**a**) LPS (10 μg, squares) or vehicle (triangles) was injected into the hemitransection site (at the level of the 9th thoracic vertebra) of the spinal cord immediately after the operation, and the spinal cord was removed. Total RNA was prepared from combined tissues of both rostral and caudal areas (5 mm length) just adjacent to the injury site, and subjected to RT-PCR for mRNA of GDNF (a) and iNOS (b). The ratio of the band intensity of GDNF or iNOS cDNA products to that of GAPDH cDNA products was calculated. The values are expressed as the means ± s.e. (n=3) of the fold-increase over the value of the control group in which total RNA samples were prepared immediately after the administration of LPS or vehicle. Significant differences from the value of the corresponding vehicle group were determined by means of Student's t-test. $^{*}p<0.05$. (**b**) LPS (10 μg) was injected into the hemitransection site of spinal cord immediately after the operation. At 12 h post injection, the spinal cord was removed from the fixed animal and processed. Sagittal sections were double stained with mouse antibody against CD11b and rabbit antibody against GDNF and visualized with rhodamine-conjugated anti-mouse IgG for CD11b (a) and FITC-conjugated anti-rabbit IgG for GDNF (b). a and b are merged in (c). Bar=50 μm.

difference in iNOS mRNA level between the mice of both genetic types. The combined action of these two types of regulators, i.e., GDNF and iNOS, with the former predominating, restored the locomotor function damaged by the spinal cord injury, suggesting that GDNF is involved in restoration processes via properly activated microglia/macrophages, i.e., via optimal inflammation reactions.

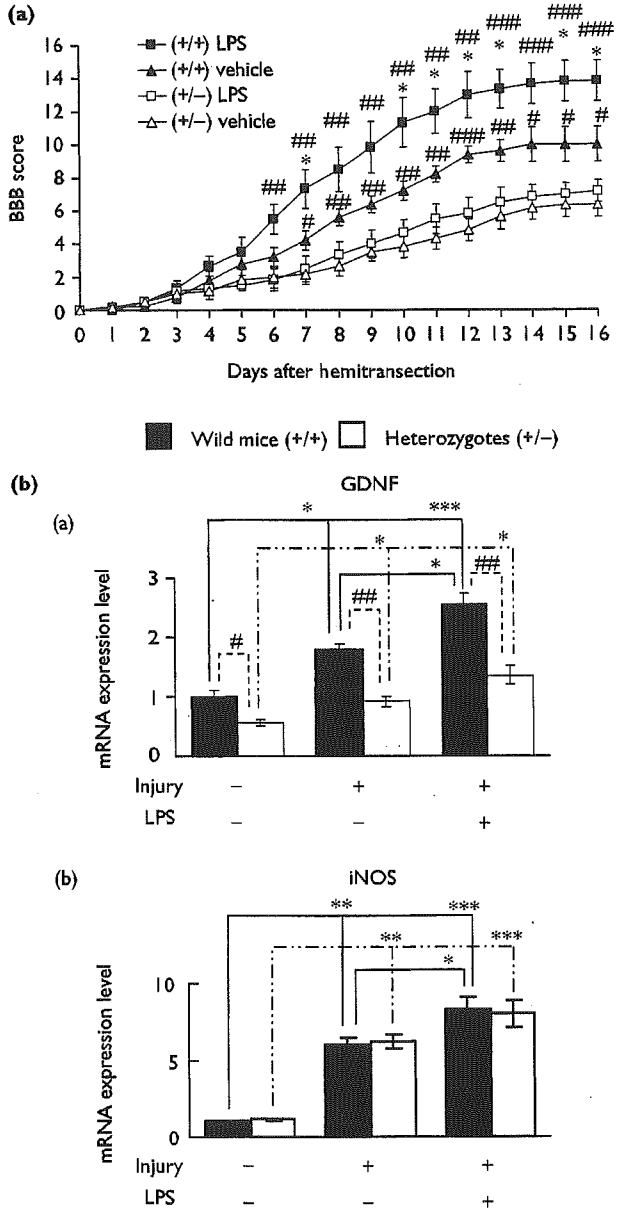

Fig. 3. Effects of LPS on locomotor function after spinal cord injury (**a**) and effects of LPS and/or spinal cord injury on the expression of GDNF and iNOS mRNAs (**b**) of wild-type mice (+/+) and GDNF-gene heterozygous mutant mice (+/−). The left side of the spinal cord was transected or not at the level of the 9th thoracic vertebra, and LPS (2 μg) or vehicle was injected into the injury site immediately after the operation. (**a**) Locomotor function of the hind limb on the injured side was evaluated for +/+ (solid) and +/− (open) mice daily after injection of LPS (squares) or vehicle (triangles). The values are expressed as the means ± s.e. (n=6). Significance, $^{*}p<0.05$ *vs* the value of the vehicle group of the corresponding genotype, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs* the value of similarly treated +/+ mice (Student's t-test). (**b**) mRNA expression of GDNF (a) and iNOS (b) in the injury site of the spinal cord of +/+ (closed columns) and +/− (open columns) mice was examined by RT-PCR 12 h after the injection of LPS (2 μg) or vehicle into the hemitransection site. The ratio of the intensity of GDNF or iNOS cDNA products to that of GAPDH cDNA products was calculated. The values are expressed as the means ± s.e. (n=3) of the fold-increase over the value of vehicle-administered +/+ mice. Statistically significant differences between groups are indicated by brackets: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (ANOVA with Newman-Keuls' *post hoc* test); $^{\#}p<0.05$, $^{\#\#}p<0.01$ (Student's t-test).

DISCUSSION

Inflammatory reactions have opposing roles via production of neurotoxic or neurotrophic molecules. Previous reports described the activation or increase in the number of macrophages by LPS and inhibition of secondary injury after spinal cord injury by daily LPS injection [18,19]. Our present results demonstrate that high-dose LPS led to functional recovery of locomotion (Fig. 1), which we interpreted to be due to the increase in GDNF expression by macrophages activated and increased in number in the injury site (Fig. 2). Moreover, although the optimal dose of LPS enhanced iNOS mRNA expression, the GDNF mRNA level was sustained higher than normal for a much longer time (Fig. 2a), suggesting that properly-controlled inflammatory reactions favor neurotrophic influences by GDNF rather than neurotoxic ones due to iNOS. As exogenous GDNF exerts neuroprotection after spinal cord injury [20], LPS-induced GDNF would seem to contribute positively to nerve regeneration.

As GDNF enhances the phagocytic activity of cultured macrophages [17], GDNF synthesized by macrophages would activate macrophages in an autocrine manner. Therefore, not only neuroprotection but also activation of macrophages would result from macrophage-secreted GDNF in the injured spinal cord. Such GDNF might further upregulate GDNF synthesis in the macrophages, which would promote more and more restorative macrophage function. GDNF may be involved in an activation loop to form an adequate neurotrophic environment for nerve regeneration. Inflammatory reactions are sometimes harmful because of the production of NO, reactive oxygen species, and cytokines [6–8]; and therefore anti-inflammatory therapies are effective for spinal cord injury [21,22]. However, such therapies may simultaneously reduce the level of neurotrophic factors. Thus a proper balance is critical for establishing circumstances favorable for nerve regeneration.

Functional recovery of the GDNF-gene deficient heterozygous mice (+/−) was inferior to that of the wild-type mice (+/+) irrespective of LPS administration (Fig. 3). GDNF mRNA expression of the +/− mutant mice was about a half of that of the +/+ ones regardless of the treatment, whereas there was no difference in iNOS mRNA expression between both genetic mice. That is, GDNF produced by microglia/macrophages after spinal cord injury was important for recovery of locomotor function, except in the following case: Namely, LPS administration to the +/− mice enhanced their GDNF mRNA expression, but did not facilitate their functional recovery. This may have been due to insufficient expression of GDNF to elicit neurotrophic effects.

CONCLUSIONS

Properly activated microglia/macrophages in the injured spinal cord synthesize and secrete the neurotrophic factor GDNF, which is involved in restoring locomotor function damaged by a spinal cord injury.

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ARTICLE

Effects of single and repeated administration of methamphetamine or morphine on neuroglycan C gene expression in the rat brain

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Abstract

The rearrangement of neural networks associated with the behavioural sensitization and tolerance induced by psychostimulants is poorly understood. We have investigated the effects of repeated administration of methamphetamine (chronic MAP), which induces behavioural sensitization, or morphine (chronic morphine), which induces tolerance to its antinociceptive effect, on the mRNA levels of neural network-related genes in the rat brain. A gene of special interest was that for neuroglycan C (NGC), a neural tissue-specific transmembrane chondroitin sulphate proteoglycan. Single MAP (acute MAP) administration significantly decreased NGC mRNA levels in the frontal cortex, ventral tegmental area (VTA), and amygdala compared to vehicle-treated groups. Repeated MAP (chronic MAP) administration significantly increased NGC mRNA levels in the frontal cortex, nucleus accumbens (NAc), striatum, hippocampus, VTA, and amygdala compared to acute MAP treatment. Single morphine (acute morphine) administration significantly increased NGC mRNA levels in the NAc, striatum, hippocampus, VTA, and amygdala compared to vehicle-treated groups. Chronic morphine administration significantly decreased NGC mRNA levels in the NAc, striatum, VTA, and amygdala compared to acute treatment. In addition, the NGC protein level in the NAc was increased after chronic MAP and acute morphine treatment. Dopamine and opioid receptor antagonists attenuated the effect of MAP and morphine respectively on NGC mRNA levels. These results suggest that the sensitization to MAP is associated with up-regulation of NGC gene expression, while the tolerance to the morphine-induced analgesic effect is associated with the down-regulation of NGC gene expression.

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Key words: Methamphetamine, morphine, neuroglycan C, sensitization, tolerance.

Introduction

The repeated, intermittent, passive administration of drugs of abuse, including cocaine, methamphetamine (MAP) and morphine, can induce a progressive increase in the sensitization of certain behavioural effects of these drugs in laboratory rodents (Kalivas et al., 1998; Pierce and Kalivas, 1997), and the behavioural alterations persist for an extremely long time. Furthermore, repeated administration of morphine results in the development of both tolerance to its antinociceptive effects and locomotor sensitization (Eitan et al., 2003). Drug addiction, which constitutes a serious threat to public health, is a multifaceted disorder involving tolerance, dependence, craving, and relapse (Nestler, 2002). Some current theories of drug addiction argue that the cellular neuroadaptations mediating behavioural sensitization are relevant to certain aspects of drug addiction (Kalivas et al., 1998;

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