influence macrophage function through activation of Src family kinase in a c-Ret-independent manner. This implies an autocrine/paracrine action of GDNF on macrophages.

It is likely that GDNF stimulated phagocytosis of macrophages by the mediation of GDNF-induced chemokines, such as MCP-1. However, MCP-1 mRNA was expressed at a similar level in both +/+ and mutant +/– macrophages before or after stimulation (Fig. 7Bc) in spite of the lower expression of GDNF mRNA in the mutant macrophages (Fig. 7Ba). Therefore, GDNF mRNA expression is correlated better than MCP-1 mRNA expression with induction of the phagocytic activity, which may be supported by our preliminary result showing that the mutant macrophages secreted about half the GDNF protein secreted by the wild-type mice. It is possible that GDNF directly participates in the activation processes of the macrophages.

Exogenously infused GDNF can exert behavioral and anatomic neuroprotection against spinal cord injury (Cheng et al., 2002). Therefore, GDNF produced after a spinal cord injury is likely to exert predominantly neurotrophic effects on injured neurons. It is also conceivable that GDNF produced from microglia/macrophages in the injured spinal cord induces more activation of macrophages and more expression of GDNF and MCP-1 from these activated macrophage and that the increased GDNF acts as a neurotrophic factor for injured neurons. However, successful regeneration after a spinal cord injury is difficult, because the inflammatory response, such as activation of macrophages, is delayed and limited. In fact, GDNF might not have been produced so much because of the limited number of activated macrophages. Zeev-Brann et al. (1998) showed that activation of microglia/macrophages was enhanced when the cells were exposed to sciatic nerve segments but was inhibited in the presence of optic nerve segments, suggesting the existence of substances in the CNS inhibitory toward the activation of macrophages. Such inhibition may reduce the neurotrophic influence of GDNF in the injured site and result in failed regeneration. Macrophages are also known to have not only beneficial but also harmful effects, the latter caused by the production of nitric oxide. Indeed, infusion of a chemokine antagonist attenuated macrophage infiltration and induced axonal sprouting in the injured spinal cord (Ghirnikar et al., 2001). This is contradictory to the beneficial effects of macrophages. We propose that a delayed inflammatory response may have a harmful influence on a spinal cord injury but that an early one may be beneficial for neuronal regeneration because of an increased production of neurotrophic factors such as GDNF (Fig. 1).

In summary, we demonstrate here that GDNF was up-regulated in microglia/macrophages after spinal cord transection and enhanced phagocytic activity and expression of GDNF or chemokine mRNA of cultured rodent peritoneal macrophages. GDNF produced after a spinal cord injury might not only have a beneficial influence on the injury but might also participate in the inflammatory response.

REFERENCES

Adams DH, Lloyd AR. 1997. Chemokines: leucocyte recruitment and activation cytokines. Lancet 349:490–495.

Babcock AA, Kuziel WA, Rivest S, Owens T. 2003. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. J Neurosci 23:7922–7930.

Batchelor PE, Liveratore GT, Wong JY, Porritt MJ, Frerichs F, Donnan GA, Howells DW. 1999. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19:1708–1716.

Blight AR. 1985. Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. CNS Trauma 2:299–315.

Blight AR. 1992. Macrophages and inflammatory damage in spinal cord injury. J Neurotrauma 9(Suppl 1):S83–S91.

Cheng H, Wu JP, Tzeng SF. 2002. Neuroprotection of glial cell line-derived neurotrophic factor in damaged spinal cords following contusive injury. J Neurosci Res 69:397–405.

Che X, Ye W, Panga L, Wu DC, Yang GY. 2001. Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. Brain Res 902:171–177.

Fawcett JW, Asher RA. 1999. The glial scar and central nervous system repair. Brain Res Bull 49:377–391.

Flugel A, Hager G, Horvat A, Spitzer C, Singer GM, Graeber MB, Kreutzberg GW, Schwaiger FW. 2001. Neuronal MCP-1 expression in response to remote nerve injury. J Cereb Blood Flow Metab 21:69–76.

Franzen R, Schoenen J, Leprince P, JoostenE, Moonen G, Martin D. 1998. Effects of macrophage transplantation in the injured adult rat spinal cord: a combined immunocytochemical and biochemical study. J Neurosci Res 51:316–327.

Ghirnikar RS, Lee YL, Eng LF. 2001. Chemokine antagonist infusion promotes axonal sparing after spinal cord contusion injury in rat. J Neurosci Res 64:582–589.

Glabinski AR, Tani M, Aras S, Stoler MH, Tuohy VK, Ransohoff RM. 1995. Regulation and function of central nervous system chemokines. Int J Dev Neurosci 13:153–165.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vandlen RA, Simmons L. 1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266:1062–1064.

Huang D, Han Y, Sandhya Rani MR, Glabinski A, Trebst C, Sorensen T, Tani M, Wang J, Chien P, O'Bryan S, Bielecki B, Zhou ZL, Majumder S, Ransohoff RM. 2000. Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. Immunol Rev 177:52–67.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis J-C, Hu S, Altrock BW, Fox GM. 1996. GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 85:1113–1124.

Kobayashi H, Mizisin AP. 2001. Nerve growth factor and neurotrophin-3 promote chemotaxis of mouse macrophages in vitro. Neurosci Lett 305:157–160.

Lee TL, Shih K, Bao P Ghirnikar RS, Eng LF. 2000. Cytokine chemokine expression in contused rat spinal cord. Neurochem Int 36:417–425.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132.

Lu R, Pitha PM. 2001. Monocyte differentiation to macrophage requires interferon regulatory factor 7. J Biol Chem 276:45491–45496.

McTigue DM, Tani M, Krivacic K, Chernosky A, Kelner GS, Maciejewski D, Maki R, Ransohoff RM, Stokes BT. 1998. Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury. J Neurosci Res 53:368–376.

Murakami Y, Furukawa S, Nitta A, Furukawa Y. 2002. Accumulation of nerve growth factor protein at both rostral and caudal stumps in the transected rat spinal cord. J Neurol Sci 198:63–69.

Paratcha G, Ledda F, Ibanez CF. 2003. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113:867–879.

Pezeshki G, Franke B, Engele J. 2001. Evidence for a ligand-specific signaling through GFRα-1, but not GFRα-2, in the absence of RET. J Neurosci Res 66:390–395.

Picher JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H. 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382:73–76.

Popovich PG, Wei P, Stokes BT. 1997. The cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 377:443–464.

Rollins BJ. 1997. Chemokines [review; 268 references]. Blood 90:909–928.

Satake K, Matsuyama Y, Kamiya M, Kawakami H, Iwata H, Adachi K, Kiuchi K. 2000. Up-regulation of glial cell line-derived neurotrophic factor (GDNF) following traumatic spinal cord injury. Neuroreport 11: 3877–3881.

Schiff DE, Kline L, Soldau K, Lee JD, Pugin J, Tobias PS, Ulevitch RJ. 1997. Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism. J Leuk Biol 62:786–794.

Susaki Y, Shimizu S, Katakura K, Watanabe N, Kawamoto K, Matsumoto M, Tsudzuki M, Furusaka T, Kitamura Y, Matsuda H. 1996. Functional properties of murine macrophages promoted by nerve growth factor. Blood 88:4630–4637.

Trupp M, Scott R, Whittemore SR, Ibáñez CF. 1999. Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. J Biol Chem 274:20885–20894.

Wei GW, Wu GC, Cao XD. 2000. Dynamic expression of glial cell line-derived neurotrophic factor after cerebral ischemia. Neuroreport 11:1177–1183.

Widenfalk J, Lundströmer K, Jubran M, Brené S, Olson L. 2001. Neurotrophic facrors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. J Neurosci 21:3457–3475.

Zeev-Brann AB, Lazarov-Spiegler O, Brenner T, Schwartz M. 1998. Differential effects of central and peripheral nerves on macrophages and microglia. Glia 23:181–190.

Zhang Z, Krebs CJ, Guth L. 1997. Experimental analysis of progressive necrosis after spinal cord trauma in the rat: etiological role of the inflammatory response. Exp Neurol 14:141–152.

Available online at www.sciencedirect.com

SCIENCE DIRECT®

European Journal of Pharmacology 507 (2005) 229–237

www.elsevier.com/locate/ejphar

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

Mika Miyoshi[a], Masayuki Nadai[b], Atsumi Nitta[c], Jun Ueyama[a], Akemi Shimizu[a], Kenji Takagi[a], Toshitaka Nabeshima[c], Kenzo Takagi[a], Kuniaki Saito[d], Takaaki Hasegawa[e,*]

[a]*Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-867, Japan*
[b]*Faculty of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan*
[c]*Department of Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan*
[d]*Department of Laboratory Medicine, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan*
[e]*Department of Hospital Pharmacy and Pharmacokinetics, Aichi Medical University School of Medicine, Nagakute-cho, Aichi-gun, Aichi 480-1195, Japan*

Received 12 November 2004; accepted 16 November 2004
Available online 15 December 2004

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

* Corresponding author. Tel.: +81 561 63 1011; fax: +81 561 63 1028.
E-mail address: takahase@aichi-med-u.ac.jp (T. Hasegawa).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.035

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 $3000\times g$ for 10 min at 4 °C. The supernatant was centrifuged at $30{,}000\times g$ 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 $6000\times g$ 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

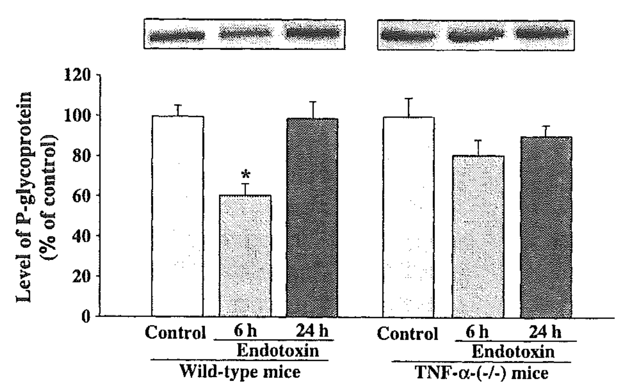

Fig. 1. Time-dependent effects of endotoxin on hepatic expression of P-glycoprotein in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control ($P<0.05$).

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).

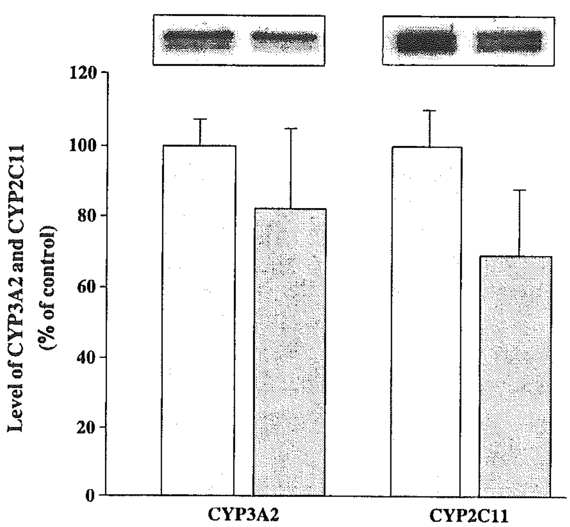

Fig. 2. Constitutive expression of CYP3A2 and CYP2C11 in liver of wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6).

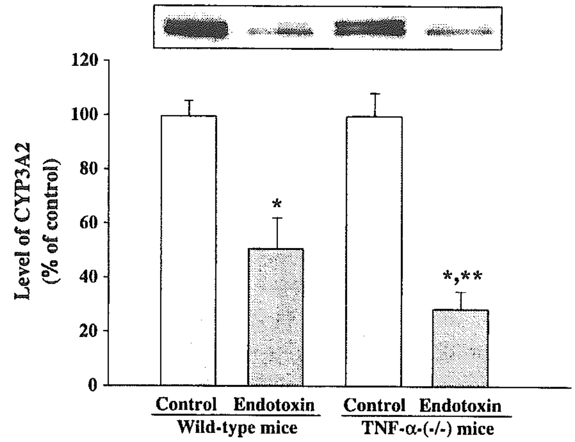

Fig. 3. Effect of endotoxin on hepatic expression of CYP3A2 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type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

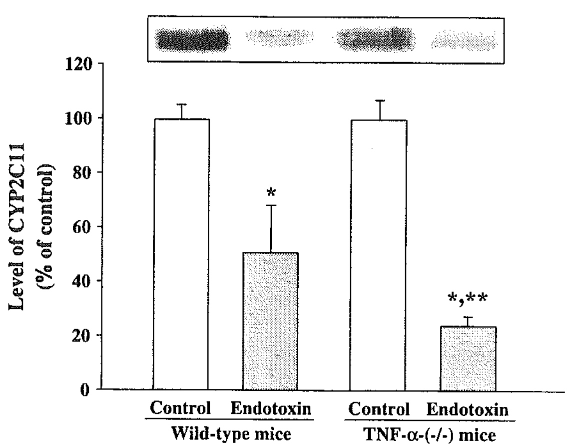

Fig. 4. Effect of endotoxin on hepatic expression of CYP2C11 in wild-type and TNF-$\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (n=4–6). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type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

3.5. Effect of endotoxin on antipyrine elimination in wild-type and TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ mice, a significant difference was observed between endotoxin-treated wild-type mice and TNF-$\alpha^{-/-}$ mice.

3.6. Effect of endotoxin on plasma levels of nitrate/nitrite (NO_x) in wild-type and TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ mice. Endotoxin significantly increased the plasma levels of NO_x by 15-fold and 4-fold in wild-type and TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ mice.

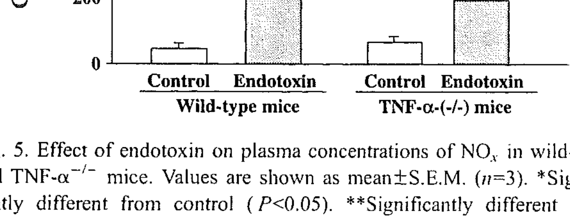

Fig. 5. Effect of endotoxin on plasma concentrations of NO_x in wild-type and TNF-$\alpha^{-/-}$ mice. Values are shown as mean±S.E.M. (n=3). *Significantly different from control (P<0.05). **Significantly different from endotoxin-treated wild-type mice (P<0.05).

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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (15590484) and a Grant-in-Aid for Scientific Frontier Research Project of Meijo University from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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.

Inflammation-induced GDNF improves locomotor function after spinal cord injury

Manabu Hashimoto, Atsumi Nitta,[1] Hidefumi Fukumitsu, Hiroshi Nomoto, Liya Shen[2] and Shoei Furukawa[CA]

Laboratory of Molecular Biology, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585; [1]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan; [2]Laboratory of Mammalian Genes and Development, National Institutes of Health, Bethesda, MD 20983, USA

[CA]Corresponding Author: furukawa@gifu-pu.ac.jp

Received 23 November 2004; accepted 29 November 2004

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.

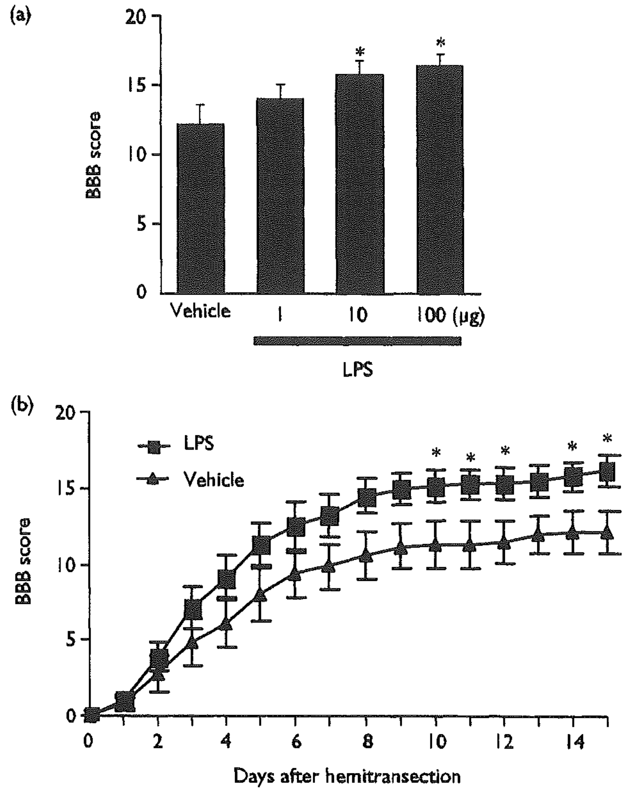

Fig. 1. Effect of LPS on locomotor function of rats with spinal cord injury. The left hemisphere of the spinal cord was transected, and a single injection (10 μl) of 1, 10 or 100 μg LPS or vehicle (PBS) was then administered to the injury site. **(a)** Locomotor function of the hind limb on the injured 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

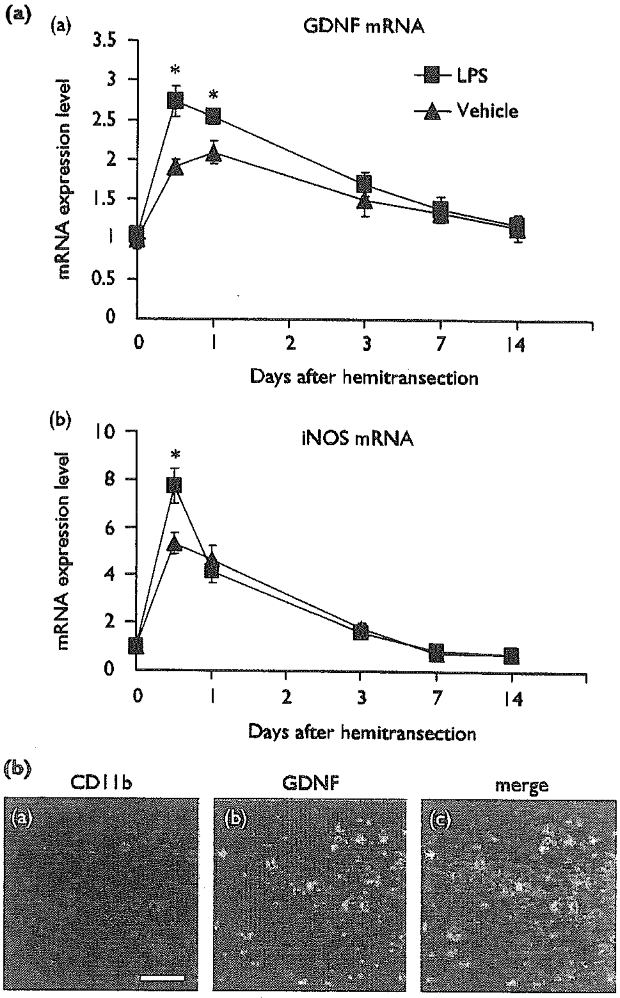

Fig. 2. Expression of GDNF and iNOS mRNAs (a) and colocalization of GDNF-ir in the CD11b-positive cells (b) in the injured rat spinal cord to which LPS or vehicle was administered. (a) LPS (10 μg, squares) or vehicle (triangles) was injected into the hemitransection site (at the level of the 9th thoracic 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.

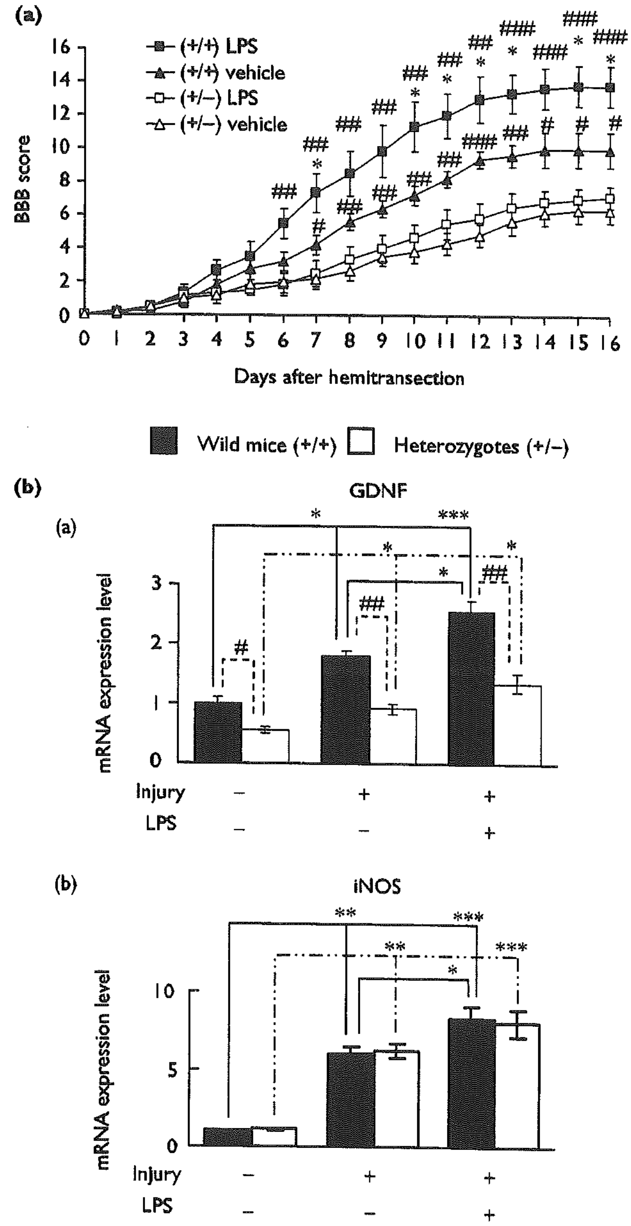

Fig. 3. Effects of LPS on locomotor function after spinal cord injury (a) and effects of LPS and/or spinal cord injury on the expression of GDNF and iNOS mRNAs (b) of wild-type mice (+/+) and GDNF-gene heterozygous mutant mice (+/−). The left side of the spinal cord was transected or not at the level 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.

REFERENCES

1. Fawcett JW and Asher RA. The glial scar and central nervous system repair. *Brain Res Bull* 1999; **49**:377–391.
2. Widenfalk J, Lundströmer K, Jubran M, Brené S and Olson L. Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. *J Neurosci* 2001; **21**:3457–3475.
3. Franzen R, Schoenen J, Leprince P, Joosten E, Moonen G and Martin D. Effects of macrophage transplantation in the injured adult rat spinal cord: a combined immunocytochemical and biochemical study. *J Neurosci Res* 1998; **51**:316–327.
4. Adams DH and Lloyd AR. Chemokines: leucocyte recruitment and activation of cytokines. *Lancet* 1997; **349**:490–495.
5. Rollins BJ. Chemokines. *Blood* 1997; **90**:909–928.
6. Blight AR. Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. *CNS Trauma* 1985; 2:299–315.
7. Zhang Z, Krebs CJ and Guth L. Experimental analysis of progressive necrosis after spinal cord trauma in the rat: etiological role of the inflammatory response. *Exp Neurol* 1997; **14**:141–152.
8. Martiney JA, Cuff C, Litwak M, Berman J and Brosnan CF. Cytokine-induced inflammation in the central nervous system revisited. *Neurochem Res* 1998; **23**:349–359.
9. Perry VH, Brown MC and Anderson PB. Macrophage responses to central and peripheral nerve injuries. *Adv Neurol* 1993; **59**:309–314.
10. Zeev-Brann AB, Lazarov-Spiegler O, Brenner T and Schwartz M. Differential effects of central and peripheral nerves on macrophages and microglia. *Glia* 1998; **23**:181–190.
11. Lin LF, Doherty DH, Lile JD, Bektesh S and Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993; **260**:1130–1132.
12. Henderson CE, Phillips HS, Pollock RA, Davies AM and Lemeulle C. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* 1994; **266**:1062–1064.
13. Satake K, Matsuyama Y, Kamiya M, Kawakami H and Iwata H. Up-regulation of glial cell line-derived neurotrophic factor (GDNF) following traumatic spinal cord injury. *Neuroreport* 2000; **11**:3877–3881.
14. Wei GW, Wu GC and Cao XD. Dynamic expression of glial cell line-derived neurotrophic factor after cerebral ischemia. *Neuroreport* 2000; **11**:1177–1183.
15. Basso DM, Beattie MS and Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995; **12**:1–21.
16. Picher JG, Shen L, Sheng HZ, Granholm AC and Drago J. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 1996; **382**:73–76.
17. Hashimoto M, Nitta A, Fukumitsu H, Nomoto H, Shen L and Furukawa S. Involvement of glial cell line-derived neurotrophic factor in activation processes of rodent macrophages. *J Neurosci Res* 2004; in press.
18. Guth L, Zhang Z, DiProspero NA, Joubin K and Fitch MT. Spinal cord injury in the rat: treatment with bacterial lipopolysaccharide and indomethacin enhances cellular repair and locomotor function. *Exp Neurol* 1994; **126**:76–87.
19. Lazar DA, Ellegala DB, Avellino AM, Dailey AT and Andrus K. Modulation of macrophage and microglial responses to axonal injury in the peripheral and central nervous systems. *Neurosurgery* 1999; **45**: 593–600.
20. Cheng H, Wu JP and Tzeng SF. Neuroprotection of glial cell line-derived neurotrophic factor in damaged spinal cords following contusive injury. *J Neurosci Res* 2002; **69**:397–405.
21. Labombarda F, Gonzales SL, Gonzales DM, Guennoun R and Schumacher M. Cellular basis for progesterone neuroprotection in the injured spinal cord. *J Neurotrauma* 2002; **19**:343–355.
22. Bao F, Chen Y, Dekaban GA and Weaver LC. Early anti-inflammatory treatment reduces lipid peroxidation and protein nitration after spinal cord injury in rats. *J Neurochem* 2004; **88**:1335–1344.

Acknowledgements: This work was supported in part by a grant-in-aid (grant no. 14658252) from the Ministry of Education, Science, and Culture of Japan.

神経毒によるパーキンソン病モデル：細胞死機序の解明と神経保護薬の開発

直井　信*，丸山　和佳子**

抄　録　パーキンソン病は環境および遺伝要因により加齢とともに黒質のドパミン細胞の変性をもたらす疾患である。その病因に関する仮説の1つとして神経毒があり，6-hydroxydopamine (6-OHDA) と 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) を用いた動物と細胞モデルが本疾患の病態の解明に多くの寄与をしてきた。我々はドパミンよりヒト脳内で生成される *N*-methyl(*R*)salsolinol がドパミン神経に対する選択的な神経毒であることを動物および細胞モデルの作成と，臨床サンプルの分析から証明してきた。神経毒によるモデル系はドパミン神経の選択的な細胞死の機序の研究有用な手段であった。最近内在性神経毒による細胞モデルを用いて神経細胞の変性を阻止する神経保護薬の開発とその作用機序の研究が進められている。本稿では細胞死と保護の機序に関する我々の研究成果を紹介する。

脳の科学 (2004年増刊号) 157-164, 2004

Key words: *neurotoxin, apoptosis, neuroprotection, N-methyl(R)salsolinol, mitochondria*

はじめに

孤発性パーキンソン病 (Parkinson's disease, PD) はアルツハイマー病に次いで頻度の高い高齢者の神経変性疾患であり，黒質ドパミン神経細胞の選択的な細胞死と特異な封入体Lewy小体の存在が病理像の特徴である。しかしその原因は未だに解明されておらず，複数の環境および遺伝要因が関与する多因子疾患であると考えられている。PD患者のほとんどを占める孤発性患者に関して疾病発症に関与する遺伝因子は見出されていない。従ってPDの原因を解明するためには疾患の病態を理解することが必須であり，そのための疾患モデルが動物，細胞レベルで報告されている。特に1979年1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) がヒトにPD様症状を発症させるとの報告以来，MPTPモデルを用いたドパミン神経の細胞死の研究が急速に発展することとなった。現在MPTPはヒト脳内には存在しない合成化合物で孤発性PDの原因ではないと考えられている。しかし，MPTP類似のドパミン神経に選択性と毒性を持つ低分子化合物が存在しPDの細胞死に関与する可能性がある。

筆者らはドパミンより脳内で酵素的に生成される *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] が内在性のMPTP様の神経毒であることを動物，細胞モデルを用いて証明し，さらに臨床サンプルの

Models of Parkinson's disease induced by neurotoxins: its application for the studies on intracellular mechanism of cell death and development of neuroprotective agents.
*岐阜県国際バイオ研究所脳神経研究部門
〒504-0838 岐阜県各務原市那加不動丘1-1]
Makoto Naoi : Department of Neurosciences, Gifu International Institute of Biotechnology, 1-1 Naka-Fudogaoka, Kakamigahara, Gifu, 504-0838 Japan.
**国立長寿医療センター老化機構研究部生化学・代謝研究室
Wakako Maruyama : Department of Basic Gerontology, National Institute of Longevity Sciences.

表 1　ドパミン神経細胞死に関与するとされる神経毒

神経毒	動物、細胞モデルの特徴	細胞死とその機序
ドパミン由来の内在性化合物		
6-OHDA	行動、薬理、病理も PD に類似、封入体は認められない	選択的、necrosis/apoptosis、酸化ストレス
*N*M(*R*)Sal	行動、薬理、病理も PD に類似、封入体は認められない	選択的、apoptosis、Mit の細胞死機構の活性化
内在/環境由来の化合物		
β-Carboline	行動、薬理、病理も PD に類似、封入体は認められない	選択的、細胞死、機序は未定（Mit 障害？）
TaClo	行動、薬理、病理も PD に類似、封入体は認められない	選択的、細胞死、機序は未定（Mit 障害？）
環境由来の神経毒		
農薬		
Rotenone	行動、薬理、病理も PD に類似、封入体を認める	非選択的 necrosis/apoptosis、Mit 障害、酸化ストレス
Paraquat	PD との類似性は低い、封入体は認められない	非選択的、necrosis/apoptosis、酸化ストレス
Dieldrin	PD との類似性は低い、封入体は認められない	非選択的、necrosis/apoptosis、酸化ストレス
合成神経毒		
MPTP	行動、薬理、病理も PD に類似、封入体は認められない	選択的、necrosis/apoptosis、酸化ストレスと Mit 障害
金属イオン		
鉄	PD との類似性は低い、封入体は認められない	非選択的、necrosis/apoptosis、酸化ストレス
マンガン		

解析により PD 病因への関与を証明してきた。現在神経毒の研究はドパミン神経細胞に選択的な細胞死の機序に関する研究と新たな PD の治療法，特に神経保護法の開発に向かっている。このため，本稿においては *N*M(*R*)Sal を用いた PD の病因に関する従来の結果とあわせ，細胞死の細胞内機序と神経保護薬剤の開発に関する最近の我々の研究を述べる。

Ｉ．神経毒を用いた疾患モデル：PD 病因との関連

6-hydroxydopamine（6-OHDA）と MPTP による動物モデルは既に各々40，25 年の歴史があり，病理，行動，薬物への反応性も PD に類似のものとして一般に用いられている[6]。しかし，Lewy body 様の封入体は認められず，老化とともに進行する PD の完璧なモデルとはなりえない。このため，最近ミトコンドリア（Mit）呼吸鎖の複合体 1 の阻害剤 rotenone による封入体形成を伴うモデルが報告された[3]。表 1 に現在報告されているドパミン神経の選択的な細胞死を起こす神経毒を示す。

MPTP はヒト脳内で B 型モノアミン酸化酵素（monoamine oxidase，MAO）により酸化され，1-methyl-4-phenylpyridinium ion（MPP^+）に変換される。MPP^+はドパミン神経に存在するドパミン再取込み機構により取り込まれ，Mit 内に蓄積する。最終的に MPP^+は Mit 呼吸鎖酵素 complex Ｉと α-ketoglutarate dehydrogenase を阻害することにより ATP 産生の阻害と酸化ストレスにより細胞死を惹起する。6-OHDA はドパミン細胞への選択的な取込みの後，自動酸化による superoxide の産生と c-Jun N-terminal kinase の活性化により主にアポトーシスを惹起する[5]。動物モデルの作成には MPTP は末梢からの投与が可能であるが，6-OHDA では脳内注入が必要となる。これら動物モデルの作成は動物種，投与法と量により結果が異なり，詳細は文献を参考にされたい[8,18]。

MPP^+とは異なり，paraquat 等の dipyridinium 殺虫剤で *N*－メチル化されているものは特定の輸送タンパクと結合して脳内に移行し酸化ストレスを介し神経細胞の変性を引き起こす。最近疫学的な結果からも農薬が PD の原因物質として再び注目されている。従来 paraquat や rotenone はドパミン細胞に対する選択性がないため，PD の病因物質にはなりえないと考えられていた。しかし最近 rotenone の濃度と投与法を配慮した動

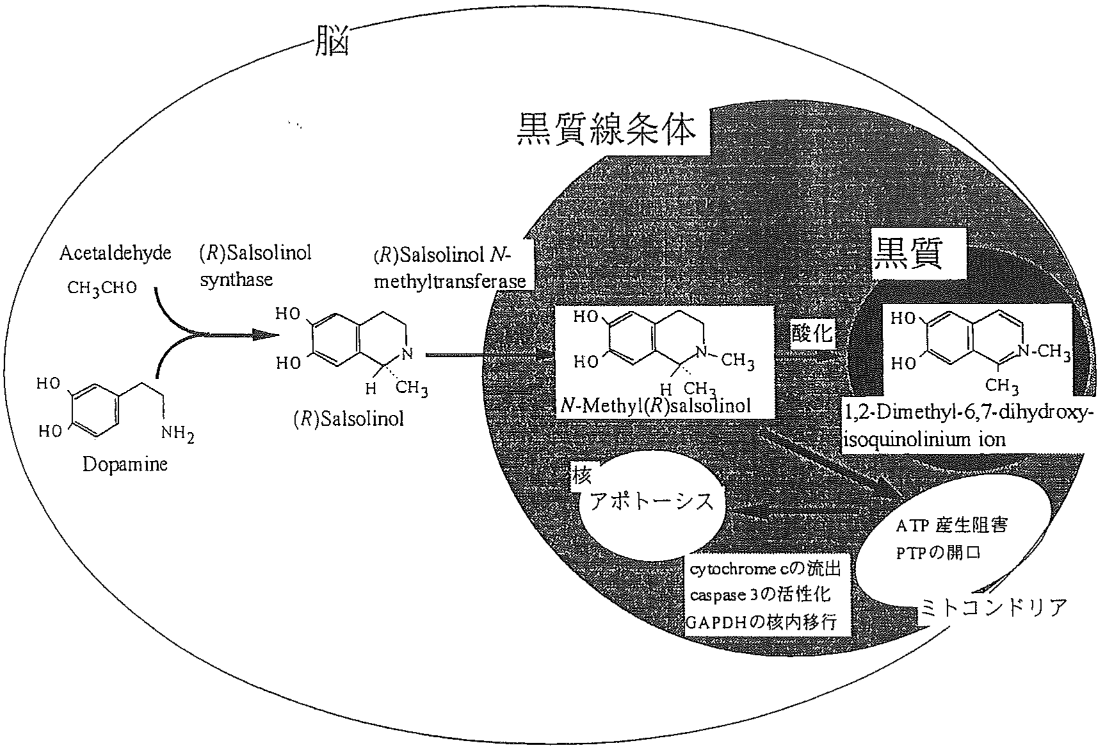

図1 ドパミン由来の神経毒 *N*-methyl(*R*)salsolinol のヒト脳内代謝と分布
ドパミンとアセトアルデヒドから (*R*)サルソリノールシンターゼにより生成される (*R*)サルソリノールは脳内に広く分布する。(*R*)サルソリノール *N*-メチル転位酵素により *N*-メチル体となり，黒質線条体に極在して存在する。さらに酵素的または非酵素的に酸化され MPP^+類似のイオン体となり黒質にのみ蓄積する。*N*M(*R*)Sal のみがミトコンドリアの膜透過性を亢進してアポトーシスを誘発する。

物モデルで黒質線条体のドパミン細胞に細胞死と Lewy body 類似の封入体が認められた[3]。この部位でのドパミン細胞が rotenone に対し脆弱であることから選択的な毒性が生ずることが示唆された。Rotenone を用いたモデルは封入体生成の研究には有用と考えられる。最近我々は rotenone による細胞死モデルで，proteasome 20 Sβ タンパクが酸化修飾され活性が低下し，構造の変化した酸化修飾タンパクが細胞内で分解されず蓄積凝集される機構を証明した[19]。他の塩素系農薬である deldrin も PD 脳内で増加し[7]，酸化ストレスを介しドパミン細胞に細胞毒性を示すことが報告されているが，今のところ上記のモデル程一般的には用いられておらず PD の病因物質としても認められてはいない。

内在性の PD と関連した神経毒としてドパミン由来のカテコールイソキノリン (salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline)，フネチルアミン由来のイソキノリン (1,2,3,4-tetrahydroisoquinoline)，インドールアミン由来の β-carboline が報告されている。我々はドパミンから脳内で酵素的に生成される salsolinol 類の研究から *N*M(*R*)Sal [1(*R*)-methyl-*N*-methy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] が PD 病因と関連する内在性神経毒であることを証明してきた。この結果を以下にまとめる。

1) ドパミンから2段階の酵素反応，即ちアルデヒドとの縮合反応により (*R*)-salsolinol が，次に (*R*)salsolinol に選択的な *N*-メチル転位酵素により，MPTP と化学構造の類似したイソキ

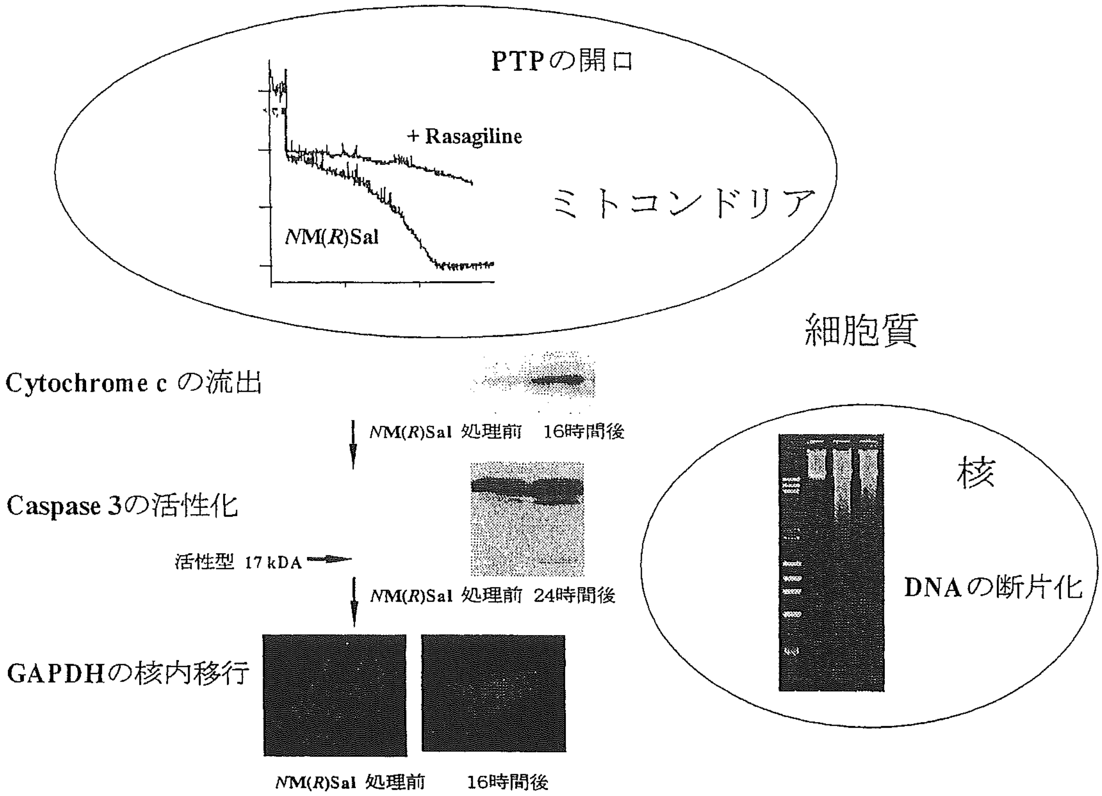

図 2　NM(R)Sal による細胞死シグナル
NM(R)Sal はミトコンドリア（Mit）のメガチャンネル permeability transition pore（PTP）を開口し膨張させる（図は単離 Mit の光分散の測定値）。その結果チトクローム C が細胞質に流出し，カスパーゼ群が活性化する。Glyceraldehyde-3-phosphate dehydrogenase（GAPDH）は細胞質より核内に移行し，最終的に核 DNA の断片化が起りいわゆる ladder 形成が見られる。また rasagiine（図 3，4 を参照）は，Mit の PTP の開口を阻止している。

ノリン NM（R)Sal が生成される[18]。NM(R)Sal のラットへの持続投与により黒質ドパミン神経細胞に選択的な細胞死とパーキンソン症状が惹起された。一方他の光学異性体（S)Sal は脳内に認められず，NM(S)Sal は神経毒性を示さなかった[16]。

2）ヒト脳部位での分布を検討すると，NM(R)Sal は線条体に，その酸化生成物で MPP^+ と構造の類似した 1,2-dimethyl-6,7-dihydroxy-isoquinolinium ion は黒質にニューロメラニンと結合して選択的に蓄積していた[10]。図 1 に脳内の代謝と分布の関連を示す。

3）NM(R)Sal 濃度は未治療 PD 患者の脳脊髄液で増加しており[9]，その機序は PD 患者リンパ球で証明したように，N-メチル化反応を触媒する（R)salsolinol N-メチル転位酵素活性が著明に増加する為と考えられる[17]。

(R)体のみがヒト脳内で生成され N-メチル化によりドパミン細胞への選択的な神経毒性を持つことから，NM(R)Sal の代謝に関与する酵素系が PD の原因となっている可能性が考えられる。同様の結果は β-carboline においても認められ，2,9-N-N-dimethyl-norharman と 2,9-N，N-dimethylated harman cation が PD の CSF で増加しており，β-carboline N-メチル転位酵素の活性が PD の脳内で高いとの報告がある[15]。またドパミンから MAO による酸化により生成する

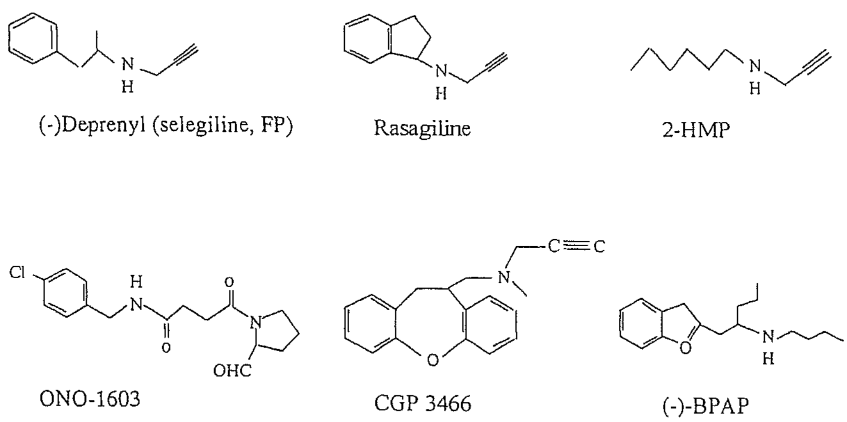

図 3　神経保護活性が認められている薬剤の化学構造
多くの薬剤は propargyl 基　$-CH_2C \equiv CH$ を持ち，Mit 膜の安定化に働く。

ドパアルデヒドとドパミンが縮合して tetrahydropapaveroline（THP）となる。THP は salsolinol と異なり（*S*）体のみがヒト脳で内在し，酸化体がアポトーシスを，還元体がネクローシスを起こす[16]。THP は L-dopa 療法を受けている PD 患者でドパミン細胞の細胞死を増加させる可能性が指摘されたが，証明はされていない。

またトリプタミンとクロラールの縮合で生成する trichloromethyl-1,2,3,4-tetrahydro-β-carboline（TaClo）がクロラールを使用した患者で存在し，ドパミン神経に対する細胞毒性が認められた。しかし PD の病因に関与する可能性は低いと考えられている。またガソリンに添加されているマンガンのカボニイル体が，可逆的なパーキンソン症候を惹起することが報告されている。しかし鉄，マンガンともに最近の疫学調査では PD の危険因子ではないとされている。

II．内在性神経毒 *N*M(*R*)Sal を用いた細胞死機序の解明

PD におけるドパミン神経の細胞死がアポトーシスによるのかネクローシスによるかとの議論は未だ最終的な決着を見ていない。しかし，最近の PD 患者脳の分析結果によるとアポトーシスに関与する細胞死シグナルの活性化が認められている[20]。我々は内在性神経毒 *N*M(*R*)Sal がアポトーシスを惹起することを見出し，その機構をヒトドパミン系神経芽細胞腫 SH-SY 5 Y 細胞を用い検討した。*N*M(*R*)Sal による細胞死の機序に関する我々の結果を図 2 に示し概説する。

1）*N*-Methylsalsolinol［*N*MSal］には *R*，*S* 体の光学異性体があるが，*R* 体のみが SH-SY 5 Y 細胞にアポトーシスを惹起する。ネクローシスは殆ど認められない。

2）細胞死は Mit 依存性の細胞死シグナルにより引き起こされる。即ち Mit 膜に存在するメガチャンネル permeability transition pore（PTP）の開口により，膜電位（$\Delta\Psi$m）の低下，cytochrome c の細胞質への流出，caspase 3 の活性化，glyceraldehyde-3-phosphate dehydrogenase（GAPDH）の細胞質から核への移行と段階的にアポトーシスシグナルが活性化し DNA の断片化に至る[1,12,13]。

3）単離 Mit を用いた研究から *R* 体の *N*MSal のみが PTP を開口する[1]。これは SH-SY 5 Y 細胞において *R* 体のみが $\Delta\Psi$m を低下させることと一致し[13]，この神経毒が Mit に直接し細胞死カスケイドを活性化することを示している。

4）Bcl-2 を過剰発現させた SH-SY 5 Y 細胞[13]と単離 Mit を[1]を用いた実験で，Mit 膜に存在する Bcl-2 タンパクがこの PTP 開口を抑制し下流の細胞死シグナルを制御していることを証明