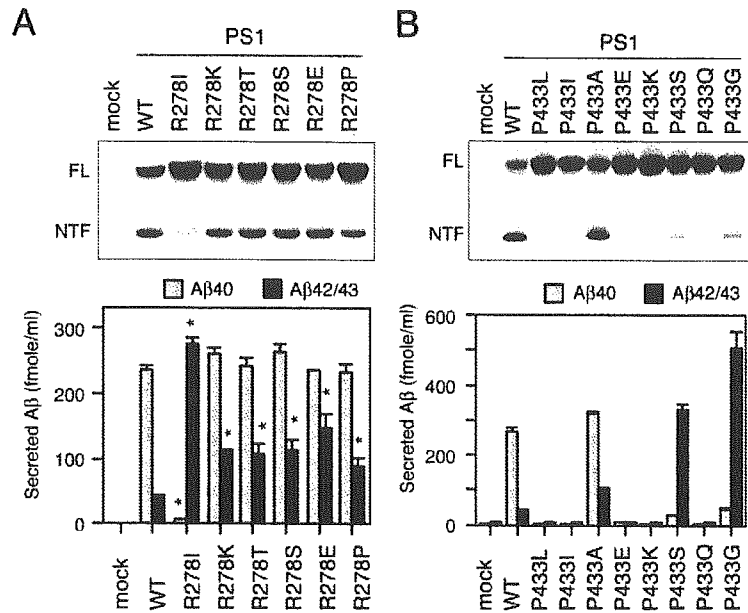


FIG. 5. γ -Secretase activity of PS1 with a mutation at residue 278 or 433. A, PS1 mutants containing six different substitutions at Arg²⁷⁸ were transfected into PS-null MEFs. An immunoblot for transfected PS1 is shown in the upper panel. The graph in the lower panel shows A β levels in the conditioned media as estimated by ELISA. Values represent the means \pm S.D. ($n = 3$). *, $p < 0.001$ by one-way analysis of variance with Dunnett's post-hoc test. FL, full-length. B, PS1 mutants containing eight different substitutions at Pro⁴³³ were transfected into PS-null MEFs. An immunoblot for PS1 is shown in the upper panel. The graph in the lower panel shows A β levels in the conditioned media. Values represent the means \pm S.D. ($n = 3$).



abolished in MEFs expressing R278I-PS1 and, in agreement with a previous report (33), was relatively attenuated in FAD-linked I143F-PS1 MEFs compared with WT-PS1 MEFs (maximum -fold increases of 0.96 ± 0.01 , 1.76 ± 0.08 , and 2.06 ± 0.14 (means \pm S.D., $n = 3$), respectively; $p < 0.001$ by Bonferroni's post-hoc test).

Sulindac sulfide, a nonsteroidal anti-inflammatory drug, preferentially inhibits γ -42/43 activity (31, 34, 37, 38). Fig. 6B shows that this inhibitory effect was obviously reduced in R278I-PS1 MEFs and was intermediately decreased in I143F-PS1 MEFs compared with WT-PS1 MEFs (-fold decreases at 60 μ M of 0.96 ± 0.08 , 0.49 ± 0.01 , and 0.12 ± 0.08 (means \pm S.D., $n = 3$), respectively; $p < 0.001$ by Bonferroni's post-hoc test). Paradoxically, A β ₄₀ secretion was enhanced by sulindac sulfide in R278I-PS1 MEFs (maximum -fold increase of 3.30 ± 0.28 (mean \pm S.D., $n = 3$)). The inhibition profile of DFK167 or sulindac sulfide in L435H-PS1 MEFs was similar to that in R278I-PS1 MEFs (data not shown). Based on these results, it appears that DFK167 and sulindac sulfide have opposite effects; specifically, DFK167 preferentially inhibited γ -40 activity in PS1^{LA} cells and enhanced γ -42/43 activity in WT-PS1 cells, whereas sulindac sulfide paradoxically enhanced γ -40 activity in PS1^{LA} cells and inhibited γ -42/43 activity in WT-PS1 cells. These results suggest an incomplete reciprocal relationship between γ -40 and γ -42/43 activities. Additionally, it appears that PS1^{LA} mutations cause similar but exaggerated modification of A β secretion compared with FAD-linked mutations.

It has been reported that DFK167 inhibits PS1 endoproteolysis in an *in vitro* assay system (39). However, when DFK167 was applied to cells expressing WT-PS1 or the FAD-linked I143F-PS1 mutant, endoproteolysis was unaffected (Fig. 6C). Unexpectedly, when DFK167 was applied to cells expressing the R278I-PS1 or L435H-PS1 mutant, endoproteolysis was activated in a dose-dependent manner (Fig. 6C). This paradoxical activation was highly specific to these mutants; DFK167 had no effect on the failed endoproteolysis of loss-of-function mutants such as D385A-PS1 and P433L-PS1.

DISCUSSION

In this study, we found six missense mutations of PS1 by a random mutagenesis screen for PS1 endoproteolysis-impaired

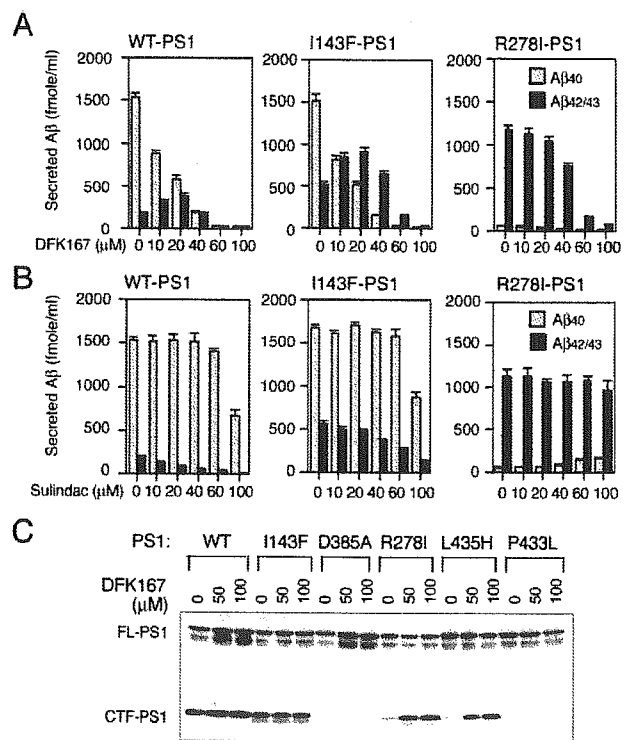


FIG. 6. Effect of DFK167 and sulindac sulfide on A β secretion and PS1 endoproteolysis. A and B, PS-null MEFs cotransfected with human APP and WT-PS1, I143F-PS1, or R278I-PS1 were treated for 12 h with the indicated concentrations of DFK167 or sulindac sulfide, and A β ₄₀ and A β _{42/43} in the media were measured by ELISA. Data represent the means \pm S.D. ($n = 3$). C, after DFK167 treatment of mutant PS1-expressing MEFs for 12 h, PS1 endoproteolysis was estimated by immunoblotting with anti-PS1 CTF antibody. The results are representative of three independent experiments. FL, full-length.

mutants. These mutations fell into two categories: PS1^{LA} and the loss-of-function mutant P433L-PS1. These mutations did not significantly affect the complex assembly and stabilization of uncleaved mutant holoproteins. Presenilase cleavage is tightly related to γ -secretase activity, although PS endoprote-

olysis is not absolutely required for the activation of γ -secretase (12, 40). Both activities require two intramembranous aspartyl residues at the putative catalytic sites of PS (Asp²⁵⁷ and Asp³⁸⁵ of PS1) (12), and certain γ -secretase inhibitors block presenilinase cleavage as well (41, 42). However, the profiles of their response to inhibitors are different from one another (39, 41). Additionally, a recent report proved that the D257A-PS1 or D385A-PS1 holoprotein is cleaved, although with low efficiency, without any concomitant γ -secretase activity when stably expressed in PS-null cells (43). Nevertheless, in most cases, except mutants harboring mutations at the cleavage site (e.g. M292D-PS1) (40, 44, 45), the previously reported cleavage-defective mutations simultaneously confer definitive modification of γ -secretase activity and/or specificity (12, 16, 17, 25, 46). This is the case for PS1^{LA} mutants, which are distinctive in mediating exclusively γ -43 cleavage of APP, but not self-endoproteolysis, γ -40 cleavage, and ϵ -cleavage. A β ₄₃ is a minor A β product of normal γ -secretase activity, but the longest identified so far, and its secretion, like that of A β ₄₂, is associated with pathogenic PS1 mutations (33) or DFK167 treatment (36).

Together with previous work, our results suggest that PS mutations allow the existence of at least four activity states of γ -secretase. The lowest activity state is exemplified by loss-of-function mutations such as D257A, D385A, G384K, and P433L (12, 16, 17). The next lowest activity state is typified by the PS1^{LA} mutants, in which there is residual self-endoproteolysis, γ -40 cleavage, and ϵ -cleavage, but relatively high γ -42/43 activity. Another stage of activity, generally supporting normal self-endoproteolysis and γ -40 activity, but reduced ϵ -cleavage and enhanced γ -42/43 activity, is represented by many FAD mutants (5, 18, 33). Finally, WT-PS1 represents the fully functional state, wherein PS1 supports self-endoproteolysis, ϵ -cleavage, predominant γ -40 activity, and minor γ -42/43 activity. A few FAD mutants have been reported to show reduced self-endoproteolysis and decreased A β ₄₀ secretion as well as enhanced A β _{42/43} secretion (41, 47), which somewhat resemble the activity state of PS1^{LA} mutants. Meanwhile, during the preparation of this manuscript, the R278I-PS1^{LA} mutation has been reported to co-segregate with family members affected with early-onset atypical FAD (48). This suggests that PS1^{LA} mutants have similar but exaggerated effects on γ -secretase activity compared with typical FAD mutants. PS1 mutations appear to result in a wide spectrum of γ -secretase activity that is associated with a shift in cleavage specificity.

Two of the five PS1^{LA} mutations and the P433L loss-of-function mutation were clustered at the highly conserved PAL sequence in the C-terminal tail. Arg²⁷⁸ is remote on the sequence from the PAL motif, but can be located close to the PAL motif near the cytoplasmic face of the membrane on the tertiary conformation based on the eight-TM model of PS1 proteins (Fig. 1B) (49). These residues may compose a conformational domain that is important not only for the activity but also for the cleavage specificity of γ -secretase. However, the two mutations S141R and T245I, which had a weaker effect, were not located in close proximity, suggesting that multiple domains can affect the γ -secretase activity and specificity. On the other hand, our results indicate that different amino acid substitutions at the same residue of PS1 can cause distinct modifications of γ -secretase activity. These results confirm and extend previous studies showing that several mutations at residue 166 or 384 differentially affect the secreted A β level and the A β ₄₂/total A β ratio (16, 18). These observations suggest that the effect of PS mutations on the γ -secretase activity and specificity is not mediated by simple loss of function of a restricted domain or motif.

A recent study suggests that γ -secretase complexes contain dimerized PS1 or PS2 and that two interfaces with a dias-

partyl combination in the TM region form catalytic sites (47). Noncompetitive inhibition by transition state analog inhibitors further indicates that the catalytic sites of γ -secretase are separated from the substrate-docking site(s) (50). Our mutagenesis study identified no restricted domain responsible for the cleavage specificity. Rather, our results imply that the mutational effects are dependent not only on the position of mutation but also on substituting amino acids. Additionally, the effects of PS1^{LA} mutations were accomplished without obvious changes in the composition or subcellular localization of the PS1 complex. These results are consistent with the hypothesis that the γ -secretase complex may have several conformational states linked to distinct γ -secretase activity and specificity. Speculatively, as a result of subtle conformational change by PS1^{LA} or FAD-linked missense mutations, substrates are presented in an aberrant position relative to the catalytic site, causing preferential cleavage at residue 42/43. This effect must be subtle and highly specific because different amino acid substitutions at the same residues in PS1 cause distinct modifications of γ -secretase activity.

DFK167 and sulindac sulfide are thought to shift the predominant cleavage site, probably through allosteric effects induced by direct PS binding (38, 47, 50, 51). Several of our observations suggest that the effects of PS1 mutations and the allosteric effects of γ -secretase inhibitors are likely to be tightly related, and we speculate that these effects might even be mediated by a shared mechanism. First, our results and previously published data (32–34) show that PS1 mutations clearly modify the response of γ -secretase to these inhibitors. Second, γ -42/43 cleavage enhanced by PS1^{LA} mutations is not further augmented by DFK167 treatment, suggesting that the effects of PS1 mutations and DFK167 treatment on A β _{42/43} up-regulation share the common plateau level. Third, similar to the case of the γ -secretase inhibitor effect, the effect of PS1 mutations on γ -42/43 cleavage is distinct from that on γ -40 cleavage and ϵ -cleavage. Fourth, the paradoxical effect of γ -secretase inhibitors can recover the activities that are inactivated by PS1 mutations. For example, DFK167 activates the endoproteolysis of PS1^{LA} holoproteins and activates γ -42/43 cleavage by WT-PS1, whereas sulindac sulfide activates γ -40 cleavage suppressed by PS1^{LA} mutations.

Taking these observations together, we speculate that various missense mutations of PS1 and the allosteric effects of γ -secretase inhibitors could modulate the cleavage specificity of γ -secretase by the same mechanism, namely a conformational change in the complex. Very recent pharmacological and indirect morphological assessment also indicates that nonsteroidal anti-inflammatory drugs alter PS1 conformation (51, 52). Furthermore, our results suggest a possible reciprocal relationship between γ -40 and γ -42/43 activities. Taken together, these results also suggest that small molecules could be designed to induce alternative PS complex conformations that are more favorable to the generation of A β ₄₀ rather than pathogenic A β _{42/43}. Such a strategy would obviate the complications of current γ -secretase inhibitor strategies that block all PS-dependent cleavage, including the cleavage of biologically important molecules like the Notch receptors.

Acknowledgments—We thank Drs. Raphael Kopan and Toshio Kitamura for Notch constructs and retrovirus vector, respectively, and Miwako Nishikawa for technical assistance. We acknowledge the Central Laboratory of the Shiga University of Medical Science for assistance in the DNA sequencing.

REFERENCES

- Haass, C., and Steiner, H. (2002) *Trends Cell Biol.* **12**, 556–562
- De Strooper, B. (2003) *Neuron* **38**, 9–12
- Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) *Cell* **100**, 391–398

4. Wang, R., Sweeney, D., Gandy, S. E., and Sisodia, S. S. (1996) *J. Biol. Chem.* **271**, 31894–31902
5. Chen, F., Gu, Y., Hasegawa, H., Ruan, X., Arawaka, S., Fraser, P., Westaway, D., Mount, H., and St George-Hyslop, P. (2002) *J. Biol. Chem.* **277**, 36521–36526
6. Selkoe, D. J. (2002) *J. Clin. Investig.* **110**, 1375–1381
7. Wolfe, M. S., and Haass, C. (2001) *J. Biol. Chem.* **276**, 5413–5416
8. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) *Neuron* **17**, 181–190
9. Podlisny, M. B., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, E., Fraser, P., Haass, C., Koo, E. H., Seubert, P., St George-Hyslop, P., Teplow, D. B., and Selkoe, D. J. (1997) *Neurobiol. Dis.* **3**, 325–337
10. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebbersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P., and St George-Hyslop, P. (2000) *Nature* **407**, 48–54
11. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) *Dev. Cell* **3**, 85–97
12. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) *Nature* **398**, 513–517
13. Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduveil, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafer, J. A., and Gardell, S. J. (2000) *Nature* **405**, 689–694
14. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) *Nature* **422**, 438–441
15. Edhauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) *Nat. Cell Biol.* **5**, 486–488
16. Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J., Capell, A., Meyn, L., Grim, M. L., Baumeister, R., Fichteler, K., and Haass, C. (2000) *Nat. Cell Biol.* **2**, 848–851
17. Tomita, T., Watabiki, T., Takikawa, R., Morohashi, Y., Takasugi, N., Kopan, R., De Strooper, B., and Iwatsubo, T. (2001) *J. Biol. Chem.* **276**, 33273–33281
18. Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C., and Steiner, H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 8025–8030
19. Wrigley, J. D., Nunn, E. J., Nyabi, O., Clarke, E. E., Hunt, P., Nadin, A., De Strooper, B., Shearman, M. S., and Behr, D. (2004) *J. Neurochem.* **90**, 1312–1320
20. Herreman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L., and De Strooper, B. (2002) *Nat. Cell Biol.* **2**, 461–462
21. Kitamura, T., Onishi, M., Kinoshita, S., Shibuya, A., Miyajima, A., and Nolan, G. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 9146–9150
22. Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D. M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Duthie, M., St George-Hyslop, P. H., and Fraser, P. E. (1998) *J. Biol. Chem.* **273**, 16470–16475
23. Schroeter, E. H., Kissinger, J. A., and Kopan, R. (1998) *Nature* **393**, 382–386
24. Komano, H., Shiraiishi, H., Kawamura, Y., Sai, X., Suzuki, R., Serneels, L., Kawauchi, M., Kitamura, T., and Yanagisawa, K. (2002) *J. Biol. Chem.* **277**, 39627–39633
25. Wang, J., Brunkan, A. L., Hecimovic, S., Walker, E., and Goate, A. (2004) *Neurobiol. Dis.* **15**, 654–666
26. Hartmann, T., Bieger, S. C., Bruhl, B., Tienari, P. J., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unsicker, K., and Beyreuther, K. (1997) *Nat. Med.* **3**, 1016–1020
27. Cook, D. G., Forman, M. S., Sung, J. C., Leight, S., Kolson, D. L., Iwatsubo, T., Lee, V. M., and Doms, R. W. (1997) *Nat. Med.* **3**, 1021–1023
28. Greenfield, J. P., Tsai, J., Gouras, G. K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S. S., Greengard, P., and Xu, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 742–747
29. Citron, M., Diehl, T. S., Gordon, G., Biere, A. L., Seubert, P., and Selkoe, D. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13170–13175
30. Zhang, L., Song, L., Terracina, G., Liu, Y., Pramanik, B., and Parker, E. (2001) *Biochemistry* **40**, 5049–5055
31. Weggen, S., Eriksen, J. L., Das, P., Sagi, S. A., Wang, R., Pietrzik, C. U., Findlay, K. A., Smith, T. E., Murphy, M. P., Bulter, T., Kang, D. E., Marquez-Sterling, N., Golde, T. E., and Koo, E. H. (2001) *Nature* **414**, 212–216
32. Xia, W., Ostaszewski, B. L., Kimberly, W. T., Rahmati, T., Moore, C. L., Wolfe, M. S., and Selkoe, D. J. (2000) *Neurobiol. Dis.* **7**, 673–681
33. Ikeuchi, T., Dolios, G., Kim, S. H., Wang, R., and Sisodia, S. S. (2003) *J. Biol. Chem.* **278**, 7010–7018
34. Weggen, S., Eriksen, J. L., Sagi, S. A., Pietrzik, C. U., Ozols, V., Fauq, A., Golde, T. E., and Koo, E. H. (2003) *J. Biol. Chem.* **278**, 31831–31837
35. Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B., Rahmati, T., Donkor, I. O., and Selkoe, D. J. (1999) *Biochemistry* **38**, 4720–4727
36. Behr, D., Wrigley, J. D., Owens, A. P., and Shearman, M. S. (2002) *J. Neurochem.* **82**, 563–575
37. Takahashi, Y., Hayashi, I., Tominari, Y., Rikimaru, K., Morohashi, Y., Kan, T., Natsugari, H., Fukuyama, T., Tomita, T., and Iwatsubo, T. (2003) *J. Biol. Chem.* **278**, 18664–18670
38. Behr, D., Clarke, E. E., Wrigley, J. D., Martin, A. C., Nadin, A., Churcher, I., and Shearman, M. S. (2004) *J. Biol. Chem.* **279**, 43419–43426
39. Campbell, W. A., Reed, M. L., Strahle, J., Wolfe, M. S., and Xia, W. (2003) *J. Neurochem.* **85**, 1563–1574
40. Steiner, H., Romig, H., Pesold, B., Philipp, U., Baader, M., Citron, M., Loetscher, H., Jacobsen, H., and Haass, C. (1999) *Biochemistry* **38**, 14600–14605
41. Behr, D., Wrigley, J. D., Nadin, A., Evin, G., Masters, C. L., Harrison, T., Castro, J. L., and Shearman, M. S. (2001) *J. Biol. Chem.* **276**, 45394–45402
42. Campbell, W. A., Iskandar, M. K., Reed, M. L., and Xia, W. (2002) *Biochemistry* **41**, 3372–3379
43. Nyabi, O., Bentahir, M., Horre, K., Herreman, A., Gottardi-Littell, N., Van Broeckhoven, C., Merchiers, P., Spittaels, K., Annaert, W., and De Strooper, B. (2003) *J. Biol. Chem.* **278**, 43430–43436
44. Jacobsen, H., Reinhardt, D., Brockhaus, M., Bur, D., Kocyba, C., Kurt, H., Grim, M. G., Baumeister, R., and Loetscher, H. (1999) *J. Biol. Chem.* **274**, 35233–35239
45. Shirota, K., Takahashi, K., Araki, W., Maruyama, K., and Tabira, T. (2000) *J. Biol. Chem.* **275**, 3681–3686
46. Mercken, M., Takahashi, H., Honda, T., Sato, K., Maruyama, M., Nakazato, Y., Noguchi, K., Imahori, K., and Takashima, A. (1996) *FEBS Lett.* **389**, 297–303
47. Schroeter, E. H., Ilagan, M. X., Brunkan, A. L., Hecimovic, S., Li, Y. M., Xu, M., Lewis, H. D., Saxena, M. T., De Strooper, B., Coonrod, A., Tomita, T., Iwatsubo, T., Moore, C. L., Goate, A., Wolfe, M. S., Shearman, M., and Kopan, R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13075–13080
48. Godbolt, A. K., Beck, J. A., Collinge, J., Garrard, P., Warren, J. D., Fox, N. C., and Rossor, M. N. (2004) *Neurology* **63**, 1702–1704
49. Doan, A., Thinakaran, G., Borchelt, D. R., Slunt, H. H., Ratovitsky, T., Podlisny, M., Selkoe, D. J., Seeger, M., Gandy, S. E., Price, D. L., and Sisodia, S. S. (1996) *Neuron* **17**, 1023–1030
50. Tian, G., Sobotka-Briner, C. D., Zysk, J., Liu, X., Birr, C., Sylvester, M. A., Edwards, P. D., Scott, C. D., and Greenberg, B. D. (2002) *J. Biol. Chem.* **277**, 31499–31505
51. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Diehl, T. S., Moore, C. L., Tsai, J. Y., Rahmati, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) *Nat. Cell Biol.* **2**, 428–434
52. Lleo, A., Berezovska, O., Herl, L., Raju, S., Deng, A., Bacskai, B. J., Frosch, M. P., Irizarry, M., and Hyman, B. T. (2004) *Nat. Med.* **10**, 1065–1066

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

NAN ZENG,^a AKIRA NAKAJIMA,^a JUE HE,^a ATSUMI NITTA,^a KIYOFUMI YAMADA,^b AND TOSHITAKA NABESHIMA^a

^a*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan*

^b*Division of Life Science, Kanazawa University Graduate School of Natural Science & Technology, Kanazawa 920-1192, Japan*

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-

Address for correspondence: Toshitaka Nabeshima, Ph.D., Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8560, Japan. Voice: +81-52-744-2674; fax: +81-52-744-2682. tnabeshi@med.nagoya-u.ac.jp

Ann. N.Y. Acad. Sci. 1025: 236–241 (2004). © 2004 New York Academy of Sciences.
doi: 10.1196/annals.1316.029

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, $23 \pm 0.5^\circ\text{C}$, $50 \pm 0.5\%$ 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
Somatosensory	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 mm². 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 learning¹³ and in behavioral response to motivationally significant stimuli 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.

ACKNOWLEDGMENTS

This study was supported in part by Special Coordination Funds for Promoting Science and Technology, the Target-Oriented Brain Science Research Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Grant-in-Aid for Health Science Research from the Ministry of Health, Labour and Welfare of Japan, by the Japan Society for the Promotion of Science Joint Research Project under the Japan-Korea Basic Scientific Cooperation Program, and by a Smoking Research Foundation Grant for Biomedical Research.

REFERENCES

1. MUNZAR, P. & S.R. GOLDBERG. 2000. Dopaminergic involvement in the discriminative-stimulus effects of methamphetamine in rats. *Psychopharmacology* **148**: 209–216.
2. KAMIEN, J.B. *et al.* 1993. Drug discrimination by humans compared to nonhumans: Current status and future directions. *Psychopharmacology* **111**: 259–270.
3. MORI, A. *et al.* 2001. Phencyclidine-induced discriminative stimulus is mediated via phencyclidine binding sites on the N-methyl-D-aspartate receptor-ion channel complex, not by signal receptors. *Behav. Brain Res.* **119**: 33–40.
4. MORGAN, J.I. & T. CURREN. 1991. Stimulus-transcription coupling in the nervous system: involvement of inducible proto-oncogenes fos and jun. *Annu. Rev. Neurosci.* **14**: 421–451.
5. UMINO, A. *et al.* 1995. Methamphetamine-induced nuclear c-Fos in rat brain regions. *Neurochem. Int.* **26**: 85–90.
6. NAMIMA, M. *et al.* 1998. Lithium inhibits the reverse tolerance and c-Fos expression induced by methamphetamine in mice. *Brain Res.* **782**: 83–90.
7. COLE, R.L. *et al.* 1995. Neuronal adaptation to amphetamine and dopamine molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* **14**: 813–823.
8. HE, J. *et al.* 2002. A role of Fos expression in the CA3 region of the hippocampus in spatial memory formation in rats. *Neuropsychopharmacology* **26**: 259–268.
9. YAMADA, K. *et al.* 1996. Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats. *Neurosci. Res.* **24**: 393–402.
10. MUNZAR, P. *et al.* 1999. Effects of dopamine and serotonin-releasing agents on methamphetamine discrimination and self-administration in rats. *Psychopharmacology* **141**: 287–296.
11. SCHULTZ, W. 1998. Predictive reward signal of dopamine neurons. *J. Neurophysiol.* **80**: 1–27.
12. HOLLERMAN, J.R. & W. SCHULTZ. 1998. Dopamine neurons report an error in the temporal prediction of reward during learning. *Nat. Neurosci.* **1**: 304–309.

13. KELLY, A.E. *et al.* 1997. Response-reinforcement learning is dependent on N-methyl-D-aspartate receptor activation in the nucleus accumbens core. *Proc. Natl. Acad. Sci. USA* **94**: 12174–12179.
14. BASSAREO, V. & G. DI CHARA. 1999. Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. *Neuroscience* **89**: 637–641.
15. PARKINSON, J.A. *et al.* 1999. Dissociation in effects of lesions of the nucleus accumbens core and shell in appetitive Pavlovian approach behavior and the potentiation of conditioned reinforcement and locomotor activity by D-amphetamine. *J. Neurosci.* **19**: 2401–2411.



Role of tumor necrosis factor- α in down-regulation of hepatic cytochrome P450 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,*}

^aDepartment of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-867, Japan

^bFaculty of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan

^cDepartment of Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan

^dDepartment of Laboratory Medicine, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

^eDepartment 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 P450 (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 .

© 2004 Elsevier B.V. All rights reserved.

Keywords: Tumor necrosis factor- α (TNF- α); Cytochrome P450 (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 P450 (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).

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) (Haghighi 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; Wachter 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, 6 β -hydroxytestosterone, 16 α -hydroxytestosterone, and 2 α -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- α ^{-/-} 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- α ^{-/-} mice 6 h after injection of endotoxin (10 mg/kg) or saline. Antipyrine (20 mg/kg) was administered intravenously in wild-type and TNF- α ^{-/-} 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 anesthetized 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 concen-

tration 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 MgCl₂, 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 μ l of 100 mM phosphate-buffered incubation medium (pH 7.4) containing 3.3 mM MgCl₂, 1.5 mM β -NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase (G6PD), and 50 μ l 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 μ l 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 μ l of 250 mM 11 α -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 μ g) 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-10Aadvp, 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 5C₁₈ (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-α^{-/-} 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-α^{-/-} 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-α^{-/-} mice.

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

The time-dependent effects of endotoxin on the expression of P-glycoprotein in wild-type mice and TNF-α^{-/-} 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-α^{-/-} 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-α^{-/-} mice. To evaluate in vivo whether the function of P-glycoprotein is maintained

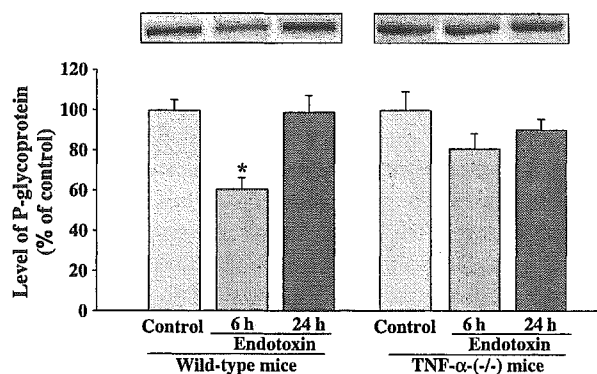


Fig. 1. Time-dependent effects of endotoxin on hepatic expression of P-glycoprotein in wild-type and TNF-α^{-/-} mice. Results are represented as percent of control levels. Values are shown as mean±S.E.M. (*n*=4–6). *Significantly different from control (*P*<0.05).

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 $\mu\text{g/ml}$, 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, 6 β -hydroxytestosterone, and both 16 α -hydroxytestosterone and 2 α -hydroxytestosterone are indicative of CYP3A2 and CYP2C11 enzymes, respectively (Takahashi et al., 1999).

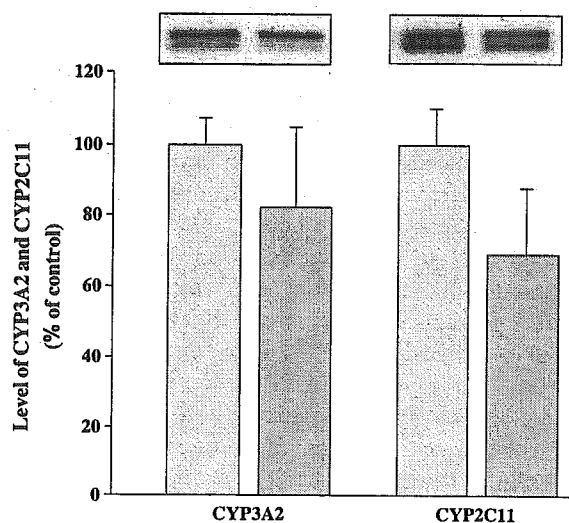


Fig. 2. Constitutive expression of CYP3A2 and CYP2C11 in liver of wild-type and TNF- $\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean \pm S.E.M. ($n=4-6$).

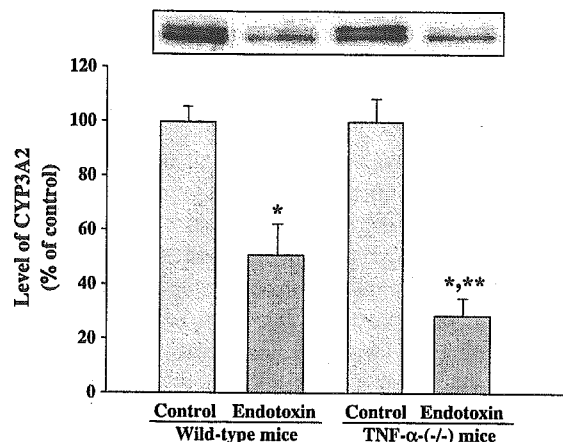


Fig. 3. Effect of endotoxin on hepatic expression of CYP3A2 in wild-type and TNF- $\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean \pm S.E.M. ($n=4-6$). *Significantly different from control ($P<0.05$). **Significantly different from endotoxin-treated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

Therefore, we measured the formation rate of 6 β -hydroxytestosterone, 16 α -hydroxytestosterone, and 2 α -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 6 β -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 16 α -hydroxytestosterone and 2 α -hydroxytestosterone. The formation rate of 16 α -hydroxytestosterone and 2 α -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.

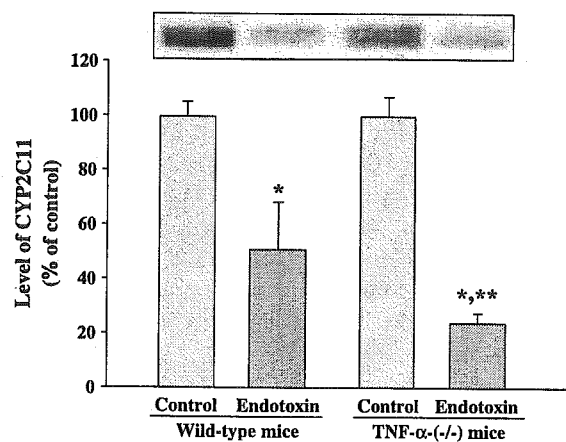


Fig. 4. Effect of endotoxin on hepatic expression of CYP2C11 in wild-type and TNF- $\alpha^{-/-}$ mice. Results are represented as percent of control levels. Values are shown as mean \pm S.E.M. ($n=4-6$). *Significantly different from control ($P<0.05$). **Significantly different from endotoxin-treated 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 $\mu\text{g/ml}$, and its value had been increased twofold by the injection of endotoxin (0.45 ± 0.08 $\mu\text{g/ml}$). On the other hand, the concentration in TNF- $\alpha^{-/-}$ mice was 0.40 ± 0.02 $\mu\text{g/ml}$, and its value had been increased threefold by injection of endotoxin (1.26 ± 0.19 $\mu\text{g/ml}$). 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.

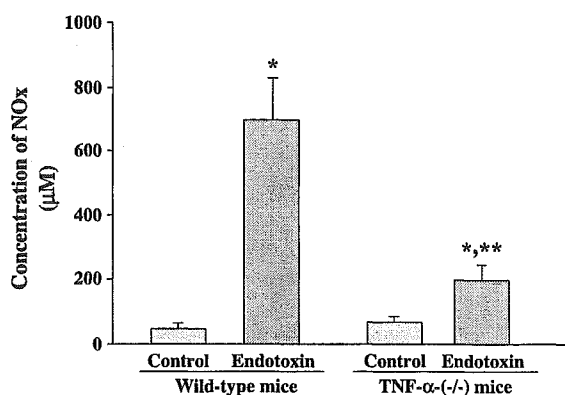


Fig. 5. Effect of endotoxin on plasma concentrations of NO_x in wild-type and TNF- $\alpha^{-/-}$ mice. Values are shown as mean \pm S.E.M. ($n=3$). *Significantly different from control ($P<0.05$). **Significantly different from endotoxin-treated wild-type mice ($P<0.05$).

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 P450-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 P450 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 P4502E1 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.
- Haghighi, 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 P450 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 P450-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 P450 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 P450: 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 P450 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 P450. *Biochem. Pharmacol.* 52, 1195–1200.
- Morgan, E.T., 1997. Regulation of cytochromes P450 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 P450 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 P450s 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 P450-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., Furuhashi, Y., Ikeda, A., Takahashi, M., Iwata, H., Kazusaka, A., Fujita, S., 1999. Characterization of hepatic cytochrome P450 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 P450 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., Koepfel, 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 P450 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 P450 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 Shen² and Shoei Furukawa^{CA}

Laboratory of Molecular Biology, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585; ¹Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan; ²Laboratory 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 hemitranssected 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'-GAGAGGAATCGGCAGGCTGCA-GCTG-3' and down-primer 5'-CAGATACATCCACATC-GTTTAGCGG-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'-CGGAGT-CAACGGATTTGGTCGTAT-3' and down-primer 5'-AGCC-TTCTCCATGGTGGTGAAGAC-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 μ g LPS into the injury site of the rat spinal cord (Fig. 1a). Administration of 10 μ g 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 μ m 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

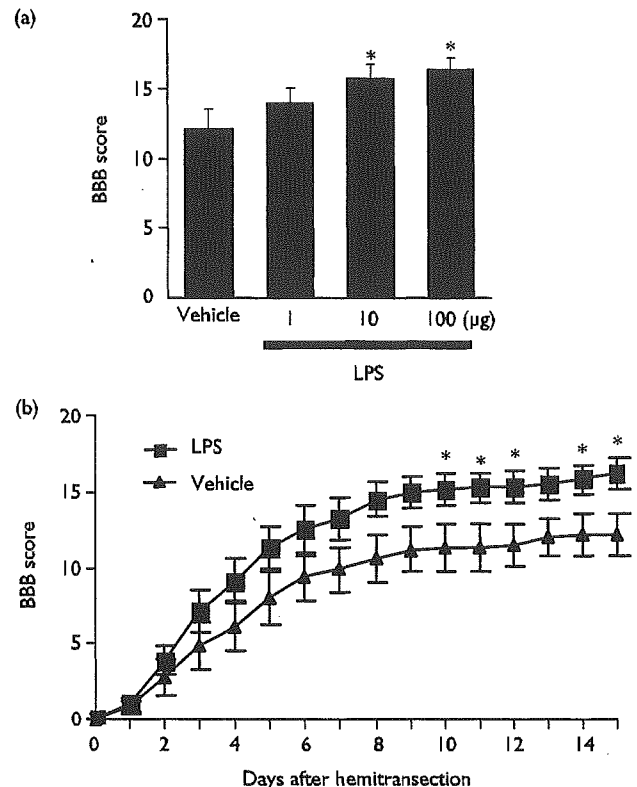


Fig. 1. Effect of LPS on locomotor function of rats with spinal cord injury. The left hemisphere of the spinal cord was transected, and a single injection (10 μ l) of 1, 10 or 100 μ g LPS or vehicle (PBS) was then administered to the injury site. (a) Locomotor function of the hind limb on the injured side was evaluated 14 days after the injury. The values are expressed as the means \pm 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 μ g; squares) or vehicle (triangles). The values are expressed as means \pm 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. * $p < 0.05$.

CD11b antigen or GDNF-ir were thus significantly increased in number after LPS administration.

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