BIOMEDICAL AND ENVIRONMENTAL SCIENCES **18**, 181-186 (2005)

Effect of Dexamethasone on Nitric Oxide Synthase and Caspase-3 Gene Expressions in Endotoxemia in Neonate Rat Brain

HUA WANG[*,1], YU-BIN WU[*], AND XIU-HUA DU[#]

[*]*Department of Pediatrics, Second Clinical College, China Medical University, Shenyang 110004, Liaoning, China*;
[#]*Department of Pediatrics, Benxi Woman and Child Hospital, Benxi 117000, Liaoning, China*

Objective To investigate the gene and protein expressions of three isoforms of nitric oxide synthase (NOS) and gene expression of Caspase-3, and effect of dexamethasone on them in neonatal rats with lipopolysaccharide (LPS)-induced endotoxemic brain damage. **Methods** Expressions of the three isoforms of NOS and caspase-3 mRNA in the brain were investigated by RT-PCR in postnatal 7-day wistar rats with acute endotoxemia by intraperitoneal administration of LPS. Regional distributions of NOSs were examined by immunohistochemical technique. **Results** nNOS and Caspase-3 mRNA were obviously detected. eNOS mRNA was faintly expressed, but iNOS mRNA was undetectable in the control rat brain. The expressions of NOS mRNA of three isoforms were weak 2 h after LPS (5 mg/mg) delivery, peaked at 6 h, and thereafter, reduced gradually up to 24 h. The expression intensity was in the order of nNOS> iNOS> eNOS. Widespread nNOS, scattered eNOS distribution and negative iNOS were identified in the control rat brain and all isoforms of NOS could be induced by LPS which reached the apex at 24 h in the order of nNOS> iNOS> eNOS as detected by immunostaining. Although Caspase-3 mRNA could be found in all groups, DNA fragmentation was only seen at 6 h and 24 h. The expressions of NOS and Caspase-3 mRNA were inhibited in the rat brain when dexamethasone was administrated. **Conclusion** LPS-induced NO production induces apoptosis of neurons through mechanism involving the Caspase-3 activation, which may play an important role in the pathogenesis of brain damage during endotoxemia, and neuro-protective effects of dexamethasone may be partially realized by inhibiting the expression of NOS mRNA.

Key words: Lipopolysaccharide; Endotoxemia; Brain; Nitric oxide synthase; Caspase-3

INTRODUCTION

Fifty percent to seventy percent of the patients are complicated with brain damage at various degrees during endotoxemia, which is characterized by damage to the neurons, microvascular injury, increased endothelial permeability, and neutrophil infiltration and accumulation in the brain. It has been recognized that bacterial endotoxin (lipopolysaccharide, LPS) plays important roles in this pathophysiological process, but the mechanism and approach to prevention of brain damage during endotoxemia are not well understood.

Nitric oxide (NO) is a transient gaseous second messenger molecule functioning in vascular regulation, immunity, and neurotransmission, which is formed from L-arginine by nitric oxide synthase (NOS). Hitherto, at least three isoforms of NOS have been identified, namely neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). In the brain, the three isoforms of NOS are expressed constitutively or inducibly, and implicated in a number of physiological and pathophysiological functions[1].

The purpose of the present study was to investigate the effect of dexamethasone on the expression of NOS isoforms in the brain in a LPS-induced endotoxemic neonatal rat animal model. Meanwhile, Caspase-3 mRNA expression was also observed to clarify the relationship between expression of NOS and apoptosis of cells during LPS endotoxemia.

METHODS

Animal Model Preparation and Grouping

Sixty eight healthy wistar rats on postnatal day

[1]Correspondence should be addressed to Hua WANG, Tel: 86-24-83955505. E-mail: wanghua62@hotmail.com or hwang@mail.cmu.edu.cn

Biographical note of the first author: Hua WANG, female, born in 1962, M. D. and Ph. D., vice head of the section of pediatric neurology, associate professor, mainly engaged in the study on regeneration and renovation of neural tissue.

0895-3988/2005
CN 11-2816/Q

7(PND7) of either sex with body weight ranging 17 ±3 g were provided by Animal Breeding Center of our university. Animals were randomly divided into three groups with 20 rats in each group. Eight rats expired during the experiment and were excluded from the experiment. Control group: 100 μL saline was given by ip to each rat. LPS group: rats were injected with *Escherichia coli* LPS (serotype 055:B5, Sigma, USA) at 5 mg/kg body weight in 100 μL saline by ip. LPS plus dexamethasone (DXM) group: 5 mg/kg LPS and DXM (10 mg/kg) in 100 μL saline were given. Animals were sacrificed by cervical vertebral dislocation 2, 4, 6, and 24 h after saline, LPS or LPS plus DXM administration, respectively. Total cerebral tissue was quickly isolated and snap-frozen in liquid nitrogen and kept at -80℃ for use.

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) and PCR

Total RNA was extracted from the cerebral tissue by the guanidinium thiocyanate-phenol-chloroform method. After RNA concentration was determined, cDNA was synthesized from total RNA (5 μg) using 5 μL 2× buffer, 2 μL 2 mol.L1 $MgSO_4$, 0.5 μL 10 mol.L1 dNTPs, 0.25 μL (40 u/μL) RNase inhibitor, 0.5 μL Bca BEST polymerase with total reaction volume of 10 μL.

PCR was performed using 3 μL of cDNA, 0.5 μL Taq polymerase (TaKaRa, Japan), 2 μL of dNTPs, and 1 μL of aimed oligonucleotide primer in a total 25 μL reaction volume. All primers were designed according to the published cDNA sequences and synthesized by the AOKE Biotechnology Co. Ltd., Peking, China. nNOS forward: GAATACCAGCCT-GATCCATGGAA; nNOS reverse: GTACGCCACCT-GTGGGAGGACCTCCT. eNOS forward: TACGGAGCAGCAAATCCAC; eNOS reverse: CTAGTTTCCTGACGTCGGAC. iNOS forward: CTACCTACCTGGGGAACACCTGGGGG; iNOS reverse: GGCGATGATAGGTAGCCGAGGAGG. Caspase-3 forward: TGAGCATTGACACAATACAC; Caspase-3 reverse: CTACTTCTCAAAGCCGAA. β-actin forward: TGTATGCCTCTGGTCGTACCAC; β-actin reverse: GAGGACTCGCGTTCATGAGACA. All PCR procedures were carried out for each cycle with denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 2 min. After 35 cycles there was an extension step at 72℃ for 7 min. As PCR control, redistilled water was substituted for reverse transcriptase. The PCR products were electrophoresized in 2% agarose gel containing ethidium bromide and visualized under UV illumination. Product concentration was analyzed by an automatic analyser (Kodak, type 1D) and photograghed by an Olympus camera.

Immunohistochemical Staining

Rat brains were collected and fixed in 4% ice-cold paraformaldehyde buffered with PBS. Five μm thick paraffin sections were made. Immunohistochemical staining was performed with rabbit anti-rat nNOS, iNOS or eNOS polyclonal antibody (IgG, Santa Cruz, USA) as the primary antibody, respectively. The sections were visualized by streptavidin-biotin peroxidase method with DAB as the chromogen. Nuclei were counter-stained with hematoxylin. As negative control, the primary antibodies were replaced by either non-immunized rabbit IgG or PBS at each staining, and no false positive staining was detected.

Statistical Analysis

Data were displayed as $\bar{x} \pm s$. Statistical significance was assessed with Student's *t* test using SPSS 10.0 for Windows. $P<0.05$ was considered statistically significant.

RESULTS

Induction of Three Isoforms of NOS mRNA Expression in the Brain by LPS Treatment

nNOS mRNA was obviously detected. eNOS mRNA was faintly expressed, but iNOS mRNA was undetectable in the control rat brain. Two h after LPS treatment, the three isoforms of NOS mRNA began to increase, reached peak at 6 h, and persisted to 24 h after LPS treatment. The expression of nNOS mRNA was the most abundant as compared with the other two isoforms, followed by that of iNOS mRNA with the least eNOS mRNA (Table 1, Fig. 1).

Regional Distribution of NOS Isoform Protein Expression After LPS Treatment

Immunohistochemical assay revealed a widespread nNOS distribution in the control rat brain. However, the staining intensity of nNOS varied with different parts of the brain. The most intensive staining was mainly localized in hypothalamus and paraventricular nuclei. Only several pyramidal cells revealed positive immunostaining in cerebral cortex, whereas eNOS protein was sporadically detected. iNOS protein expression was not detectable in any region of the control rat brain. Expressions of the three NOS isofrom proteins were induced by LPS treatment. The time course showed that LPS-induced nNOS and eNOS expressions were increased 6 h after LPS treatment, and reached maximum at 24 h. iNOS was

sparsely expressed 6 h after LPS treatment, and strongly expressed at 24 h with its intensity in order of nNOS>iNOS>eNOS. In addition, stronger staining of eNOS was detected at hypothalamus, paraventricular nuclei, and less intensive staining in the hippocampus, cerebral cortex (including pyramidal and multiform neurons), and endothelial cells of blood vessels (Fig. 2).

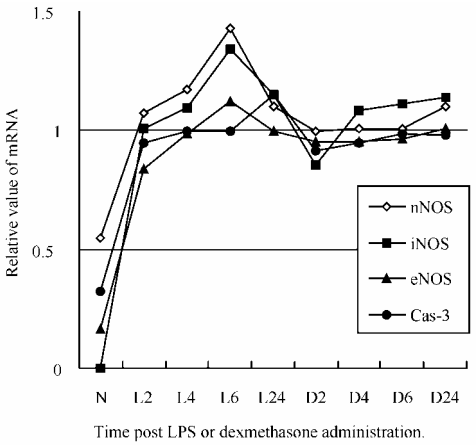

FIG. 1. Time-dependent expressions of three isoforms of NOS and Caspase-3 mRNA during endotoxemia in neonate rat brain.

TABLE 1

Changes of nNOS mRNA Expression During Endotoxemia in Neonate Rat Brain

Group	*n*	0 h	2 h	4 h	6 h	24 h
LPS	5	0.563±0.058	1.071±0.035	1.174±0.027	1.428±0.063	1.098±0.048
t Value				4.509	3.87	1.03
P Value				<0.05	<0.05	>0.05
DEX	5		0.875±0.033	1.004±0.056	1.011±0.078	1.088±0.064
t Value			8.117	1.705	1.326	0.571
P Value			<0.01	>0.05	>0.05	>0.05

Note. The table shows comparison of LPS groups at 4, 6, 24 h or DEX groups at 2, 4, 6, 24 h with LPS group at 2 h. *t* values of all LPS and DEX groups ranged 17.305-5.121 with *P* values<0.01 as compared with control group.

Effect of DXM on the LPS-induced NOS Expression

The expressions of the three isoforms of NOS mRNA were distinctively inhibited 2-4 h after LPS plus DXM administration, and the expressions of the three NOS isoform proteins were remarkably inhibited 6 h after LPS plus DXM treatment, which persisted up to 24 h. The strongest DXM inhibiting action was seen on the expression of iNOS, followed by nNOS with least effect on eNOS. Effects of DXM on the LPS-induced NOS protein expression were the same as the effect of DXM on NOS mRNA expression (Figs. 1 and 2).

Effect of DXM on LPS-induced Caspase-3 mRNA Expression

Caspase-3 mRNA was faintly expressed in the brain of control rats, and increased 2, 4, and 6 h after LPS-treatment, reached peak at 24 hrs time point. Caspase-3 mRNA expression was inhibited partially 2 h after DXM administration, and the inhibition was gradually decreased. Twenty-four hrs after DXM administration, the expression of Caspase-3 mRNA was still lower than the group without DXM administration (Figs. 1 and 2).

DISCUSSION

In the present study, we demonstrated that LPS activated all the three types of NOS gene in the brain, and protein expressions were detected by immunohistochemical assay. Although peripheral administration of LPS was reported to increase the expression of NOS mRNA in the brain, a detailed analysis of three NOS isoform genes and protein expression and distribution in the normal brain and

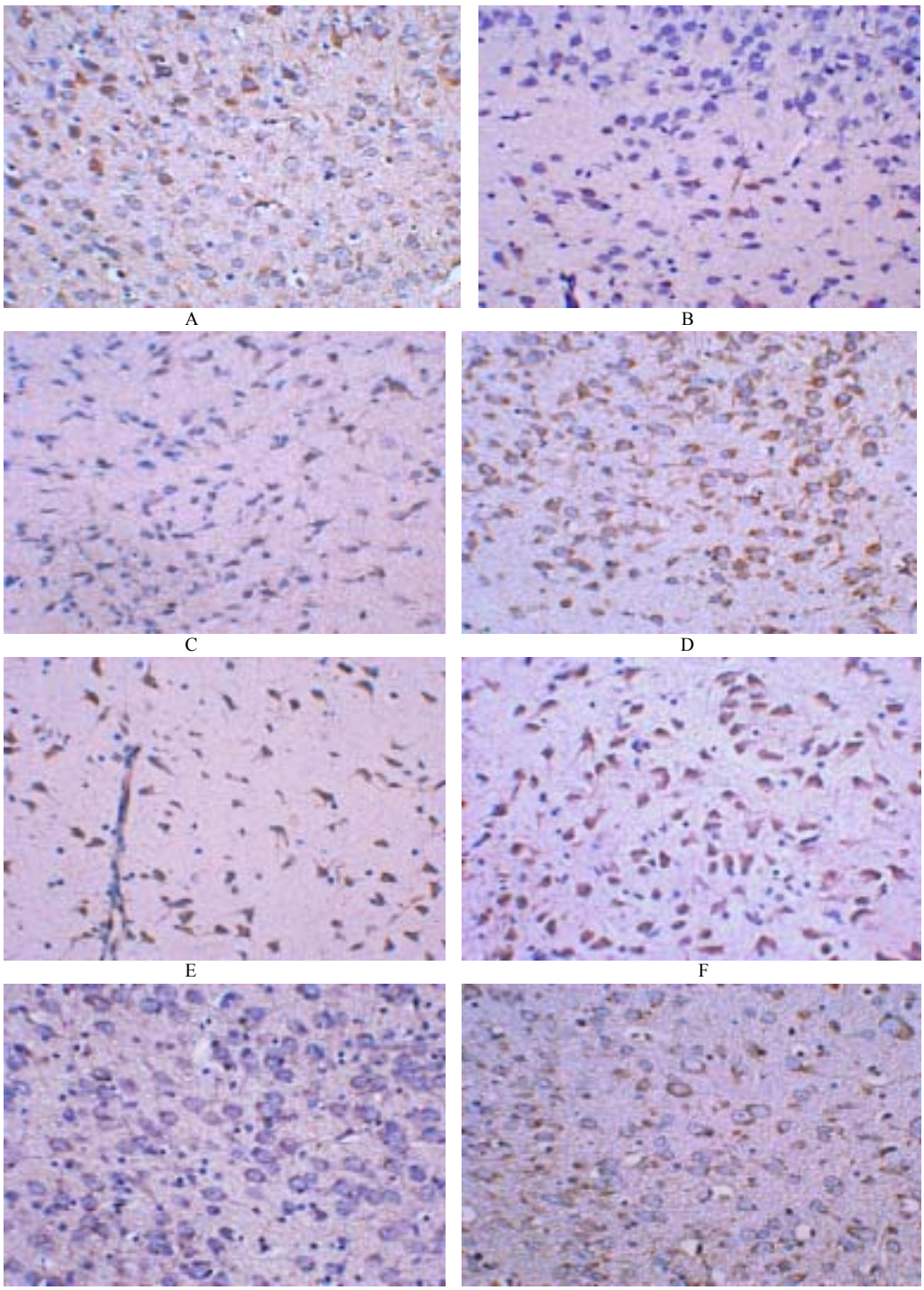

FIG. 2. Immunohistochemical detection of three isoforms of NOS protein during endotoxemia in neonate rat brain (DAB, original magnification ×400). A: Scattered expression of nNOS in pyramidal cells of cerebral cortex in control neonate rat brain. B: Faint expression of eNOS detected at paraventricular nuclei in control neonate rat brain. C: Expression of nNOS at paraventricular nuclei of cerebral cortex in LPS group at 24 h in neonate rat brain. D: Expression of iNOS in cerebral cortex in LPS group at 6 h in neonate rat brain. E: Expression of iNOS detected at paraventricular nuclei in LPS group at 24 h in neonate rat brain. F: Expression of eNOS detected at paraventricular nuclei in LPS group at 24 h in neonate rat brain. G: Expression of nNOS in pyramidal cells of cerebral cortex in DEX group at 6 h in neonate rat brain. H: Decreased expression of eNOS detected in cerebral cortex in DEX group at 24 h in neonate rat brain.

the brain after LPS-treatment has not yet been documented. Our results showed that nNOS mRNA was expressed in normal neonate rat brain. In addition, a widespread nNOS and eNOS protein distribution throughout the PND7 control rat brain was detected by the nNOS and eNOS antibody, but the latter was in a less extent. The present study indicated that, in the forebrain and midbrain, the distribution pattern of nNOS- or eNOS-containing neurons was fundamentally completed at birth, which was consistent with previous studies[1-2].

A number of studies on NOS expression induced by LPS treatment have been reported recently. Kawahara *et al.* reported that iNOS mRNA in murine microglial MG5 cells was induced 2 h after 1 μg/mL LPS treatment, reached a maximum at 6-12 h, and then decreased slowly, but remained at high levels at 24-48 h. iNOS protein began to increase 6 h after LPS stimulation, increased up to 24 h and was somewhat decreased at 48 h, suggesting that iNOS protein was induced later than its mRNA[3]. The time-course of iNOS and nNOS was different, and iNOS expression was delayed in comparison with nNOS expression. nNOS mRNA was significantly increased at 2 h and peaked at 5 h, while significantly increased induction of iNOS was first detected at 3h. Differential dose dependence in induction of brain eNOS and iNOS mRNAs by LPS also existed. eNOS mRNA was substantially induced with LPS at 2.5 μg/g, reaching a plateau level at 10 μg/g. On the other hand, only a slight increase in iNOS mRNA was detected at 10 μg/g, and a maximum increase at 50 μg/g[4], indicating that in the pathophysiological dose range, higher amount of LPS is required for induction of iNOS mRNA than in that of eNOS mRNA. We observed three isoforms of NOS in PND7 rat brain at 5 mg/kg LPS level, and the results revealed that NOSs mRNA was induced by LPS with its intensity in order of nNOS> iNOS> eNOS, whereas immunohistochemical staining showed that iNOS protein expression was less induced than that of nNOS and eNOS, with its intensity in order of nNOS> eNOS >iNOS at the same LPS level. The reason for this phenomenon is not known, but it might be due to the immature of blood-brain-barrier compared with adult rat brain.

The mechanism underlying NOS expression has not been clarified. It is known that activities of nNOS and eNOS are highly dependent on prevailing Ca^{2+} concentrations. Glutamine binding to N-methyl-asparate (NMDA) receptors could increase the levels of intracellular Ca^{2+}, which in turn could activate nNOS and eNOS via calmodulin for the mediation of a series of rapid events[5]. On the other hand, iNOS is relatively independent of the calcium melieu. In addition, iNOS has promotor elements, which confer LPS inducibility. Such a distinct manner of gene expression may contribute to differential temporal expression patterns and distributions of NOS isoforms in immune challenge[6].

Many experiments have demonstrated that induction of NOS by LPS and pro-inflammatory cytokines could result in brain damage, and the severity was positively related with the amount of LPS and cytokines[7]. NO mediated neuronal cell death occurs through both necrotic and apoptotic cell death pathways. In cortical culture, mild excitation free radicals insults could lead to delayed cell death dominated by apoptotic features, whereas intense exposure to a high concentration of NMDA or $ONOO^-$ might induce necrotic cell death. Activity of nNOS by LPS could cause early neuronal cell death, and activation iNOS might contribute to delayed neuronal cell death, but expression of eNOS is neuroprotective[8]. In the present study, an increase in expression of Caspase-3 mRNA was detected and NOSs expression was found after LPS-treatment, in addition to the faint expression of Caspase-3 mRNA under normal condition. Foreign researchers have also found the relationship between Caspase-3 mRNA expression and the dosage and duration of LPS[9-10].

Neuro-protection of DEX has attracted the attention of researchers. Zhang *et al.* found that DEX could attenuate iNOS mRNA expression and NO production induced by LPS through inhibiting NF-κB activity by the induction of IκB synthesis[11], and reduce neurotoxicity caused by NO production[12-13]. Our study showed that DXM could inhibit expressions of nNOS, iNOS mRNA induced by 5 mg/kg, with a little effect on that of eNOS. DXM attenuated Caspase-3 mRNA expression, peaked at 2-6 h, and persistent to 24 h after DXM administration, suggesting that neuroprotection role of DXM was realized by preventing NO and apoptosis pathways. The mechanism of this role of DXM is not known till now. Some researchers found that DXM could up-regulate bcl-x mRNA expression which inhibited apoptosis[14] and down-regulate mRNA expression of early-rapid reaction gene c-jun[15]. But the exact mechanism remains to be further studied.

REFERENCES

1. Doris, B.D., Heinemann, T., Lan, J., Pederrsen, V., Leblhuuuber, F., Paulus, W., Riederr, P., and Gerlach, M. (1999). Characterization and regional distribution of nitric oxide synthase in the human brain during normal ageing. *Brain Res.* **834**(1), 128-135.

2. Terada, H., Nagai T, Okada S Kimura H., and Kitahama, K. (2001). Ontogenesis of neurons immunoreactive for nitric oxide synthase in rat forebrain and midbrain. *Brain Res. Dev. Brain Res*. **128**(2), 121-137.
3. Kawahara, K., Gotoh, T., Oyadomari, S., Kajizono, M., Kuniyasu, A., Ohsawa, K., Imai, Y., Kohsaka, S., Nakayama, H., and Mori, M. (2001). Co-induction of argininosuccinate synthase, cationic amino acid transporter-2, and nitric oxide synthase in activated morine microglial cells. *Brain Res. Mol. Brain Res*. **90**(2), 165-173.
4. Harada, S., Imaki, T., Chikada, N., Naruse, M., and Demura, H. (1999). Distinct distribution of time-course changes in neuronal nitric oxide synthase and iNOS in the laraventricular nucleus following LPS injection. *Brain Res*. **821**(1-2), 322-332.
5. Iwase, K., Miyanaka, K., Shimizu, A., Nagasaki, A., Gotoh, T., Mori, M., and Takiguchi, M. (2000). Induction of endothelial nitric oxide synthase in rat brain astrocytes by systemic LPS treatment. *J. Biol. Chem*. **275**(16), 11929-11933.
6. Son H., Hawkins R.D., Martin K, Kiebler, M., Huang, P. L., Fishman, M. C., and Kandel, E. R. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* **87**(6), 1015-1023.
7. Araki, E., Forster, C., Dubinsiky, J. M., Ross, M. E., and Iadecola, C. (2001). Cyclooxygenase-2, inhibitor ns-398 protects neuronal cultures from LPS-induced neurotoxicity. *Stroke* **32**(10), 2370-2375.
8. Sandani, A. F., Dawson, T. M., and Dawson, V. L. (1997). Nitric oxide synthase in models of focal ischemia. *Stroke* **28**(6), 1283-1289.
9. Heneka, M. T., Loschmann, P. A., Gleichmann, M., Weller, M., Schulz, J. B., Wullner, U., and Klockgether, T. (1998). Induction of nitric oxide synthase and nitric oxide-mediated apoptosis in neuronal PC12 cells after stimulation with tumor necrosis factor-alpha/lipopolysaccharide. *J. Neurochem*. **71**(1), 88-94.
10. Lee, J., Hur, J., Lee, P., Kim, J. Y., Cho, N., Kim, S. Y., Kim, H., Lee, M., and Suk, K. (2001). Dual role of inflammatory stimuli in activation-induced cell death of mouse microglial cells. *J. Biol. Chem*. **276**(35), 32956-32965.
11. Zhang, W. Y., Gotoh T, Oyadomari, S., and Mori, M. (2000). Coinduction of inducible nitric oxide synthase and arginine recycling enzymes in cytokine-stimulated PC12 cells and high output production of nitric oxide. *Brain Res. Mol. Brain Res*. **83**(1-2), 1-8.
12. Rieger, J., Stander, M., Loschmann, P. A., Heneka, M., Dichgans, J., Klockgether, *T.*, and Weller, M. (1998). Synthesis and biological effects of NO in malignant glioma cells: modulation by cytokines including CD95L and TGF-beta, dexamethasone, and p53 gene transfer. *Oncogene* **17**(18), 2323-2332.
13. Flavin, M. P., Ho, L. T., and Coughlin, K. (1997). Neurotoxicity of soluble macrophage products *in vitro*-influence of dexamethasone. *Exp. Neurol*. **145**(2pt1), 462-470.
14. Gorman, A. M., Hirt, U. A., Orrenius, S., and Ceccatelli, S. (2000). Dexamethasone pre-treatment interferes with apoptotic death in glioma cells. *Neuroscience* **96**(2), 417-425.
15. Macaya, A., Munell, F., Ferrer, I., de Torres, C., and Reventos, J. (1998). Cell death and associated c-jun induction in perinatal hypoxia-ischemia. Effects of the neuroprotective drug dexamethasone. *Brain Res. Mol. Brain Res*. **56**(1-2), 29-37.

(*Received December* 21, 2003 *Accepted May* 10, 2004)