ELSEVIER

European Journal of Pharmacology 321 (1997) 343–347

ejp

Interleukin-6 increases the levels of cyclic GMP and nitrite in rat hippocampal slices

Tiancai Ma, Xingzu Zhu *

Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China

Received 18 July 1996; revised 28 November 1996; accepted 3 December 1996

Abstract

We examined the effect of interleukin-6 on the levels of cGMP and nitrite in rat hippocampal slices. Interleukin-6 at 400 ng/ml time dependently increased the content of cGMP of slices after incubation for 1, 2, 3, and 4 h, and the effect of interleukin-6 was maximal at 2 h post-incubation. In addition, exposure of slices to interleukin-6 at 80, 400 and 2000 ng/ml or at 16, 80 and 400 ng/ml for 2 h significantly elevated the cGMP level and nitrite level, respectively, in a concentration-dependent manner. Also, 0.1 mM N^G-nitro-L-arginine alone showed no effect on either the cGMP level or the nitrite level. However when incubated in conjunction with 400 ng /ml interleukin-6 for 2 h, N^G-nitro-L-arginine apparently prevented the increase in cGMP and nitrite induced by 400 ng /ml interleukin-6. The present results show that NO mediates the increase in cGMP induced by interleukin-6 and suggest that interleukin-6 may exert an inducible effect on the NO synthase in hippocampal slices.

Keywords: Nitric oxide (NO); cGMP; Interleukin-6; Hippocampus; Sodium nitroprusside; N^G-Nitro-L-arginine

1. Introduction

Interleukin-6 is a multifunctional cytokine that is produced by essentially every injured tissue (Sehgal, 1990). Human microglial cells and astrocytes are the major source of interleukin-6 in the central nervous system (CNS) (Aloisi et al., 1992; Lee et al., 1993; Sebire et al., 1993; Yamabe et al., 1994). Recently, interleukin-6 has been reported to promote the survival of rat midbrain catecholaminergic neurons (Kushima et al., 1992), basal forebrain cholinergic neurons (Hama et al., 1991) and hippocampal neurons (Yamada and Hatanaka, 1994). These data indicate a neuroprotective role of interleukin-6. In contrast, this cytokine plays a pathogenic role in response to CNS injury (Woodroofe et al., 1991) and cerebral ischemia (Saito et al., 1996). Moreover, cerebral overexpression of interleukin-6 in transgenic mice has been found to result in neurodegeneration, astrocytosis and induction of acute-phase protein production (Campbell et al., 1993).

At present, the reasons for the neurotoxic effect of interleukin-6 are unknown. To provide some insight into what events in the brain could produce this effect of interleukin-6 on CNS neurons, the present study was undertaken to examine the effect of interleukin-6 on the content of cGMP and nitrites in rat hippocampal slices and the possible involvement of nitric oxide in the increase in cGMP induced by interleukin-6.

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats weighing 280–320 g were obtained from the Experimental Animal Center of Shanghai, Chinese Academy of Sciences. The animals were decapitated and the brain was quickly removed. Parasagittal hippocampal slices 400-mm thick were prepared with a McIlwain tissue chopper. Slices were placed in small plastic tubes (2–3 slices per tube) containing 1 ml Krebs-Ringer's solution and preincubated in a water bath at 37°C for 1 h with continuous carbogen (95% O_2/5% CO_2) aeration. Krebs-Ringer's solution had the following composition (mM): NaCl 129, $MgSO_4$ 1.3, $NaHCO_3$ 22.4, KH_2PO_4 1.2, KCl 4.2, glucose 10.0, $CaCl_2$ 1.5.

* Corresponding author. Tel.: (86-21) 6431-1833; Fax: (86-21) 6437-0269.

PII S0014-2999(96)00955-7

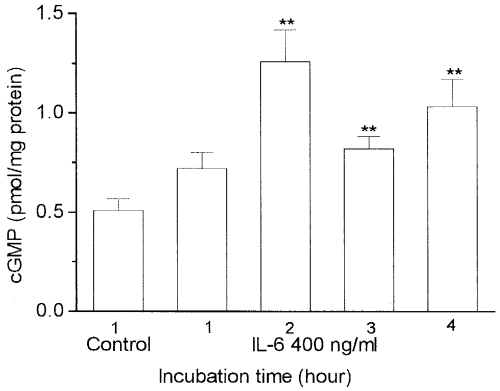

Fig. 1. Time–response effect of interleukin -6 (IL-6) on the level of cGMP in rat hippocampal slices. Each column represents the means ± S.E.M. of results from six rats. * * $P < 0.01$ vs. control slices.

2.2. Drugs

N^G-Nitro-L-arginine (NNA), 2,3-diaminonaphthalene and sodium nitroprusside were obtained from Sigma (St. Louis, MO, USA). Recombinant human interleukin-6 was a gift from Chengdu Di'ao Pharmaceutical Company of Chinese Academy of Sciences and the ^{125}I-cGMP radioimmunoassay (RIA) kit was from the Department of Radioisotopes at Shanghai University of Traditional Chinese Medicine. All other chemicals were of analytical grade. NNA, sodium nitroprusside and interleukin-6 were dissolved in the incubation medium, and 2,3-diaminonaphthalene was dissolved in 0.62 M HCl just before use. Sodium nitroprusside and 2,3-diaminonaphthalene solutions were protected from light. Double-deionized water was used throughout the nitrite assay.

2.3. Cyclic GMP and nitrite assays in hippocampal slices

After a 1-h preincubation, the incubation medium was replaced and the slices were further incubated with drugs for different periods. For the cGMP assay, after the end of incubation, the medium was rapidly aspirated and the slices were inactivated in boiling Tris-EDTA buffer for 5 min. Subsequently, the slices were homogenized in 0.5 ml of acetate buffer (50 mM) containing EDTA (4 mM). The homogenate was centrifuged at 10 000 × g for 10 min. Resulting supernatants were stored at −40°C until radioimmunoassay. Resulting pellets were digested with 0.5 ml of 1 M NaOH for the protein assay. For the nitrite assay, following the completion of incubation, the slices were transferred into small plastic tubes containing 0.5 ml of ice-cold Tris (50 mM, pH 7.4) buffer. The slices were homogenized and the homogenates were centrifuged at 25 000 × g for 30 min at 4°C. Resultant supernatants were directly used for the measurement of nitrite or kept at −40°C until assay. Resulting pellets were used for the protein assay.

The cGMP content was measured according to the instructions of the manufacturer. The limit of detection was 2 fmol. The fluorometric assay of nitrite was performed by the method of Misko et al. (1993). Briefly, samples were properly diluted with double-deionized water and nitrite standards at a range of 7 concentrations (0.05–1.0 μM) were made fresh. Samples and standards were kept on ice prior to use. One milliliter of samples or nitrite standards was mixed with 100 μl of freshly prepared 2,3-diaminonaphthalene (0.05 mg/ml). After a 10-min incubation at 20°C, the reaction was terminated with 50 μl of 2.8 M NaOH. Formation of 2,3-diaminonaphthotriazole, a fluorescent product, was measured using a fluorescence spectrophotometer (HITACHI G50-10S) with excitation at 365 nm and emission at 450 nm. The detection limit was 10 nM. Protein measurement was carried out by the modified Lowry method (Geiger and Bessman, 1972).

2.4. Statistical analysis

Results are expressed as mean cGMP or nitrite levels per milligram of protein ± S.E.M. The significance of differences among groups or between groups was determined by one-way analysis of variance (ANOVA) followed by Duncan's test for individual comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. Time–response effect of interleukin-6 on cGMP levels

Incubation of 400 ng/ml interleukin-6 with hippocampal slices for 1, 2, 3 or 4 h resulted in a time-dependent increase in the level of cGMP ($F(4,25) = 7.56$, $P < 0.01$). Except for 1-h incubation, interleukin-6 markedly elevated the level of cGMP in slices after 2, 3 and 4-h incubations. The optimal period of incubation was 2 h (Fig. 1).

3.2. Concentration–response effect of inteleukin-6 on cGMP levels

Because the increase in cGMP produced by interleukin-6 was maximal after a 2-h incubation, we investigated the

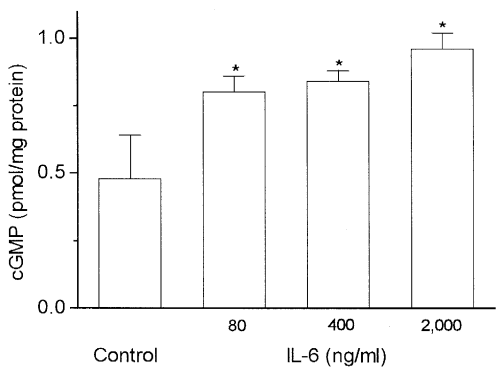

Fig. 2. Concentration–response effect of interleukin-6 (IL-6) on the content of cGMP in rat hippocampal slices. Slices were incubated with IL-6 for 2 h at 37°C. Values are the means ± S.E.M. of results from 6 rats. * $P < 0.05$ vs. control slices.

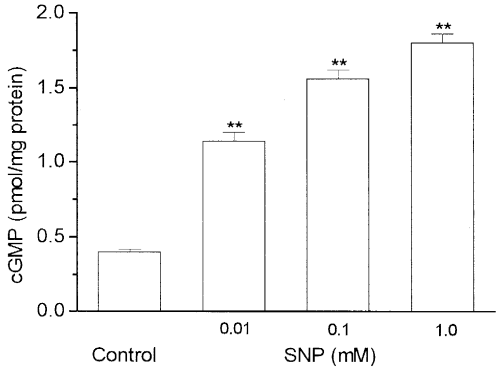

Fig. 3. Concentration–response effect of sodium nitroprusside (SNP) on the level of cGMP in rat hippocampal slices. Slices were incubated with SNP for 5 min at 37°C. Values are the means ± S.E.M. of results from 6 rats. ** $P < 0.01$ vs. control slices.

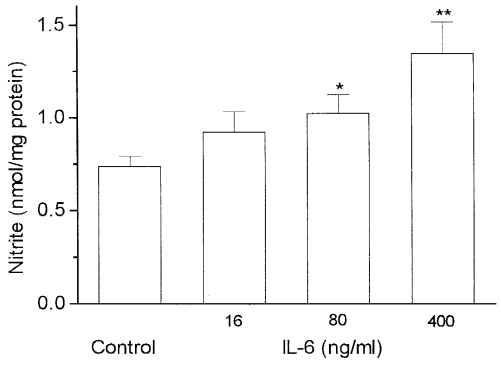

Fig. 5. Concentration–response effect of interleukin-6 (IL-6) on the content of nitrite in rat hippocampal slices. Slices were incubated with IL-6 for 2 h at 37°C. Values are the means ± S.E.M. of results from 6 rats. * $P < 0.05$, ** $P < 0.01$ vs. control slices.

effect of different levels of interleukin-6 on the content of cGMP after 2-h exposure. The level of cGMP was concentration dependently increased significantly by treatment with 80, 400 and 2,000 ng/ml interleukin-6 ($F(3,20) = 21.29$, $P < 0.01$). These concentrations of interleukin-6 all elevated cGMP levels about 1-fold above basal values (Fig. 2).

3.3. Concentration–response effect of sodium nitroprusside on cGMP levels

Significant concentration-dependent increases in cGMP levels were observed after incubation of the slices with three levels of sodium nitroprusside for 5 min ($F(3,20) = 130.15$, $P < 0.01$). The 0.01, 0.1 and 1 mM concentrations of sodium nitroprusside increased the cGMP levels by about 2-, 3-, or 3.5-fold, respectively. Furthermore, their effect on the content of cGMP showed a marked dose–response difference ($F(2,15) = 29.86$, $P < 0.01$) (Fig. 3).

3.4. Effect of NNA on interleukin-6-induced cGMP increases

Exposure of slices to NNA, interleukin-6 or interleukin-6 + NNA to slices for 2 h caused a significant change in the level of cGMP ($F(3,20) = 6.23$, $P < 0.01$). Whereas 400 ng/ml interleukin-6 noticeably increased the cGMP level, 0.1 mM NNA alone showed no obvious effects. However NNA significantly prevented interleukin-6-induced cGMP increases when added in conjunction with interleukin-6 (Fig. 4).

3.5. Concentration–response effect of interleukin-6 on nitrite levels

After a 2-h incubations, interleukin-6 resulted in a significant concentration-dependent increase in the nitrite content of slices ($F(3,20) = 4.82$, $P < 0.05$). Higher concentrations of interleukin-6 (80 and 400 ng/ml) markedly elevated the nitrite content, whereas a lower concentration of interleukin-6 (16 ng/ml) had no effect on the nitrite content (Fig. 5).

3.6. Effect of NNA on interleukin-6-induced nitrite increases

Following a 2-h incubation of slices with chemicals, the levels of nitrite were significantly changed ($F(3,20) = 5.13$,

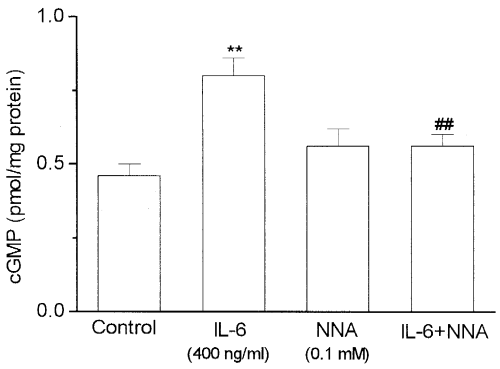

Fig. 4. Effect of interleukin-6 (IL-6), N^G-nitro-L-arginine (NNA) and IL-6 + NNA on the level of cGMP in rat hippocampal slices. Slices were incubated with chemicals for 2 h. Values are the means ± S.E.M. of results from 6 rats. ** $P < 0.01$ vs. control slices and ## $P < 0.01$ vs. IL-6 slices.

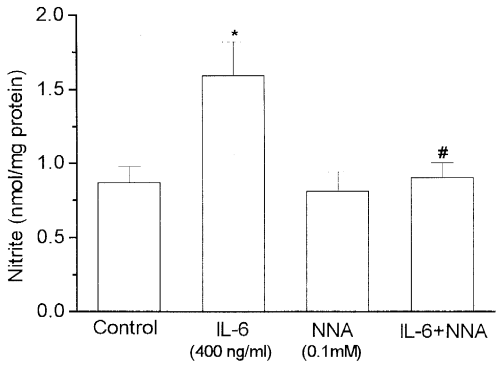

Fig. 6. Effect of interleukin-6 (IL-6), N^G-nitro-L-arginine (NNA) and IL-6 + NNA on the level of nitrite in rat hippocampal slices. Slices were incubated with chemicals for 2 h. Values are the means ± S.E.M. of results from 6 rats. * $P < 0.05$ vs. control slices and # $P < 0.05$ vs. IL-6 slices.

$P < 0.01$). Interleukin-6 400 ng/ml obviously increased nitrite levels, while 0.1 mM NNA exerted no effect on the levels. NNA in combination with interleukin-6 apparently reversed the increase in nitrite produced by interleukin-6 (Fig. 6).

4. Discussion

The present results demonstrated that the levels of cGMP in rat hippocampal slices are increased in a time- and concentration-dependent manner by interleukin-6. In addition, sodium nitroprusside, a nitric oxide (NO) donor, also concentration dependently elevated the level of cGMP. Furthermore, interleukin-6 concentration dependently elevated the nitrite level, and NNA, a NO synthase inhibitor, significantly reversed the increases in cGMP and nitrite levels induced by interleukin-6. These results indirectly and directly demonstrate the inducible effect of interleukin-6 on the NO synthase in hippocampal slices.

Recently, Boulton et al. (1994) described the nitric oxide-cGMP pathway present in rat hippocampal slices. In their study, sodium nitroprusside (1 mM) significantly increased cGMP levels ~5-fold above basal levels by stimulating soluble guanylate cyclase, which is similar to our present finding.

Nitrite is thought to be a main oxidation product of NO and the assay of nitrite can be used as a measurement of NO (Butler et al., 1995). Therefore, nitrite is also a useful parameter in monitoring NO synthase function. The inhibitive effect of NNA on interleukin-6-induced increases in cGMP was only an indirect index of the induction of NO synthase by interleukin-6. In order to explore the direct effect of interleukin-6 on the NO synthase, we measured the nitrite levels. The observation that interleukin-6 80 and 400 ng/ml significantly increased the nitrite levels and NNA reversed this interleukin-6 effect more directly confirmed the induction of NO synthase by interleukin-6. To our knowledge, the present findings are the first data concerning the inducible effect of interleukin-6 on the NO synthase in the hippocampus. As for the observation that the levels of cGMP did not largely increase along with the increase in interleukin-6 doses, one possible explanation is that the activity of cGMP-specific phosphodiesterase increases as cGMP accumulates in slices in response to the higher doses of interleukin-6. In fact, significant concentration-dependent increases in nitrite induced by interleukin-6 indirectly support the mentioned-above explanation.

It has been suggested that NO may play a dual role as a messenger in the CNS. Some studies have found nitric oxide to have a neuroprotective role both in vitro and in vivo (Lipton et al., 1993; Morikawa et al., 1992; Zhang and Iadecola, 1993). Conversely, most studies have emphasized the neurotoxic effect of nitric oxide (Buisson et al., 1992; Caldwell et al., 1994; Moncada et al., 1992; Nagafuji et al., 1992; Nowicki et al., 1991; Ohno et al., 1994; Trifiletti, 1992). Additionally, the mRNAs of interleukin-6 and interleukin-6 receptors have been reported to be located in the hippocampal formation and also in the dentate (Schöbitz et al., 1992). Recent studies have demonstrated that the activity of NO synthase and the level of interleukin-6 are significantly increased in the hippocampus of gerbils after cerebral ischaemia (Caldwell et al., 1994; Saito et al., 1996). Taking the inducible effect of interleukin-6 on NO synthase into account, we postulate that the neurotoxic effect of interleukin-6 may be involved in the neurotoxicity of NO in the CNS. Further studies are in progress to test this hypothesis.

In addition, the following observations should be emphasized and explained. Firstly, interleukin-6 at the dose of 16 ng/ml had no effect on the NO synthase. Therefore, low levels or normal levels of interleukin-6 may not exert a neurotoxic effect. Secondly, that NNA alone did not affect basal cGMP and nitrite levels suggests that basal neuronal NO synthase activity is very limited on the one hand and that NNA mainly inhibits inducible NO synthase activity on the other. Finally, according to our pilot studies (not shown data), the basal levels of cGMP in hippocampal slices are quite stable after 30 min and 1–4 h of incubation. Therefore, in the study on the time–response effect of interleukin-6, we only measured the cGMP level after 1-h incubation as controls.

In conclusion, interleukin-6 elevates the levels of cGMP and nitrite in rat hippocampal slices and the cGMP-increasing effect of interleukin-6 is mediated by overproduction of nitric oxide due to the induction of NO synthase in hippocampal slices. Furthermore, our studies suggest that the neurotoxic effect of interleukin-6 may be mediated by overformation of nitric oxide in the brain.

Acknowledgements

The authors thank Chengdu Di'ao Pharmaceutical Company for providing us with interleukin-6. The skillful assistance of Mr Rengang Wang in preparing the figures is gratefully acknowledged.

References

Aloisi, F., A. Care, G. Borsellino, P. Gallo, S. Rosa, A. Bassani, A. Cabibbo, U. Testa, G. Levi and C. Peschle, 1992, Production of hemalymphopoietic cytokine (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1β and tumor necrosis factor-α, J. Immunol. 149, 2358.

Boulton, C.L., A.J. Irving, E. Southam, B. Potier, J. Garthwaite and G.L. Collingridge, 1994, The nitric oxide-cyclic GMP pathway and synaptic depression in rat hippocampal slices, Eur. J. Neurosci. 6, 1528.

Buisson, A., M. Plotkine and R.G. Boulu, 1992, The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischemia, Br. J. Pharmacol. 106, 766.

Butler, A.R., F.W. Flitney, D.L.H. Williams, 1995, NO, nitrosonium

ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective, Trends Pharmacol. Sci. 16, 18.

Caldwell, M., M. O'Neill, B. Earley and B. Leonard, 1994, N^G-Nitro-L-arginine protects against ischemia-induced increases in nitric oxide and hippocampal neuro-degeneration in the gerbil, Eur. J. Pharmacol. 260, 191.

Campbell, I.L., C.R. Abraham, E. Masliah, P. Kemper, J.D. Inglis, M.B.A. Oldstone and L. Mucke, 1993, Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6, Proc. Natl. Acad. Sci. USA 90, 10061.

Geiger, P.J. and S.P. Bessman, 1972, Protein determination by Lowry's method in the presence of sulphydryl reagents, Anal. Biochem. 49, 467.

Hama, T., Y. Kushima, M. Miyamoto, M. Kubota, N. Takei and H. Hatanaka, 1991, Interleukin-6 improves the survival of mesencephalic catecholaminergic and septal cholinergic neurons from postnatal two-week-old rats in cultures, Neuroscience 40, 445.

Kushima, Y., T. Hama and H. Hatanaka, 1992, Interleukin-6 as a neurotrophic factor for promoting the survival of cultured catecholaminergic neurons in a chemically defined medium from fetal and postfetal rat midbrains, Neurosci. Res. 13, 267.

Lee, S.C., W. Liu, D.W. Dickson, C.F. Brosnan and J.W. Berman, 1993, Cytokine production by human fetal microglia and astrocytes: differential induction by lipopolysaccharide and IL-1β, J. Immunol. 150, 2659.

Lipton, S.A., Y.B. Choi, Z.H. Pan, S.Z. Lei, H.S.V. Chen, N.J. Sucher, J. Loscalzo, D.J. Singel and J.S. Stamler, 1993, A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds, Nature 364, 626.

Misko, T.P., R.J. Schilling, D. Salvemini, W.M. Moore and M.G. Currie, 1993, A fluorometric assay for the measurement of nitrite in biological sampales, Anal. Biochem. 214, 11.

Moncada, C., D. Lekieffer, B. Arvin and B. Meldrum, 1992, Effect of NO synthase inhibition on NMDA- and ischemia-induced hippocampal lesions, Neuroreport 3, 530.

Morikawa, E., Z. Huang and M.A. Moskowitz, 1992, L-Arginine decreases infarct size caused by middle cerebral arterial occlusion in SHR, Am. J. Physiol. 263, H1632.

Nagafuji, T., T. Matsui, T. Koide and T. Asano, 1992, Blockade of nitric oxide formation by N^{ω}-nitro-L-arginine mitigates ischaemic brain edema and subsequent cerebral infarction in rats, Neurosci. Lett. 147, 159.

Nowicki, J.P., D. Duval, H. Poignet and B. Scaton, 1991, Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse, Eur. J. Pharmacol. 204, 339.

Ohno, M., T. Yamamoto and S. Watanabe, 1994, Intrahippocampal administration of the NO synthase inhibitor L-NAME prevents working memory deficits in rats exposed to transient cerebral ischemia, Brain Res. 634, 173.

Saito, K., K. Suyama, K. Nishida, Y. Sei and A.S. Basile, 1996, Early increases in TNF-α, IL-6 and IL-1β levels following transient cerebral ischemia in gerbil brain, Neurosci. Lett. 206, 149.

Schöbitz, B., D.A.M. Voorhuis and E.R. De Kloet, 1992, Localization of interleukin-6 mRNA and interleukin 6 receptor mRNA in rat brain, Neurosci. Lett. 136, 189.

Sebire, G., D. Emilie, C. Wallon, C. Hery, O. Dervegne, J.-F. Delfraissy, P. Galanaud and M. Tardien, 1993, In vitro production of IL-6, IL-1b, and tumor necrosis factor-α by human embryonic microglial and neural cells, J. Immunol. 150, 1517.

Sehgal, P.B., 1990, Interleukin-6: molecular pathophysiology, J. Invest. Dermatol. 94, 2S.

Trifiletti, R.R., 1992, Neuroprotective effects of N^G-nitro-L-arginine in focal stroke in the 7-day old rat, Eur. J. Pharmacol. 218, 197.

Woodroofe, M.N., G.S. Sarna, M. Wadhwa, G.M. Hayes, A.J. Loughlin, A. Tinker and M.L. Cuzner, 1991, Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production, J. Neuroimmunol. 33, 227.

Yamabe, T., G. Dhir, E.P. Cowan, A.L. Wolf, G.K. Bergey, A. Krumholz, E. Barry, P.M. Hoffman and S. Dhib-Jalbut, 1994, Cytokine-gene expression in measles-infected adult human glial cells, J. Neuroimmunol. 49, 171.

Yamada, M. and H. Hatanaka, 1994, Interleukin-6 protects cultured rat hippocampal neurons against glutamate-induced cell death, Brain Res. 643, 173.

Zhang, F. and C. Iadecola, 1993, Sodium nitroprusside improves blood flow and reduces brain damage after focal ischemia, Neuroreport 4, 559.