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Expression of inducible nitric oxide synthase by neurones following exposure to endotoxin and cytokine

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In the CNS, nitric oxide (NO) has been implicated as both a mediator of neurotoxicity and a neuromodulator. The inducible NO synthase (iNOS), thought to mediate toxic effects of NO, has been attributed to glial cells in the CNS. We now report that cerebellar granule cell neurones can be stimulated by lipopolysaccharide and interferon-γ to express iNOS *in vitro*, as demonstrated by reverse transcription-polymerase chain reaction and fluorescent *in situ* hybridisation. The expression of both constitutive NO synthase (cNOS) and iNOS by neurones suggests that NO has diverse functions in the brain, and supports the possibility that iNOS plays a role in neuronal damage and inflammation following activation of brain microglia and production of cytokines.

Keywords: Cerebellar granule cells; macrophage; cerebellum; interferon-γ; lipopolysaccharide

Introduction Two forms of nitric oxide synthase (NOS) have been described: a constitutive form (cNOS), expressed tonically, and an inducible form (iNOS), only present following exposure to agents such as cytokines and endotoxins. NO formed by cNOS functions in intercellular interactions, whereas NO produced by iNOS is mainly a cytotoxic agent (Moncada *et al.*, 1991). In the CNS, NO functions as a neuromodulator, but also mediates N-methyl-D-aspartate (NMDA) excitotoxicity on cortical neurones in culture (Dawson *et al.*, 1991). Induction of iNOS has not been demonstrated in neurones, but attributed to glial cells (Murphy *et al.*, 1993). Because of the potential role of iNOS in neurotoxicity, we have examined whether iNOS could be induced in neurones by cytokines, which increase in the brain following various insults (Morganti-Kossmann *et al.*, 1992).

Methods Cerebellar granule cells (CGC) were prepared from 8-day old rat pups (Novelli *et al.*, 1988) and used after 10–12 days *in vitro*. Previous characterization of these cultures has shown that greater than 95% of the cells are CGC. The absence of microglia and astrocytes in cultures (<1/100 cells) was verified by staining with antibodies to ED-1 and glial fibrillary acidic protein respectively. A macrophage cell line, P388D1 (Koren *et al.*, 1975), was used as a positive control for the induction of the iNOS gene. Lipopolysaccharide (LPS, $10\,\mu g\,ml^{-1}$ *E. coli* serotype 0128:B12, Sigma) and mouse interferon-γ (IFN-γ, $100\,u\,ml^{-1}$, Genzyme) were used to induce iNOS.

RNA was prepared using RNAzol (Tel-Test). RNA-specific (Shuldiner *et al.*, 1991) or standard reverse transcription – polymerase chain reaction (RS- or RT – PCR) were used to amplify iNOS mRNA, or the mRNA of the unchanging gene cyclophilin, which served as a quantity control, respectively. The primer for the reverse transcription of iNOS mRNA (GenEMBL No. M84373) corresponded to the antisense sequence of nucleotides 3554-3573, with the addition of the T30 tag (Shuldiner *et al.*, 1991). PCR primers for the amplification of iNOS cDNA corresponded to nucleotides 3440-3461 (upstream) and the T30 tag (downstream, Shuldiner *et al.*, 1991). RT-PCR primers for the gene encoding cyclophilin (GenEMBL No. M19533) corresponded to nucleotides 53–71 (upstream) and 679–696 (downstream). Each PCR cycle consisted of: 1'-94°C, 1'-59°C, 1'-72°C (30 cycles). Aliquots of the amplification products were electrophoresed, visualised by ethidium bromide staining, and transferred onto a nylon membrane. The blot was hybridised with ^{32}P-labelled iNOS cDNA from activated macrophages, washed under conditions of high stringency (0.1 × SSC, 0.1% SDS at 60°C), and exposed to X-ray film. Negative controls that underwent the same procedures without reverse transcriptase, or without RNA, did not yield PCR products.

CGC grown on glass slides, with or without application of LPS-IFN-γ for 4 h prior to fixation, were used for fluorescent *in situ* hybridisation (FISH). The slides were hybridised with $350\,ng\,ml^{-1}$ of each of two biotinylated complementary or identical oligonucleotide probes to the iNOS mRNA (nucleotides 3440–3461 and 3554–3573), in a solution of 50% formamide, 4 × SSC, 1 × Denhardt's solution, $25\,\mu g\,ml^{-1}$ tRNA, $50\,\mu g\,ml^{-1}$ salmon sperm DNA, overnight at 37°C. Following hybridisation, the slides were washed 4 times in 2 × SSC and 50% formamide at 40°C for 15 min, twice for 1 h in 1 × SSC at 22°C. Following rinsing in ethanol, the slides were incubated with Streptavidin fluorescein (Amersham), washed, mounted, and examined by confocal laser scanning microscopy (CLSM), as described (Tsarfaty *et al.*, 1992).

Results Treatment of CGC with LPS-IFN-γ resulted in induction of transcription of the iNOS gene, as evidenced by RS-PCR. The iNOS PCR product in stimulated macrophages and CGC was the expected size (164 bases including the T30 tag) and hybridised with a macrophage iNOS cDNA, but not with a brain cNOS cDNA. iNOS mRNA was detectable 2–6 h following application of LPS-IFN-γ, in CGC as well as in macrophages, but not in untreated cultures (Figure 1a). Sequence of the PCR product from stimulated CGC revealed greater than 90% homology to the mouse macrophage sequence and 100% homology at the amino acid level. All samples gave rise to comparable amounts of PCR product for the nonchanging cyclophilin mRNA (Figure 1b).

FISH followed by CLSM demonstrated that iNOS was expressed by CGC, rather than by possible contaminating cells in the culture. This method showed iNOS mRNA in 80–90% of CGC treated with LPS-IFN-γ for 4 h (Figure 2a) but not in untreated cells (Figure 2g). Hybridisation of CGC with sense oligoprobes yielded no signal under identical microscope parameters (Figure 2e). Overlay analyses of the fluorescent hybridisation in treated CGC with the Nomarski optic morphology, which shows the typical appearance of cultured CGC (Figure 2a, b), demonstrated that iNOS mRNA is localised in the CGC cell bodies (Figure 2c). NOS

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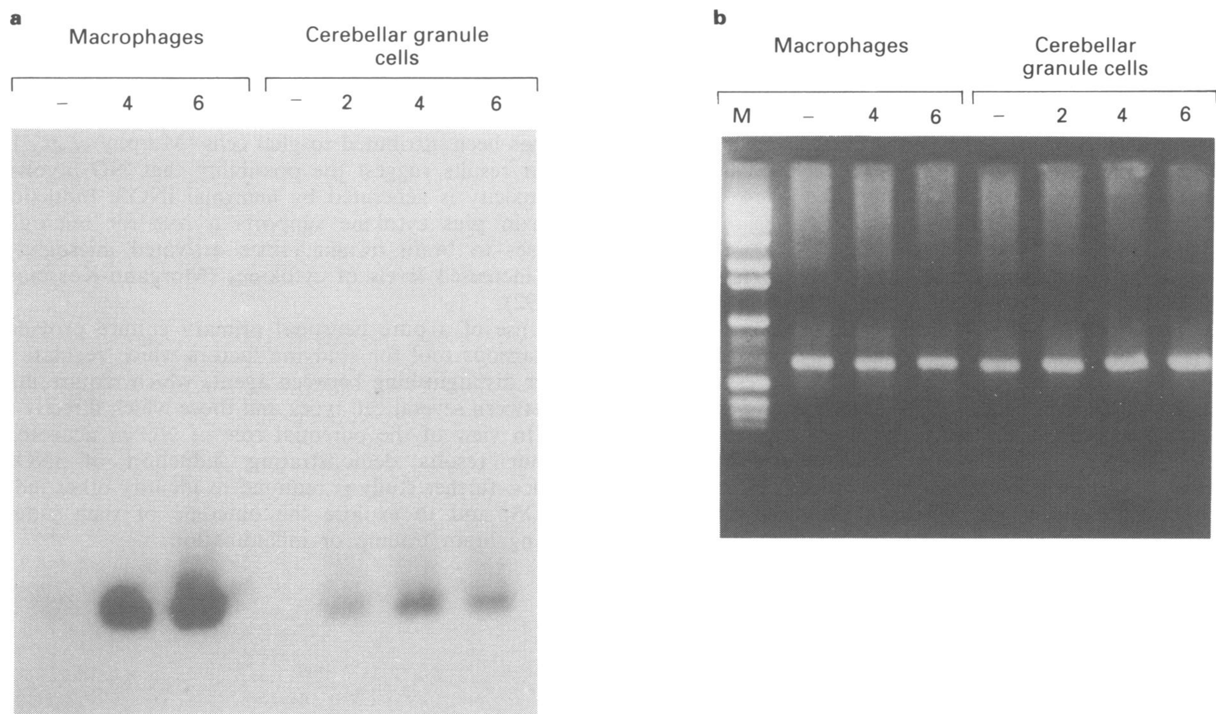

Figure 1 Inducible NO synthase (iNOS) mRNA in macrophages and in cerebellar granule cells (CGC). (a) Southern blot of RS-PCR product (164 bases) of the iNOS gene, after exposure of macrophages or CGC to LPS-IFN-γ for 0–6 h. (b) Ethidium bromide-stained gel of RT-PCR products for cyclophilin mRNA using the same RNA samples as in (a). The left lane is 1 Kb DNA ladder (GIBCO). The cyclophilin product is the expected size 644 bases. The numbers above the lanes show the hours of exposure to LPS-IFN-γ. For abbreviations, see text.

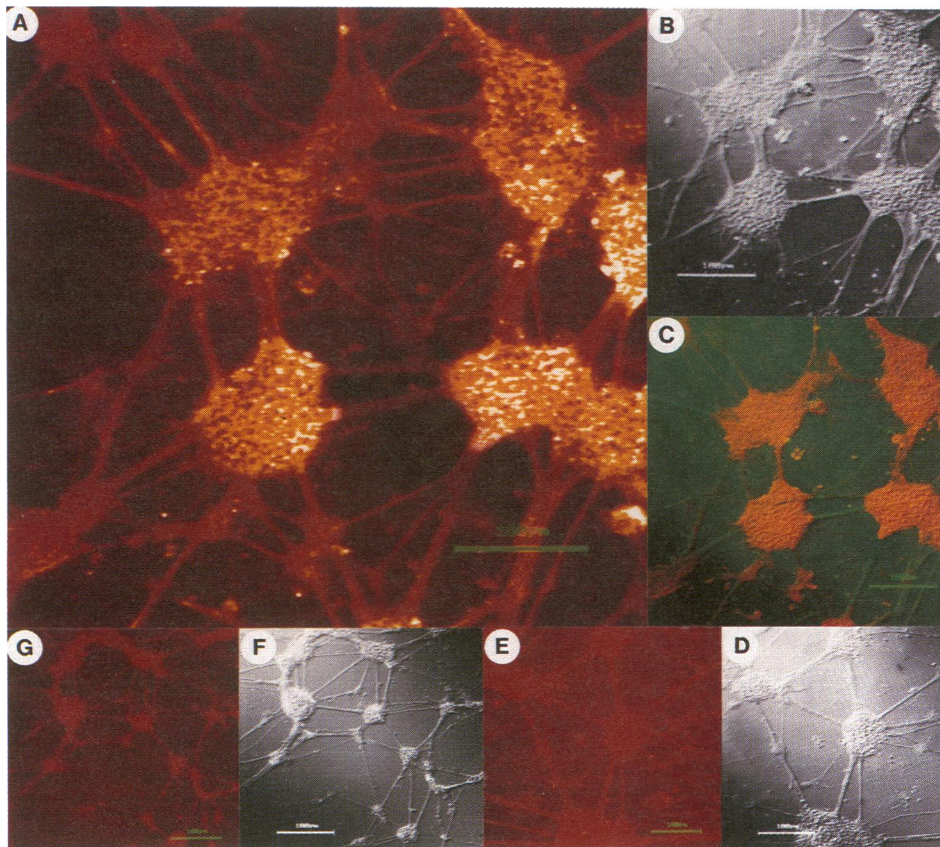

Figure 2 Fluorescent *in situ* hybridisation (FISH) analysis of inducible NO synthase (iNOS) mRNA in cerebellar granule cells (CGC) treated with lipopolysaccharide-interferon-γ (LPS-IFN-γ). FISH for the iNOS gene transcript was carried out as described in Methods. (a) Fluorescent *in situ* signal in the cell bodies. (b) Nomarski image of the same region as in (a). (c) Overlay analysis between iNOS mRNA fluorescence (a) and morphology (b) (yellow indicates overlap). (d, e) A treated sister culture hybridised with the sense probes and (f, g) an untreated sister culture hybridised with the antisense probes. Nomarski images (d, f); fluorescent images (e, g). Scale bars are 100 μm.

enzymatic activity was measured by assessing conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline. Exposure of CGC to LPS-IFN-γ increased the rate of conversion from 0.21 ± 0.02 pmol min^{-1} mg^{-1} protein in untreated cells to 0.41 ± 0.04 pmol min^{-1} mg^{-1} protein in stimulated cells, indicating that the iNOS message is translated to an active enzyme in these cultured neurones. Either LPS or IFN-γ alone increased the activity to a lesser extent (data not shown).

Discussion Our results demonstrate that CGC express iNOS following exposure to LPS-IFN-γ in culture. In contrast to neurones, macrophages contain only iNOS which requires gene transcription, and the NO produced by this enzyme function as a cytotoxic agent. The level of expression of iNOS mRNA in macrophages is higher than that in neurones (Figure 1), perhaps because cytotoxicity is a major function of macrophages. Since CGC are the richest in cNOS in the CNS, these neurones can express two different forms of NOS, supporting the view that NO has a variety of different functions in nerve cells. In addition to its function as a neuronal messenger, NO has been implicated in neurotoxicity (Dawson *et al.*, 1991). iNOS mRNA was induced in whole brain extracts in experimentally-induced models of neurological disorders (Koprowski *et al.*, 1993). NOS inhibitors attenuated NMDA excitotoxicity and reduced ischaemic damage (Nowicki *et al.*, 1991). iNOS activity in the CNS has been attributed to glial cells (Murphy *et al.*, 1993), but our results suggest the possibility that NO involved in neurotoxicity is generated by neuronal iNOS. Induction by endotoxin plus cytokine supports a role for microglia in responses to brain trauma, since activated microglia produced increased levels of cytokines (Morganti-Kossmann *et al.*, 1992).

The use of a pure neuronal primary culture provides an advantageous tool for studying factors which regulate NOS and for distinguishing between agents which require interaction between several cell types and those which directly affect NOS. In view of the potential role of NO in neurotoxicity and our results demonstrating induction of iNOS in neurones, further study is required to identify other inducers of iNOS, and to explore the outcome of such induction following brain trauma or inflammation.

References

DAWSON, V.L., DAWSON, T.M., LONDON, E.D., BREDT, D.S. & SNYDER, S.H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6368-6371.

KOPROWSKI, H., ZHENG, Y.M., HEBER-KATZ, E., FRASER, N., RORKE, L., FU, Z.F., HANLON, C. & DIETZSCHOLD, B. (1993). In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 3024-3027.

KOREN, H.S., HANDWERGER, B.S. & WUNDERLICH, J.R. (1975). Identification of macrophage-like characteristics in cultured murine tumor line. *J. Immunol.*, **114**, 894-897.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathology, and pharmacology. *Pharmacol. Rev.*, **43**, 109-142.

MORGANTI-KOSSMANN, M.C., KOSSMANN, T. & WAHL, S.M. (1992). Cytokines and neuropathology. *Trends Pharmacol. Sci.*, **13**, 286-291.

MURPHY, S., SIMMONS, M.L., AGULLO, L., GARCIA, A., FEINSTEIN, D.L., GALEA, E., REIS, D.J., MINC-GOLOMB, D. & SCHWARTZ, J.P. (1993). Synthesis of nitric oxide in CNS glial cells. *Trends Neurosci.*, **16**, 323-328.

NOVELLI, A., REILLY, J.A., LYSKO, P.G. & HENNEBERRY, R.C. (1988). Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intraceullar energy levels are reduced. *Brain Res.*, **451**, 205-212.

NOWICKI, J.P., DUVAL, D., POIGNET, H. & SCATTON, B. (1991). Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. *Eur. J. Pharmacol.*, **204**, 339-340.

SHULDINER, A.R., TANNER, K., MOORE, C.A. & ROTH, J. (1991). RNA template-specific PCR: an improved method that dramatically reduces false positives in RT-PCR. *BioTechniques*, **11**, 760-763.

TSARFATY, I., RESAU, J.H., RULONG, S., KEYDAR, I., FALETTO, D.L. & VANDE WOUDE, G.F. (1992). The met proto-oncogene receptor and lumen formation. *Science*, **257**, 1258-1261.

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