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Expression of nitric oxide synthase-2 in glia associated with CNS pathology

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Introduction

While the production of nitric oxide (NO) in the nervous system is catalyzed by three, highly homologous isoforms of NO synthase (NOS) (Forstermann et al., 1995), in this chapter we focus exclusively on NOS-2. This dimeric, heme-containing, soluble protein whose activity is independent of a rise in intracellular calcium, is variously termed 'inducible', 'immunologic', and 'macNOS' (Griffith and Stuehr, 1995). These terms are somewhat misleading because NOS-2 has been reported in a wide variety of normal and neoplastic cell types, in some cases constitutively (Xie and Nathan 1994). All CNS parenchymal cells, together with the smooth muscle and endothelium of the vascular wall, can be transcriptionally activated by a variety of agents to express NOS-2, as well as cells (neutrophils and monocytes) infiltrating the parenchyma following changes in blood–brain barrier properties (Grzybicki et al., 1997, 1998). In addition, there are reports of developmentally related NOS-2 expression in particular brain regions (Galea et al., 1995; Arnhold et al., 1997).

Initially from studies with cultured cells (Murphy et al., 1993), but now exemplified by a host of

in vivo conditions (see later), identified astrocytes have been shown to express NOS-2 upon activation. Induction can be triggered in vitro (see Table 1) by endotoxin and/or combinations of such cytokines as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ; HIV envelope proteins also activate the astrocyte gene (Koka et al., 1995). The promoter region of the glial NOS-2 gene displays a wide variety of potential response elements for transcription factors, such as AP-1, Hif, NFκB, NF-IL6, TNF, IRF, and GAS (Spitsin et al., 1997). In cultured astrocytes the transcript can be detected within a few hours of exposure to cytokines, is maximally transcribed by 4 h and then declines rapidly, such that it is not detectable at 12 h (Park and Murphy, 1994). In contrast, expression of NOS-2 in the CNS in vivo is an unexpectedly delayed event, which may, as we discuss later, have functional implications.

The observation that HIV envelope proteins can induce NOS-2 is important as it demonstrates that priming cells with IFN-γ (a cytokine not produced by resident CNS cells) is not obligatory for gene activation. In addition, cytokine-activated astrocytes, macrophages and cerebrovascular cells release TNFα which, in turn, induces NOS-2 in naive cells (Borgerding and Murphy, 1995; Shafer and Murphy, 1997). This implies that induction of NOS-2 in CNS parenchymal cells could occur

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TABLE 1

Regulators of NOS-2 expression in vitro.

Inducers	Blockers
Proinflammatory cytokines (IL-1β, TNFα, IFNγ)	Anti-inflammatory cytokines (TGFβ1, IFNβ, IL-4, IL-10)
Endotoxin	Dexamethasone
HIV env. proteins	Chemokines (MCP-1, IL-8)
Hypoxia	Neurotransmitters (norepinephrine, glutamate, ATP, angiotensin II)
β-amyloid	Heat shock protein 70 nitric oxide (NO)

in vivo following events on the luminal side of an intact blood-brain barrier. Transcriptional activation of the cerebral vasculature by endotoxin or cytokines from adhering hematogenous cells may be sufficient to induce NOS-2 in astrocytes and microglia on the parenchymal side.

The timing and extent of NOS-2 expression associated with various neuropathologies may also be related to the prior history of the cells involved. For example, heat shock protein 70 and the anti-inflammatory cytokines transforming growth factor (TGF)-β1 and IFNβ block NOS-2 induction at the transcriptional level (Park et al., 1994; Feinstein et al., 1996; Stewart et al., 1997). Activation of glutamate, ATP, angiotensin-II and β-adrenergic receptors on astrocytes blunt the NOS-2 response to endotoxin and pro-inflammatory cytokines (Kopnisky et al., 1997; Lin and Murphy, 1997; Feinstein 1998). It is clear that some of these modulatory effects result from interference with the subsequent activation of a key transcription factor, NFκB, and/or with its binding to the promoter.

Nitric oxide inhibits not only NOS-2 activity but also regulates the level of NOS-2 mRNA expression through a mechanism involving NF-κB. Some reports suggest a blockade of NF-κB activation (Colasanti et al., 1995; Peng et al., 1995), whereas others propose inhibition of binding to its response element on the NOS-2 promoter via nitrosylation of a key cysteine residue on one of

the NF-κB subunits (DelaTorre et al., 1997; Park et al., 1997). This regulation might represent a means to limit or focus NO production in discrete groups of cells in the CNS (Togashi et al., 1997). Such a feedback effect of NO has been confirmed in vivo (Luss et al., 1994), pointing to potential rebound effects with the therapeutic use of NOS-2 inhibitors.

The NOS-2 transcript is rapidly degraded, implying there may be factors co-induced in cells which are responsible for causing this instability (Park and Murphy, 1996). The 3'-untranslated region of NOS-2 has AUUUA repeats (Galea et al., 1994) which are suggested to be determinants of mRNA stability and this could represent yet another mechanism by which the amount and duration of NO production is regulated.

With all of these potential regulatory mechanisms for NOS-2 expression in mind, what is the evidence for expression in vivo? what are the roles that NO from this source play in CNS function? There are many potential targets for NO and its oxidation products (such as peroxynitrite). These include heme and non-heme iron, thiols and DNA. Such reactions account for the reported biological effects of NOS activation, such as mitochondrial dysfunction (Knowles, 1997), neuromodulation (Christopherson and Bredt, 1997), and cell and organism killing (Nathan, 1997). In addition, it is evident that NO has subtle effects on the regulation of a number of cytokine and chemokine genes (Merrill and Murphy, 1997). However, it is important to distinguish between effects due to NO and those resulting from peroxynitrite exposure. The latter is a strong oxidant with effects that, in general, are biologically deleterious; the production of NO in the absence of superoxide anion produces different effects, many of which may be beneficial (Butler et al., 1995; Nathan, 1997).

Expression of NOS-2 in vivo

Since last we reviewed this literature (Murphy and Grzybicki, 1996) there have been considerable efforts to identify specific neuropathological con-

ditions that result in the induction of NOS-2. The earlier, suggestive reports of NOS-2 mRNA and/or protein expression associated with certain types of CNS injury have now been confirmed by in situ hybridization and immunohistochemistry and provide evidence for the cellular source. There is specific evidence for glial expression of NOS-2 associated with neuronal injury and infection of the CNS, and in neurodegenerative and demyelinating diseases (see Table 2). We refer to these most recent reports for completeness, and then focus on ischemia and the evidence for glial NOS-2 expression. Finally, we speculate on the possible roles for NO in the development of pathology, and in recovery from injury.

Trauma

Direct injury in the CNS results in a reactive gliosis, characterized by induction of the glial fibrillary acidic protein gene and changes in astrocyte morphology (Eddleston and Mucke, 1993). In a freeze-lesion model, we found that NOS-2 was induced within a few hours in cells infiltrating the lesion (Grzybicki et al., 1998). Only after 24 h could we find NOS-2 expression in astrocytes delimiting the lesion edge. In a compression injury to the spinal cord, Hamada et al. (1996) report NOS-2 mRNA, which peaks at 24 h and persists for three days. The expression was associated with motor dysfunction and application

TABLE 2

Role of NOS-2 in neuropathologies.

Pathology	After NOS-2 block
Direct cortical injury	Reduced
Indirect injury	?
Hepatitis, Theiler's, HIV	?
LCM virus	Worsened
Endotoxin	Reduced
ALS	?
Retinal dystrophy	?
Alzheimer	?
EAE/MS	Reduced or worsened
Global ischemia	Reduced
Stroke	Reduced

of a NOS inhibitor reduced motor disturbance. This delayed expression of NOS-2 in parenchymal cells is of interest and we speculate on its meaning later in the chapter.

There is also suggestive evidence for NOS-2 expression in glia after indirect neuronal injury, which occurs with axotomy or chemical toxicity, and results in a marked microglial, but lesser astroglial cell response. Following a local injection of kainic acid into the cortex, Lei et al. (1996) describe neuronal degeneration, followed 2–3 days later by expression of NOS-2 in reactive astrocytes and in endothelial cells at the edge of the lesion.

Infection

How viruses cause lesions, or dysfunction without direct damage to the CNS, is unclear. Diffusible cytokines, together with reactive oxygen and nitrogen species produced as a component of the immune response presumably contribute to the pathology. In many instances, NOS-2 expression is associated with viral infection of the CNS and, in some, the resulting NO appears to be beneficial.

Intranasal inoculation of mice with the neurotropic hepatitis virus leads to expression of NOS-2 in the brain (Grzybicki et al., 1997). The cells responsible for its expression appear to be entirely infiltrating, with no evidence for parenchymal cell expression. However, this infection proves fatal within seven days. Lane et al. (1997) used a neuroattenuated strain of this virus which is cleared and the animals thus survive. They report NOS-2 in astrocytes and macrophages in the olfactory bulb. We have looked also at expression of NOS-2 in persistently infected animals, generated by raising inoculated pups with immunized dams. In these animals, which display marked demyelinating lesions and associated hindlimb paralysis, NOS-2 is expressed very prominently in astrocytes surrounding such lesions (Sun et al., 1995; Grzybicki et al., 1997). A similar expression was reported by Oleszak et al. (1997) in astrocytes surrounding necrotizing lesions in mice infected with Theiler's virus. There is also suggestive evidence that NOS-2 expression in the CNS is

associated with HIV-1 (Adamson et al., 1996) and SIV infection (Lane et al., 1996).

The study by Lane et al. (1997) suggests that, while NO from NOS-2 can block viral replication in vitro, this is not the determining factor for clearing hepatitis virus in the CNS. Campbell (1996), employing intracerebral infection with lymphocytic choriomeningitis virus, found NOS-2 expression only in macrophages. While the viral burden was unaffected by a NOS inhibitor, such treatment exacerbated clinical severity and decreased survival time, suggesting a protective role for NO.

In wild-type mice early in the course of systemic inflammation, resulting from an injection of endotoxin, there is a marked expression of NOS-2 in vascular, glial and neuronal structures adjacent to areas devoid of a blood–brain barrier (Wong et al., 1996; Htain et al., 1997). Boje (1996) found that intracisternal administration of endotoxin resulted in NO production from the meninges and alteration in blood–brain barrier properties, which could be reversed by a NOS inhibitor. It is of interest to note that mice with deletions in the NOS-2 gene are protected from death after high-doses of endotoxin (MacMicking et al., 1995).

Neurodegenerative diseases

Characteristic of such diseases is the progressive loss of a subset of neurons and there is evidence to implicate NO as causal or, at least, contributory.

In a proportion (20%) of cases of familial amyotrophic lateral sclerosis (ALS) there are multiple missense mutations in cytosolic Cu/Zn superoxide dismutase (SOD) and it is proposed that these result in either a gain of function, or increase an injurious reaction catalyzed by SOD. For example, Crow and Beckman (1995) suggest that there is enhanced nitration by NO of neurofilament protein. Evidence for nitration being causal in ALS comes from the observation that nitration blocks phosphorylation of tyrosine residues and that nitrotyrosine resembles the strongly antigenic dinitrophenol, perhaps provoking an autoimmune reaction.

Inherited retinal dystrophy results from accumulation of cellular debris in the subretinal space and visual cell degeneration. Cotinet et al. (1997) looked at the retinal Muller (astrocyte-like) cell from dystrophic rats and found that they produce abnormal amounts of TNFα and NOS-2 derived NO. As NO can decrease phagocytosis of photoreceptor segments, which underlies this dystrophy, they suggest that NO may disrupt transmission in retinal neurons.

Alzheimer's disease (AD) occurs in 10–15% of individuals over 65 and is characterized by the loss of pyramidal neurons in the hippocampus and neocortex, and of cholinergic neurons in the median forebrain. The hallmark of AD is the deposition of β-amyloid aggregates which can be toxic to neurons and also leads to NO production from glial cells (Rossi and Bianchini, 1996; Barger and Harmon, 1997) and neurons (Vodovotz et al., 1996). Weldon et al. (1998) injected soluble and fibrillar β-amyloid into rat striatum. They noted that microglia and astrocytes nearby showed a marked increase in NOS-2 expression and speculate that the resultant NO may be involved in neuronal killing.

Demyelinating diseases

Demyelinating diseases are characterized by preferential damage to myelin, with relative preservation of axons. Multiple sclerosis (MS) and animal models such as experimental autoimmune encephalomyelitis (EAE) are inflammatory demyelinating diseases characterized by early damage to the blood–brain barrier. Lymphocyte and monocyte infiltration occurs, as well as activation of indigenous glia. What produces the resulting myelin damage is unknown and both indigenous as well as infiltrating cell types may contribute to myelin destruction, either directly or indirectly by secretion of proinflammatory cytokines or NO (Merrill and Benveniste, 1996).

A number of recent reports confirm a suggested role for NOS-2 in demyelinating disease. Using a spin trap method in vivo Hooper et al. (1995) could detect NO in the CNS of EAE animals,

which correlated with the onset of neurological symptoms. Increased NO production occurs in MS as illustrated by elevated levels of nitrite and nitrate levels in CSF (Johnson et al., 1995). Using immunocytochemistry, NOS-2 and nitrotyrosine have been found in diseased brain (Okuda et al., 1995; Hooper et al., 1995; Bagasra et al., 1995; DeGroot et al., 1997; Merrill et al., 1998). Both the protein and the transcript for NOS-2 colocalize with markers for macrophages/microglia (Bagasra et al., 1995; Hooper et al., 1997; van der Veen, 1997) and/or astrocytes (Okuda et al., 1997; Tran et al., 1997; Merrill et al., 1998). Cross et al. (1996) showed that NOS-2 transcript and enzyme activity correlate with active EAE. It is of interest that MS patients have elevated circulating antibodies to S-nitrosocysteine, suggesting nitrosylation of proteins in vivo (Boullerne et al., 1995). Akin to the human situation with MS, Ding et al. (1997) find gender-relatedness in NOS-2 expression and NO production in mice with EAE.

Based on the evidence for NO production in MS and EAE, several studies have assessed roles for NO using NOS inhibitors. The results have been mixed. In one study of acute EAE inhibitors of NOS provided no benefit (Zielasek et al., 1995). Other EAE studies showed a small rise in clinical score (Ruuls et al., 1996) or a decrease in disease incidence, severity and duration (Cross et al., 1994; Zhao et al., 1996). Most recently, Hooper et al. (1997) report significant therapeutic effects on EAE with NOS inhibitors, and scavengers of NO and peroxynitrite.

The current therapeutic available for the treatment of MS is IFNβ, demonstrated to be a blocker of NOS-2 expression (Stewart et al., 1997). Hall et al. (1997) suggest that it is beneficial through its antagonism of endogenously produced IFNγ, hence impairing the activation of macrophages and microglia. Treatment of EAE animals with anti-inflammatory cytokines such as IL10, another NOS-2 modulator, inhibits disease (Willenborg et al., 1995). However, using the EAE model in NOS-2 gene deficient mice, Fenyk-Melody et al. (1998) report that disease is more severe and prolonged. This might suggest either that NO is immunosup-

pressive at the time of immunization or that, without NO, there is a loss of the normal regulation of infiltration of inflammatory cells into the CNS (De Caterina et al., 1995; Hickey et al., 1997).

Cerebral ischemia

Elucidating the sequence of physiological and biochemical events following cerebral ischemia, in an attempt to identify means to reduce the amount of damage that develops, has been the focus of extensive investigation. The early events that occur following blood flow reduction, leading to cell death, have been well characterized in animal models of cerebral ischemia (Siesjo, 1992). Briefly, ischemia induces mitochondrial injury leading to a rapid depletion of ATP levels and a disruption of normal ion homeostasis. Large amounts of glutamate are released and intracellular calcium levels rise because of NMDA-receptor activation. The massive increase in free intracellular calcium following ischemia is a precursor to a variety of events capable of promoting neuronal and glial cell death such as inflammation, gene activation, and free radical production (for a review see Iadecola, 1997). While the pathogenesis of ischemic brain injury is probably the result of the activation of multiple biochemical pathways, it is evident, given the chain of events that occur, that NO has the potential to participate in the mechanisms of cerebral ischemia.

There is sufficient evidence that NO is produced following both global and focal cerebral ischemia. Using techniques such as in vivo spin trapping (Sato et al., 1994), porphyrinic microsensor measurement (Malinski et al., 1993), and measurements of brain nitrite and cGMP levels as a reflection of increased NO production (Kader et al., 1993; Shibata et al., 1996), increased generation of NO in the brain following cerebral ischemia has been demonstrated.

NOS-2 and global ischemia

Transient global ischemia is seen clinically associated with disorders of systemic circulation, such as

heart failure or other cardiac dysfunction. Blood flow to the brain may be reduced below levels of autoregulation or may be completely interrupted for a period of time. Global ischemia induces a gliotic reaction, and delayed neuronal necrosis occurs in selectively vulnerable cells such as pyramidal neurons in the CA1 subfield of the hippocampus. NOS-2 protein expression has been shown to occur in a rat model of transient global ischemia (Endoh et al., 1994). Three days after a 10 min ischemic episode, NOS-2 protein was detected in the CA1 region of the hippocampus by immunohistochemistry, with immunoreactivity progressively increasing to day 30. Double-labeling experiments identified these NOS-2-expressing cells as reactive astrocytes, and ruled out microglial expression.

In our own studies with spontaneously hypertensive rats, we can detect NOS-2 protein by 72 h of reperfusion following 30 min of global ischemia. These cells morphologically resemble astrocytes in the corpus callosum, basal ganglia, and the hippocampus (Fig. 1). In response to global ischemia, NOS-2 induction parallels, spatially and temporally, the gliotic reaction. The induction of NOS-2 appears to be restricted to astrocytes in regions of neuronal damage, as there is no evidence for NOS-2 expression in other cell types or other regions. The mechanism of delayed NOS-2 induction, and the role of NO generated by this isoform following global ischemia remains to be determined. To date, no studies have been performed investigating global ischemic damage in mice deficient in NOS-2 expression. This may be due to the difficulties associated with mice not surviving the ischemic episode (Yang et al., 1997), or it may be due to the complications of strain variability in global ischemic outcome (Fujii et al., 1997).

NOS-2 and focal ischemia

Focal cerebral ischemia, or stroke, occurs as a regional injury and results from the prolonged interruption of blood flow due to the occlusion of a single CNS artery. A focal vascular occlusion
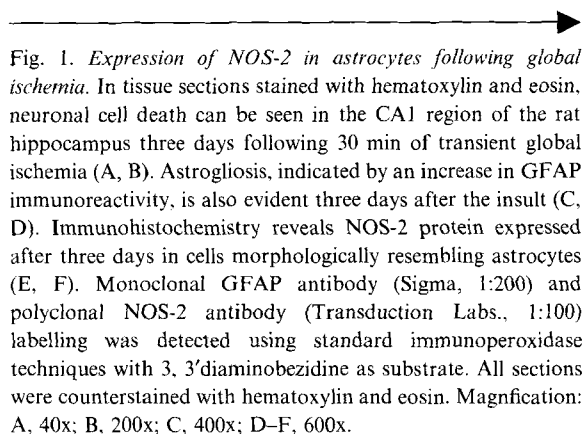

Fig. 1. *Expression of NOS-2 in astrocytes following global ischemia.* In tissue sections stained with hematoxylin and eosin, neuronal cell death can be seen in the CA1 region of the rat hippocampus three days following 30 min of transient global ischemia (A, B). Astrogliosis, indicated by an increase in GFAP immunoreactivity, is also evident three days after the insult (C, D). Immunohistochemistry reveals NOS-2 protein expressed after three days in cells morphologically resembling astrocytes (E, F). Monoclonal GFAP antibody (Sigma, 1:200) and polyclonal NOS-2 antibody (Transduction Labs., 1:100) labelling was detected using standard immunoperoxidase techniques with 3, 3'diaminobezidine as substrate. All sections were counterstained with hematoxylin and eosin. Magnfication: A, 40x; B, 200x; C, 400x; D–F, 600x.

produces a localized, destructive lesion or infarct, with necrosis of all cellular elements in the territory normally perfused by the occluded vessel. Ischemic stroke occurs most often in humans as the result of a thromboembolic vascular occlusion, secondary to atherosclerosis, in which there is a focal deposition of plasma-derived lipids that inhibits normal flow. In rodent models, focal ischemia can be induced by either permanently or transiently occluding the middle cerebral artery (MCA).

Several studies have provided biochemical, molecular, and immuno-histochemical evidence of NOS-2 induction following focal cerebral ischemia. Two separate reports demonstrated the induction of NOS-2 activity following permanent MCA occlusion in rats (Yoshida et al., 1995; Iadecola 1995a). Both studies found an increase in NOS-2 activity at 2–3 days post-occlusion, with a return to baseline by seven days. The time course of NOS-2 induction suggests that NO generated from this isoform does not contribute to the early stages of ischemic injury. Iadecola et al. (1995c; 1996) have also investigated NOS-2 mRNA and protein expression in response to focal ischemia. Following permanent MCA occlusion in rats, NOS-2 mRNA was detected by 12 h, peaked at 48 h and returned to baseline by seven days post-occlusion. Immunoreactivity for NOS-2, observed at 48–96 hours after ischemia, was detected only in neutrophils invading the infarct and was not seen in resident cells (Iadecola et al., 1996). Transient
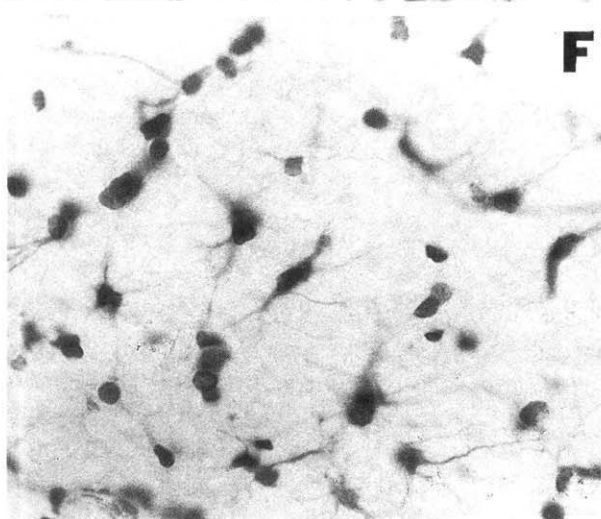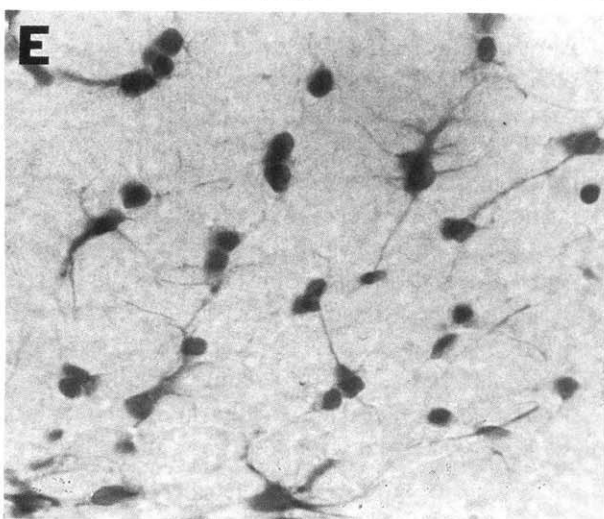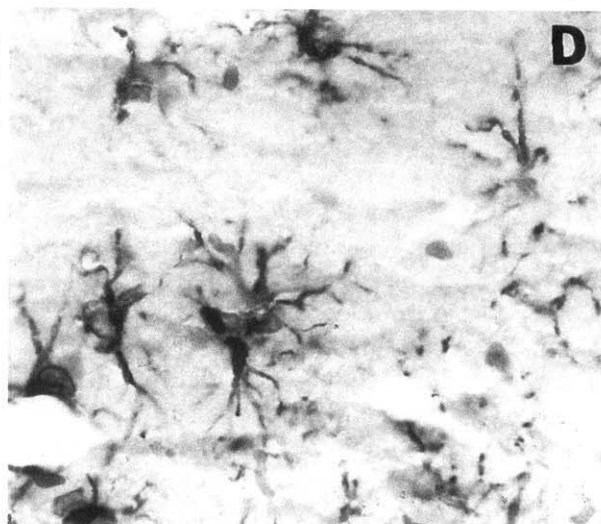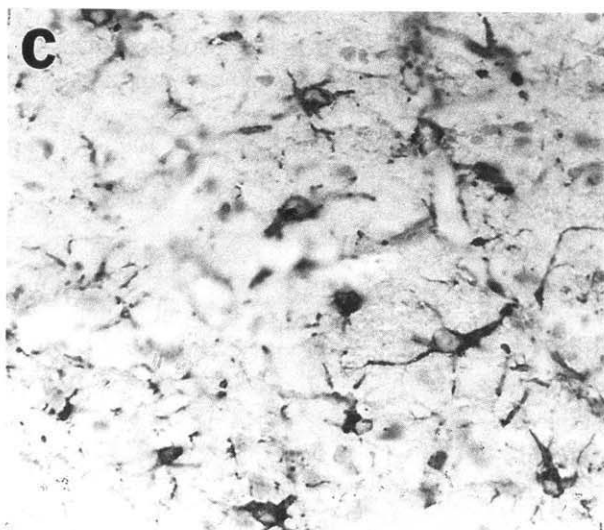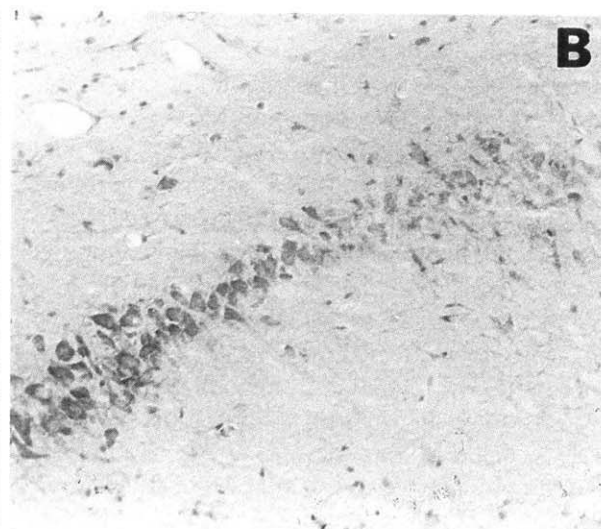
focal ischemia in rats also leads to NOS-2 induction, though with different spatial and temporal patterns of expression than seen following permanent occlusion. Two hours of MCA occlusion, followed by reperfusion, resulted in maximal expression of NOS2 mRNA at an earlier time point, and detection of protein predominantly in vascular cells, rather than in neutrophils (Iadecola et al., 1996).

In mice, permanent MCA occlusion using the filament model of ZeaLonga et al. (1989) leads to the induction of NOS-2 mRNA and protein expression. Using in situ hybridization, we were able to detect NOS-2 message at 24, 48 and 72 h (latest point studied), but not at 6 h post-occlusion (Fig. 2). At 24 hours after ischemia, NOS-2 immunoreactivity was observed primarily in what appeared, morphologically, to be inflammatory cells within the infarct (Figure 2). However, at 48 and 72 h NOS-2 immunoreactivity was also observed in cells morphologically resembling astrocytes, as well as by GFAP staining on adjacent sections (Fig. 2). These NOS-2 positive cells were located primarily in regions surrounding the infarct. In contrast to previously published reports, these data indicate that resident glial cells, in addition to infiltrating cells, express NOS-2 in the ischemic brain. The differences in our findings compared to previous reports may reflect the differences in total infarct volumes produced as a result of using different surgical procedures (cauterization versus filament occlusion). The infarct volume generated with the filament model of MCA occlusion was typically greater than 80 mm^3 (Loihl and Murphy, 1997) while infarcts produced by cauterization were generally smaller ($< 40 \text{ mm}^3$), and predominantly cortical (Iadecola et al., 1997).

Role of NOS-2 in cerebral ischemia

While it is clear that NO participates in the mechanisms of ischemia, much of the early work investigating the precise role of NO yielded contradictory results. This controversy stemmed largely from the fact that much of the research being
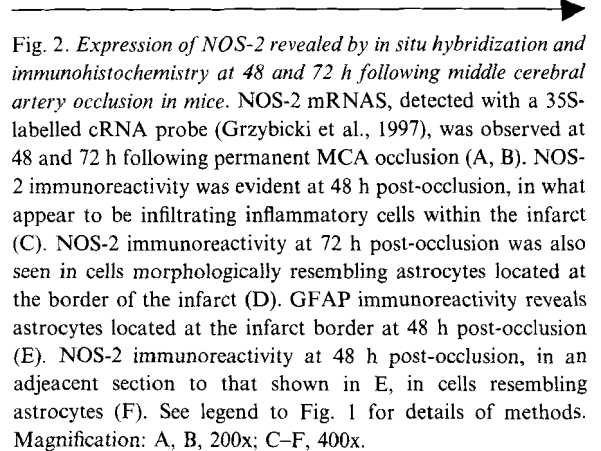
Fig. 2. *Expression of NOS-2 revealed by in situ hybridization and immunohistochemistry at 48 and 72 h following middle cerebral artery occlusion in mice.* NOS-2 mRNAS, detected with a 35S-labelled cRNA probe (Grzybicki et al., 1997), was observed at 48 and 72 h following permanent MCA occlusion (A, B). NOS-2 immunoreactivity was evident at 48 h post-occlusion, in what appear to be infiltrating inflammatory cells within the infarct (C). NOS-2 immunoreactivity at 72 h post-occlusion was also seen in cells morphologically resembling astrocytes located at the border of the infarct (D). GFAP immunoreactivity reveals astrocytes located at the infarct border at 48 h post-occlusion (E). NOS-2 immunoreactivity at 48 h post-occlusion, in an adjeacent section to that shown in E, in cells resembling astrocytes (F). See legend to Fig. 1 for details of methods. Magnification: A, B, 200x; C–F, 400x.

done involved the use of pharmacological inhibitors that, while somewhat selective, are not actually specific for a particular isoform of NOS. Experiments in vivo have shown that inhibition of NO synthesis ameliorates (Nowicki et al., 1991; Buisson et al., 1992; Nagafugi et al., 1992; Ashwal et al., 1993; Iadecola et al., 1995b, 1996; Zhang et al., 1996) and worsens (Yamamoto et al., 1992; Dawson et al., 1992; Kuluz et al., 1993) tissue damage in animal models of cerebral ischemia. Given the scope of potential biological actions of NO, and the different spatial and temporal patterns of expression of the NOS isoforms, it is likely that NO generated by the different isoforms is subserving different roles at different times following ischemic insult. Therefore, non-specifically blocking NOS activity overshadows the potential for multiple roles of NO in ischemia. To circumvent the limitations of the currently available pharmacological inhibitors, and to elucidate the contributions of the different isoforms of NOS, ischemic brain damage has been investigated in genetically altered mice, which are deficient in a particular isoform of NOS.

Evidence provided in the last few years using gene targeting strategies has resolved the debate over the roles of the constitutive isoforms of NOS in the pathophysiology of focal cerebral ischemia (Iadecola, 1997). Infarct volume, resulting from MCA occlusion, in mice deficient for NOS-1 is reduced compared to wild type mice. Infarct
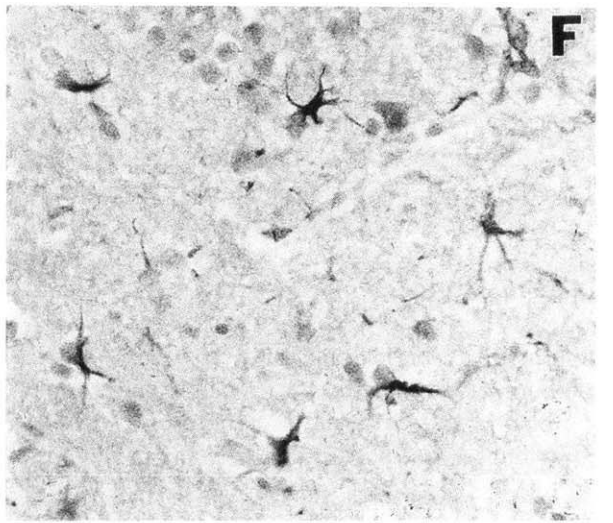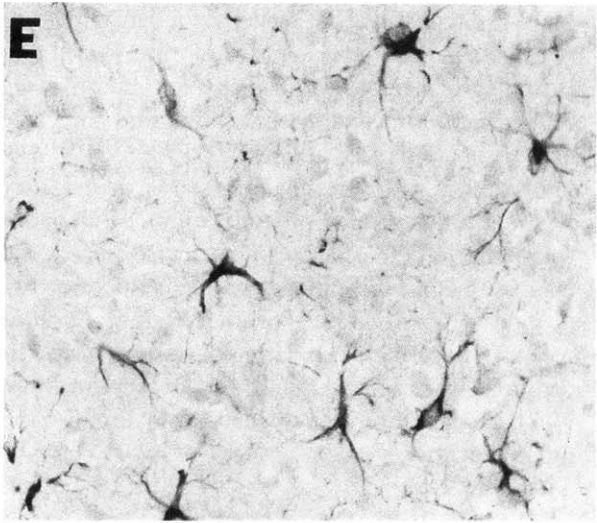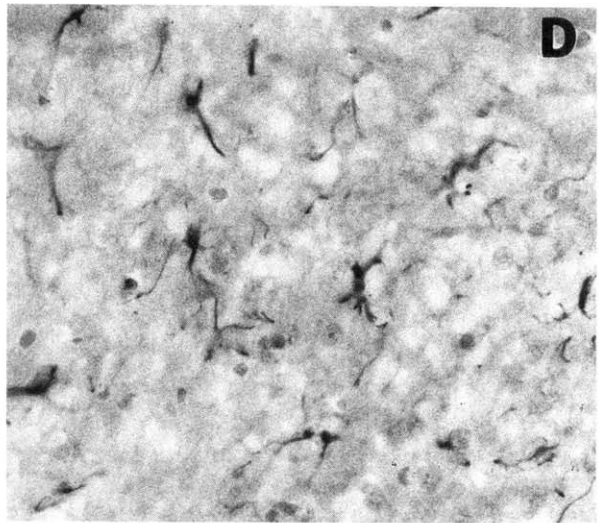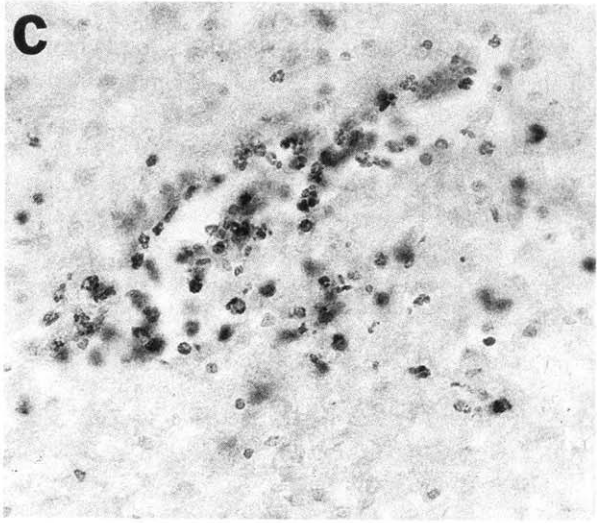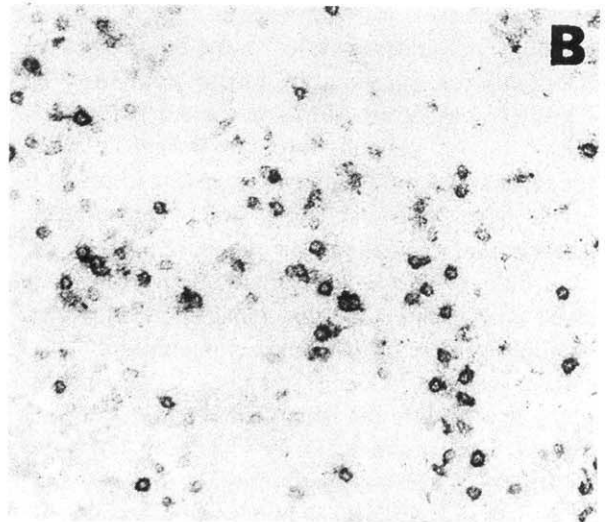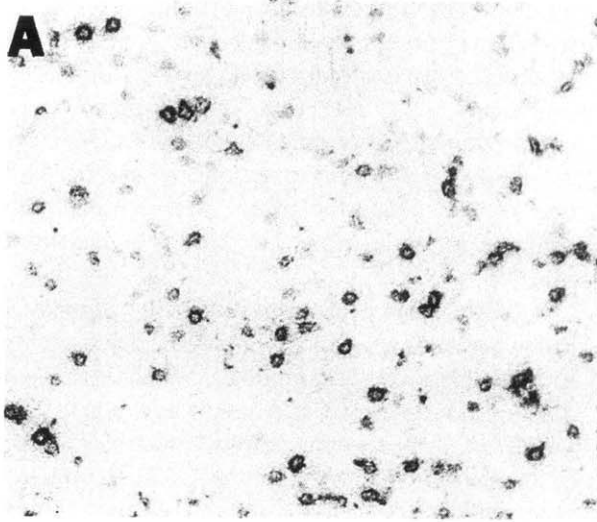
volume, however, in these mutant mice increased following administration of nitro-L-arginine, a NOS inhibitor, presumably due to inhibition of NOS-3 (Huang et al., 1994). In mice deficient for NOS-3, infarct volumes were larger than in wild type littermates. These results indicate that NO derived from NOS-1 acts early following cerebral ischemia and contributes to the development of tissue damage, while NO generated from NOS-3 serves a protective function, most likely by regulating blood flow to the ischemic penumbra.

More recently, evidence has been provided that begins to elucidate the role of the NOS-2 isoform in focal cerebral ischemia. A report by Iadecola et al. (1997) demonstrated that 24 h following MCA occlusion, infarct volumes in mice deficient for NOS-2 did not vary from infarct volumes in wildtype animals. We have found similar results in our own studies (Loihl and Murphy, 1997). This is consistent with the idea, suggested by enzyme activity studies, that NOS-2 does not contribute to the early stages of ischemic damage, and is not particularly surprising given the time course of NOS-2 induction. Iadecola et al. (1997) did find, however, that 96 h after MCA occlusion infarct volumes in NOS-2 deficient mice were 28% smaller than in wild-type controls. This smaller infarct at 96 h post-occlusion was accompanied by less severe motor deficits in mutant mice, indicating that the lack of NOS-2 correlates with improved functional outcome.

Regulation and role of astroglial NOS-2 in neuropathologies

Perhaps the only conclusions that can be drawn from the neuropathological evidence presented above are that NOS-2 is sometimes expressed in astroglial cells and that, when there is expression, its appearance is delayed (> 1 day) beyond what we might predict from in vitro observations (a few hours). This tardy appearance of NOS-2 might simply reflect development of the appropriate conditions for gene activation, such as infiltration of inflammatory cells either producing the relevant cytokines or initiating their production in resident cells. Indeed, in some pathologies other stimuli may predominate, such as hypoxia. On the other hand, we could interpret the delay in NOS-2 expression as a reflection of the presence of factors that suppress gene activation. In this case, the timing of NOS-2 expression and the production of NO from this source may represent an adaptive response, and thus hint at a beneficial function.

If NOS-2 gene expression is initially suppressed following damage, then the in vitro evidence points to a number of likely candidates. While the in vivo data are not definitive, they are at least consistent. Levels of transforming growth factor $\beta 1$ rise following ischemia (Wang et al., 1995; Krupinski et al., 1996). Extracellular glutamate and ATP are elevated and persist for a few hours following CNS injury and the activation of selective chemokine genes is an early event. Specific chemokines are elevated following trauma (Ghirnikar et al., 1996; Grzybicki et al., 1998), exposure to β-amyloid (Meda et al., 1996), with bacterial infection (Spanaus et al., 1997), in EAE and MS (Hulkower et al., 1993; Karpus et al., 1995; Miyagishi et al., 1995; Ransohoff et al., 1996) and in ischemia (Kim et al., 1995; Yamasaki et al., 1995, 1997; Takami et al., 1997; Yoshimoto et al., 1997). Not only will chemokines such as IL-8 and MCP-1 block NOS-2 induction (McCall et al., 1992; Rojas et al., 1993) but NO can also inhibit the expression of IL-8 and MCP-1 (Andrew et al., 1995; Zeiher et al, 1995; De Caterina et al., 1995). A delayed appearance of NOS-2 might therefore ensure an environment in which chemokine gene expression can proceed and the entry of hematogenous cells is unhindered by NO effects on endothelial adhesion molecule expression (Khan et al., 1996; Tsao et al., 1996; Hickey et al., 1997). That the later production of NO might then terminate infiltration of inflammatory cells into the parenchyma, or downregulate expression of pro-inflammatory cytokines, are intriguing possibilities. Together with its general role as a vasodilator, thus improving perfusion in compromised tissue and leading to remodelling, NO may therefore prove beneficial by limiting the inflammatory response.

From functional and genetic studies, it seems that the effects of NO from NOS-2 can either worsen or improve recovery from damage. Evidence from the various enzyme inhibition studies is mixed, fraught with problems regarding specificity. Evidence from studies with NOS-2 gene-deficient mice is emerging to suggest that the presence of the enzyme can be beneficial, deleterious or without consequence (Nathan 1997). In cerebral ischemia, while NOS-2 clearly does not contribute to pathology within the first 24 h (Iadecola et al., 1997; Loihl and Murphy, 1997), gene-deficient mice are reported to have smaller infarcts at 4 days (Iadecola et al., 1997). Such NOS-2 deficient animals have a greater leukocyte response in endotoxemia (Hickey et al., 1997) which might explain why the condition of such mice with EAE is made worse (Fenyk-Melody et al., 1998).

The interpretation of results from gene-deficient mice is itself not without problems because of the possibility of developmental compensation and the real potential for expression of a truncated protein, perhaps with novel or unregulated functions. However, and in the absence of truly selective enzyme inhibitors, observing neuropathological outcome in experimental models employing such NOS-2 deficient mice is a powerful indicator of the roles of NOS-2. Verification could then be sought by virally re-introducing the gene in deficient animals. It would also be informative to observe pathological outcomes in transgenic mice in which NOS-2 can be overexpressed. However, there are no reports of the generation of such animals to date.

Acknowledgements

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