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Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices

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Abstract

Nitric oxide (NO) overproduction has been postulated to contribute significantly to ischaemia-reperfusion neurotoxicity. Inducible or type II NO synthase (iNOS) synthesizes NO in large quantities for long periods of time. Therefore we investigated the expression and localization of iNOS after oxygen and glucose deprivation in rat forebrain slices. In this experimental model, calcium-independent NOS activity reached a maximum 180 min after the end of a 20 min oxygen–glucose deprivation period. During the same period of time, the calcium-independent activity was absent in control forebrain slices. To test whether this calcium-independent NOS activity was due to the expression of iNOS, the effects of the addition of dexamethasone, cycloheximide and pyrrolidine dithiocarbamate were determined. All of them inhibited the induction of the calcium-independent NOS activity measured in the rat forebrain slices after oxygen and glucose deprivation. Furthermore, oxygen and glucose deprivation caused the expression of the gene encoding iNOS in rat forebrain slices, as assessed by the detection of iNOS message and protein in these samples. A sixfold increase in the iNOS mRNA levels was observed at 180 min and the time-course of the expression of iNOS mRNA was in agreement with the temporal profile of iNOS enzymatic activity. Immunohistochemistry analysis revealed that iNOS was highly expressed in neurones, astrocytes and microglial cells. These results demonstrate for the first time that iNOS is expressed in neurones after oxygen and glucose deprivation, and that this expression occurs in short periods of time. These findings suggest that NO can play an important pathogenic role in the tissue damage that occurs after cerebral ischaemia.

Introduction

The mechanisms of ischaemic brain damage are still a matter of debate. The neurotoxic actions of glutamate and other excitatory amino acids have been implicated in the pathogenesis of brain injury (Choi & Rothman, 1990). An overproduction of nitric oxide (NO) derived from the excessive stimulation of the neuronal NO synthase (type I NOS or nNOS) has been postulated to be the link between the actions of excitatory amino acids and the subsequent cell damage (Dawson *et al.*, 1991). Data showing that mice lacking nNOS are more resistant to ischaemic damage (Huang *et al.*, 1994) are also consistent with this idea. In this context, increased NO levels have been reported after ischaemia-reperfusion (Malinski *et al.*, 1993). nNOS and endothelial NOS (type III NOS or eNOS) are constitutively expressed, require calcium and calmodulin for activation, and produce NO for short intervals. Furthermore, eNOS is also important for maintaining adequate tissue perfusion (Huang *et al.*, 1994) and its immunoreactivity is up-regulated in the cerebral vessels after ischaemia (Zhang *et al.*, 1993).

NO is also synthesized by a high-output inducible isoform of NOS (type II NOS or iNOS); this isoform is independent of calcium and calmodulin and is expressed after exposure to cytokines and/or lipopolysaccharide (LPS) (for a review, see Knowles & Moncada, 1994). This NOS isoenzyme mediates cytotoxicity in many cell systems (Moncada *et al.*, 1991; Gross & Wolin, 1995). The metabolic derangement that occurs during ischaemia and subsequent reperfusion results in the expression and release of several cytokines such as tumour necrosis factor-α and pro-inflammatory cytokines (Rothwell & Relton, 1993), important mediators which activate the expression of iNOS in different cell types, including some from the central nervous system (Galea *et al.*, 1992; Simmons & Murphy, 1992, 1993). It is therefore possible that iNOS is expressed after ischaemia and contributes to the cell damage associated with this condition. In support of this idea, it has been shown that a selective inhibitor of iNOS ameliorates damage caused by focal cerebral ischaemia (Iadecola *et al.*, 1995a) and that blood and glial cells express iNOS immunoreactivity in *in vivo* models of brain ischaemia-reperfusion (Endoh *et al.*, 1994; Wallace & Bisland, 1994; Iadecola *et al.*, 1995b). Therefore, we investigated the expression and localization of iNOS after oxygen and glucose deprivation using rat forebrain slices. Our

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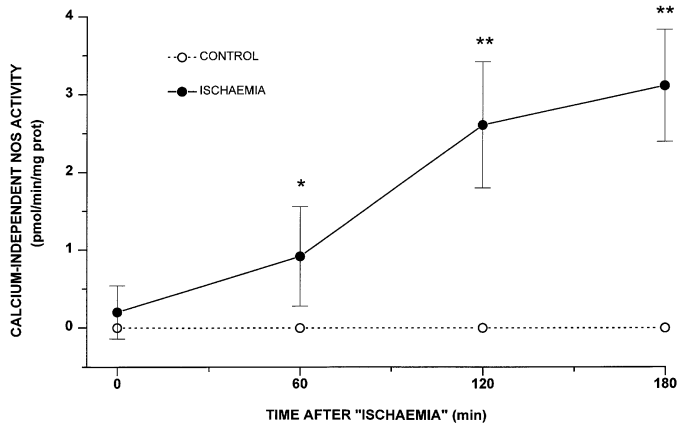


FIG. 1. Time-course of calcium-independent nitric oxide (NO) synthase activity from control and 'ischaemic' rat forebrain slices. Nitric oxide synthase (NOS) activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline (see Materials and methods). The data represent the mean ± SEM of 16 independent experiments. $*P < 0.05$; $**P < 0.01$ (Newman–Keuls test).

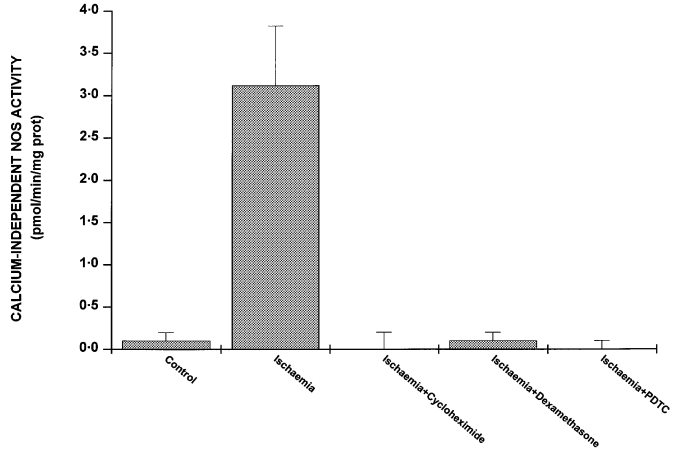


FIG. 2. Effect of cycloheximide, dexamethasone and pyrrolidine dithiocarbamate (PDTC) on calcium-independent nitric oxide (NO) synthase activity in 'ischaemic' rat brain slices collected at 180 min of 'reperfusion'. Enzymatic activity was determined as described in legend to Figure 1.

data show that iNOS is expressed not only in glial cells but also in neurones following oxygen and glucose deprivation.

Materials and methods

Preparation and incubation of slices

Male Sprague–Dawley rats (200–250 g) were killed by decapitation (according to procedures approved by Committee of Animal Care at the Universidad Complutense of Madrid), the forebrain was removed and coronally cut (2 mm anterior and 6 mm posterior to bregma), and the central portion sliced (0.4 mm slice thickness) using a Vibraslice (Campden Instruments, World Precision Instruments Ltd, Stevenage, Herts., UK) in cold (12–14 °C) modified Krebs–Henseleit solution (preincubation solution) containing (mM): NaCl, 120; KCl, 2; $CaCl_2$, 0.5; $NaHCO_3$, 26; $MgSO_4$, 10; KH_2PO_4, 1.18; glucose, 11 and sucrose 200) (Garthwaite *et al.*, 1979; Lizasoain *et al.*, 1995). Sucrose was added in order to obtain an hypertonic solution for preventing cell swelling. High magnesium and low calcium concentrations were used to prevent *N*-methyl-D-aspartate (NMDA)-receptor-mediated damage (Aitken *et al.*, 1995).

Three experimental groups of slices were performed. Some slices (control 0) were prepared for immunocytochemistry, Northern and Western blot immediately after cutting. For the other two groups, slices were preincubated in sucrose-free preincubation solution equilibrated with 95% O_2/5% CO_2, in a shaking water bath at 37 °C for 45 min. After the preincubation period, slices were incubated in a modified Krebs–Henseleit solution (incubation solution) containing (mM): NaCl, 120; KCl, 2; $CaCl_2$, 2; $NaHCO_3$, 26; $MgSO_4$, 1.19; KH_2PO_4, 1.18; glucose, 11 and 5,6,7,8-tetrahydrobiopterin (BH_4), 10 μM; bubbled with 95% O_2/5% CO_2. The slices corresponding to the second group (control) were then incubated 20 min further in the same conditions. Slices corresponding to the third experimental group ('ischaemic') were incubated 20 min in incubation solution without glucose and equilibrated with 95% N_2/5% CO_2 to mimic an ischaemic condition. After these periods of 20 min, the medium was replaced with fresh incubation solution equilibrated with 95% O_2/5% CO_2 to simulate a reperfusion period. Slices were taken out at different times: 60, 120 and 180 min (control 180 or ischaemic 180) after the 'ischaemic' period and frozen immediately with liquid nitrogen.

NOS activity

NO synthase activity was determined after homogenization of the forebrain slice (Heidolph homogenizer) at 4 °C in 5 volumes of buffer containing 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DL-dithiothreitol, 10 μg/mL leupeptin, 100 μg/mL phenylmethylsulphonyl fluoride, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 50 mM Tris brought to pH 7.0 at 20 °C with HCl. The homogenate was centrifuged at 5 °C at 12 000 ***g*** for 20 min and the pellet discarded. NO synthase activity was then determined in cell extracts under conditions (substrate and calcium concentration) of maximal activity, to assess indirectly the amount of enzyme present by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline in the postmitochondrial supernatant. The activity of the calcium-dependent NOS was calculated from the difference between the [^{14}C]citrulline produced from control samples and samples containing 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA); the activity of the calcium-independent isoform was determined from the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA and 1 mM N^G-monomethyl-L-arginine (L-NMMA) (Salter *et al.*, 1991).

The protein content of the homogenate from each slice was determined using bicinchoninic acid (Hill & Straka, 1988).

mRNA assay

Total RNA was extracted from forebrain slices by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). Aliquots of RNA (10 μg) were size-fractionated by electrophoresis (20 mA 15 h) in a 0.9% agarose gel containing 2% formaldehyde and 3- (N-morpholino)-propanesulphonic acid buffering system (Chomzynski & Sacchi, 1987). After transference of the RNA to Nytran membranes (NY 13-N, Schleicher and Schüell, Dassel, Germany) the level of iNOS mRNA was determined by hybridization using as probe an *Eco*RI–*Hin*dII fragment from the murine iNOS cDNA (donated by Dr Q.-W. Xie and Dr C. Nathan, Cornell University) labelled with [α-^{32}P]dCTP (Random Primed labelling kit; Amersham, Ibérica, Madrid, Spain). Upon hybridization the membranes were exposed to an X-ray film (Kodak-X-OMAT) and the bands were quantified by laser densitometry (Molecular Dynamics, Sunny Valley, CA, USA). Hybridization with a probe specific for β-actin was used to normalize the RNA lane charge of the blot.

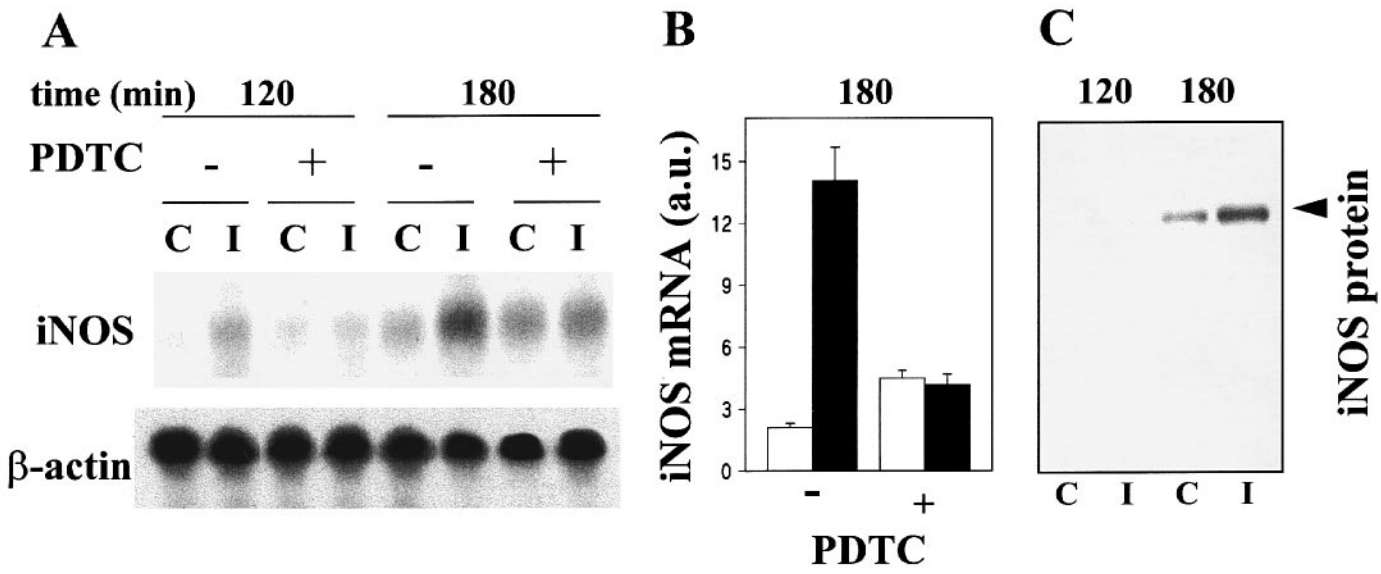


FIG. 3. (A) Northern blot analysis of the expression of inducible nitric oxide synthase (iNOS) mRNA in control -C- and 'ischaemic' brain slices -I-, collected at 120 (columns 1–4) and 180 min (columns 5–8) of 'reperfusion'. (B) Laser densitometric analysis of iNOS expression at 180 min. Effect of pyrrolidine dithiocarbamate (PDTC) on iNOS mRNA levels in control (open bars) and 'ischaemic' rat brain slices (filled bars). (C) Detection of iNOS protein by Western blot in control and 'ischaemic' brain slices. Results show the mean ± SEM of 16 samples corresponding to slices obtained at both times.

Characterization of iNOS by Western blot

Slices were homogenized in lysis buffer (10 mM Tris pH 8.0, 0.2% Nonidet P-40, 1 mM dithioerythritol) and after centrifugation in a microcentrifuge for 15 min, the proteins present in the supernatant were loaded (10 µg) and size-separated in 10% sodium dodecyl sulphate–polyacrilamide gel electrophoresis (50 mA). The gels were blotted on to a PVDF membrane (Millipore) and incubated with a specific polyclonal iNOS antibody (Transduction Laboratories, Lexington, KY, USA; 1 : 1000 dilution; Lowenstein *et al.*, 1992). iNOS isoform was revealed by ECL™-kit following manufacturers instructions (Amersham).

Tissue preparation for histochemistry

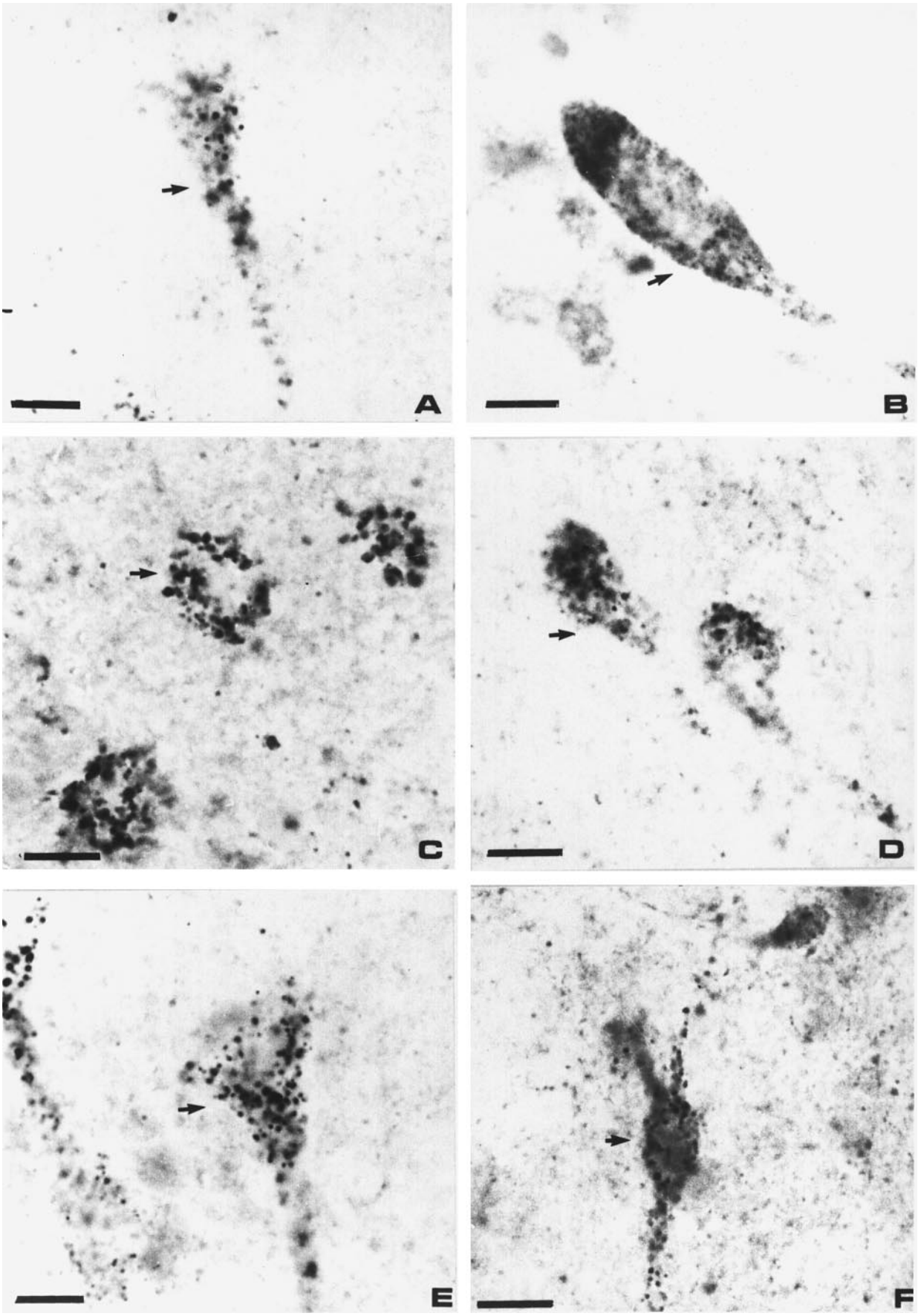
Slices were fixed for optical and electron microscopy studies (Rodrigo *et al.*, 1994). For optical microscopy slices were fixed by immersion for 4 h in a solution containing 4% *p*-formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 at room temperature. For electron microscopy slices were fixed by immersion for 30 min in 4% *p*-formaldehyde and 0.05% glutaraldehyde in the same buffer. The slices were then postfixed for 4 h in 4% *p*-formaldehyde at room temperature. After fixation, the slices were rinsed with stirring by immersion in 0.1 M PB containing 30% sucrose overnight at 4 °C.

Immunohistochemistry

Light microscopy

A. Immunohistochemistry of iNOS. Free-floating 40 µm sections were incubated first in phosphate-buffered saline (PBS) containing 3% normal goat serum (ICN Biochemicals, Costa Mesa, CA, USA) and 0.2% Triton X-100 for 30 min and subsequently with a specific antibody anti-iNOS at a 1 : 3000 dilution overnight at 4 °C. In order to ensure that the staining was specific, two rabbit antisera against iNOS were tested: an antipeptide that recognizes human hepatocyte iNOS (Evans *et al.*, 1996, a gift from J. M. Polak, Hammersmith Hospital, London UK) and a polyclonal antibody specific for the mouse macrophage iNOS (Transduction Laboratories, Lowenstein *et al.*, 1992). After washing in PBS, the sections were incubated in a biotinylated antirabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA; 1 : 200 dilution) in PBS for 1 h. After washing in PBS, the sections were incubated in a solution of peroxidase-linked ABC (ABC kit, Vector Laboratories) and the nickel-enhanced diaminobenzidine procedure was used to reveal the peroxidase activity (Shu *et al.*, 1988).

B. Double histochemistry for neurone-specific enolase (NSE)/iNOS, glial-fibrillary acidic protein (GFAP)/iNOS and for biotin conjugate of Lycopersicon esculentum agglutinin (tomato lectin)/iNOS. To double stain either for both NSE and iNOS, for both GFAP and iNOS or for both tomato lectin and iNOS, sections were washed in PBS containing 3% normal goat serum and 0.2% Triton X-100 for 30 min and then sections were incubated in the two primary antibodies for each double staining overnight at 4 °C: anti-iNOS and a mouse antineurone specific enolase antibody (Chemicon, Temecula, CA, USA; 1 : 1 dilution) to identify neurones (Marangos & Schmechel, 1987; Sommer *et al.*, 1995), anti-iNOS and a rat anti-GFAP antibody (Miles S.A., Madrid, Spain; 1 : 400 dilution) to identify astrocytes or anti-iNOS and a biotinylated tomato lectin (from *Lycopersicon esculentum*; 1 : 150 dilution) to characterize microglia and macrophages (Acarin *et al.*, 1994, 1996; Velasco *et al.*, 1995). After washing in PBS, the sections were incubated in each respective secondary antibody for 1 h. For iNOS, Cy 2™-labelled goat antirabbit IgG was used (Amersham; 1 : 1000 dilution; green colour with fluorescence maximum at 506 nm); for NSE and GFAP, the sections were incubated in a biotinylated antimouse or antirat IgG, respectively (Vector Laboratories; 1 : 100 dilution) for 1 h and then in Cy 5™-avidin (Amersham; 1 : 1000 dilution; far-red colour with fluorescence maximum at 670 nm); for tomato lectin, the sections were directly incubated in Cy 5™-avidin. The sections were mounted with PBS/glycerol and analysed with a TCS laser scanning confocal imaging system equipped with a krypton-argon ion laser (Leica, Spain).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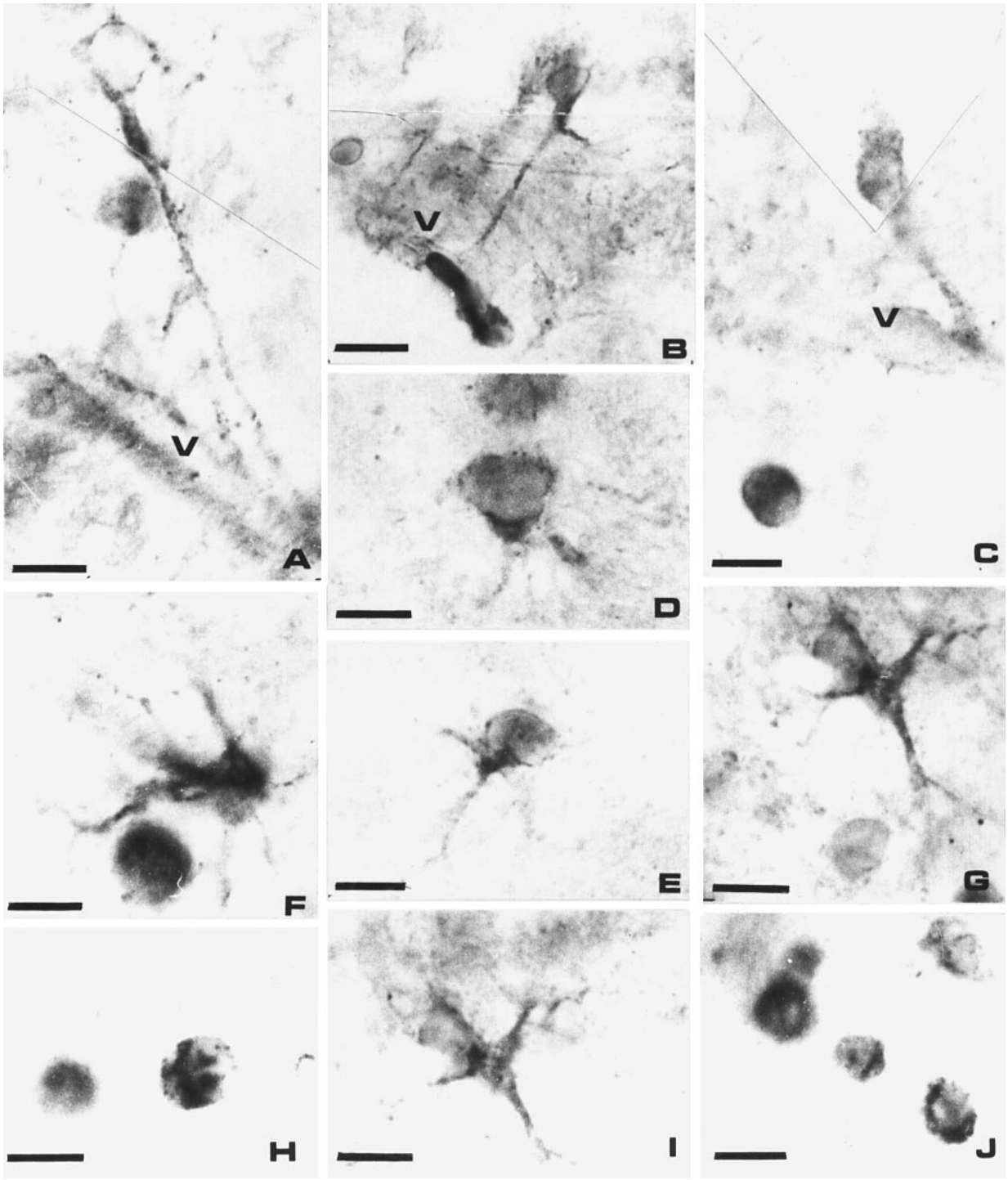


FIG. 5. Inducible nitric oxide synthase (iNOS) staining of astrocytes, microglia and macrophages in sections from rat forebrain slices exposed to oxygen and glucose deprivation at 180 min of 'reperfusion'. (A–C) Immunoreactive perivascular astrocytes with a long process ending around a small blood vessel (V). (D–G and I) Immunoreactive microglia with short processes irregularly distributed around the cytoplasm. (H and J) Small swollen cells that might correspond to transformed macrophages. The pattern of iNOS immunostaining is homogeneously distributed throughout the cytoplasm conferring a dark background. Scale bar = 6 μm.

FIG. 4. Inducible nitric oxide synthase (iNOS) staining of neuronal cells in sections from rat forebrain slices exposed to oxygen and glucose deprivation after 180 min of 'reperfusion'. (A, C–E) Immunoreactive cells with large apical dendritic processes, triangular body and large diameter, morphology that corresponds to pyramidal neurones. (B and F) Immunoreactive cells with a multipolar shape, smaller diameter and fusiform morphology typical of associative neurones. The pattern of iNOS immunostaining is granular forming clusters. Scale bar = 12 μm.

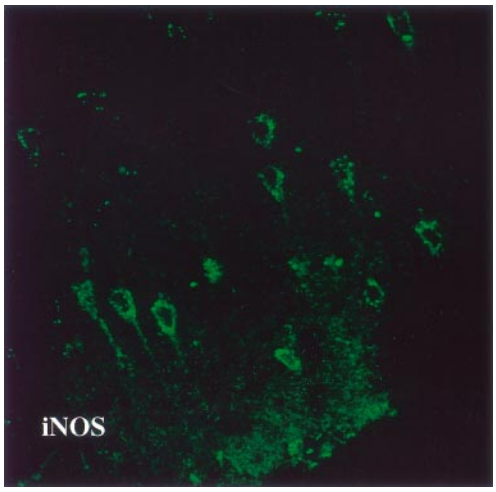
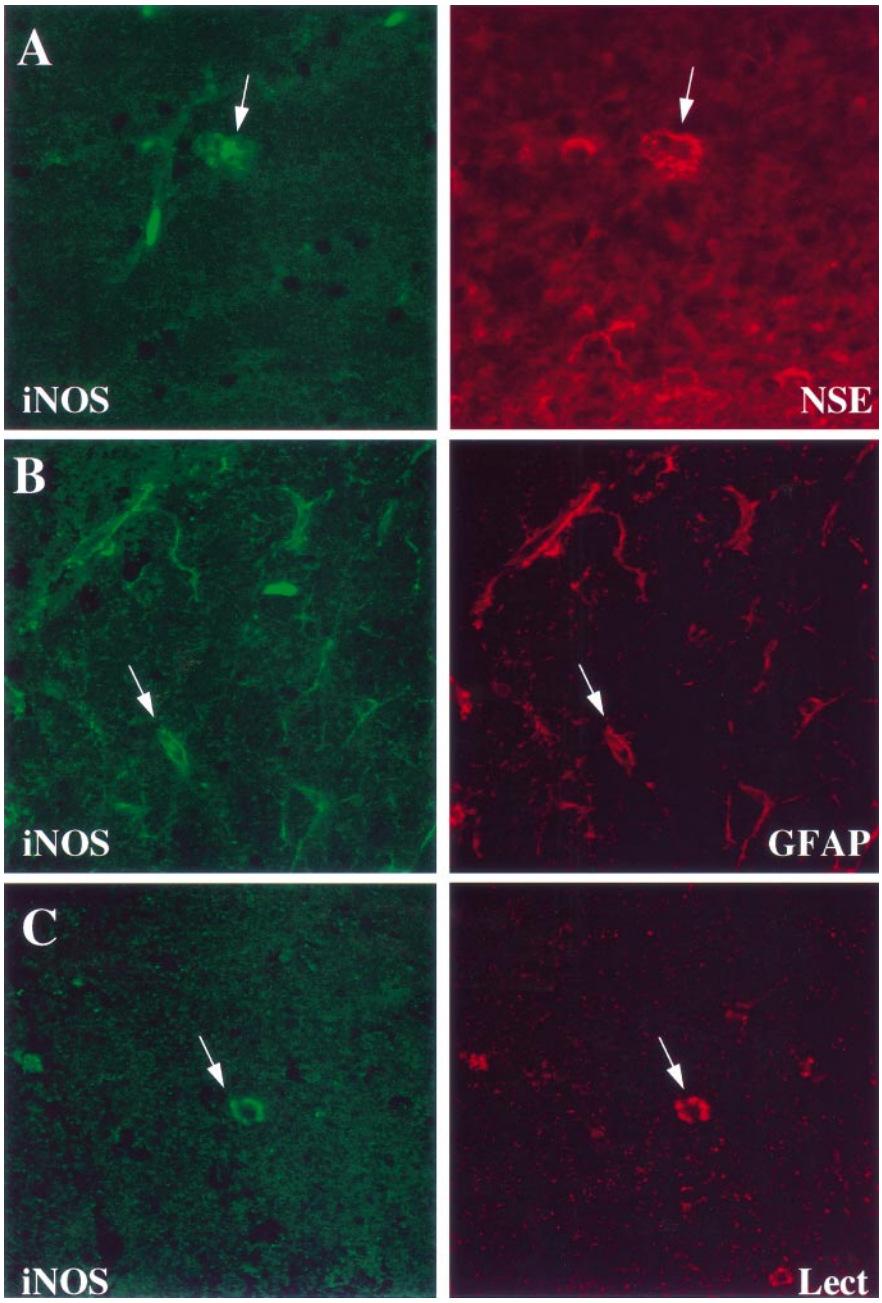


FIG. 6. Pyramidal neurones expressing inducible nitric oxide synthase immunoreactivity in sections from rat forebrain slices exposed to oxygen and glucose deprivation after 180 min of 'reperfusion'. The morphology of these neurones is similar to that shown in Figure 4.



Electron microscopy

The immunostaining was carried out as for light microscopy except for that Triton X-100 was not included in the incubation solutions. The immunocytochemical reaction was developed by incubating the tissue sections in 0.006% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in PBS for 10 min, followed by 0.003% H_2O_2 in the same buffer solution. The DAB reaction was interrupted at different times for inspection of trial sections (⊤ 8 min). Subsequently, the sections were washed in PBS, post fixed in 1% osmium tetroxide in 0.1 M PB (1 h), dehydrated in aqueous ethanol of increasing concentrations and block-stained in uranyl acetate (1% in 70% ethanol) in the dark for 40 min at room temperature. The sections were mounted on Durcupan ACM resin slides (Fluka) under a plastic coverslip and incubated for 3 days at 56 °C. Selected areas of the cortex were dissected out, re-embedded in Durcupan and semithin sections (2–2.5 μm thick) were cut. Selected sections were re-embedded in Durcupan to prepare ultrathin serial sections mounted on Formvar-coated grids, stained with lead citrate and examined in a Jeol 1200 EX electron microscope.

Immunohistochemical controls

No reactivity to endothelial or neuronal NOS was seen after incubation with both antibodies used to demonstrate the inducible isoform of NOS (Lowenstein *et al.*, 1992; Evans *et al.*, 1996). Negative controls were also performed by substitution of the primary antibody by PBS or a prebleed serum.

Chemicals and statistical analyses

L-[U-^{14}C]arginine was obtained from Amersham, BH_4 [(6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride] was obtained from RBI (Research Biochemicals International, Natick, MA, USA), and other chemicals were from Sigma or as indicated in the previous sections. Results are expressed as mean ± SEM of the indicated number of experiments, and statistical comparisons were made using a Newman–Keuls test.

Results

I. Calcium-independent NOS activity in rat forebrain slices exposed to oxygen and glucose deprivation

Oxygen and glucose deprivation for 20 min caused the appearance of a calcium-independent NOS activity in the rat forebrain slices, which increased time-dependently during the period studied. As shown in Figure 1, calcium-independent NOS activity appeared ⊤ 60 min after the 'ischaemic' period reaching the maximal activity 180 min after the onset of the 'reperfusion' period. During the same period of time, the calcium-independent NOS activity in control forebrain slices remained unchanged.

To determine whether this calcium-independent NOS activity was due to the expression of a NOS isoform, presumably iNOS, the effects of the addition of the induction inhibitor dexamethasone (1 μM), the protein synthesis inhibitor cycloheximide (10 μM) and the inhibitor of the activation of transcription factor NF-κB PDTC (pyrrolidine dithiocarbamate, 100 μM; Schreck *et al.*, 1992) were determined. All of them inhibited the induction of the calcium-independent NOS activity found in the rat forebrain slices exposed to oxygen and glucose deprivation (Fig. 2).

II. Expression of iNOS mRNA and protein in rat forebrain slices exposed to oxygen and glucose deprivation.

Oxygen and glucose deprivation caused the expression of the gene-encoding iNOS in rat forebrain slices, as assessed by the detection of iNOS message and protein in these samples. The transcription was detectable at 120 min after the end of the 'ischaemic' period and was clearly established after 180 min (Fig. 3A,B). The time-course of the expression of iNOS mRNA was in agreement with the temporal profile of iNOS enzymatic activity in rat forebrain slices exposed to 'ischaemia' (Fig. 1). Under oxygen and glucose deprivation, a sixfold increase in the iNOS mRNA levels was observed at 180 min (Fig. 3B). Interestingly, control slices (control 180) showed a minimal basal expression of both iNOS mRNA and protein (Fig. 3B,C). As shown in Figure 3(A),(B), the addition of PDTC produced a decrease in iNOS mRNA levels in the rat forebrain slices at both times studied.

III. iNOS immunoreactivity in rat forebrain slices exposed to oxygen and glucose deprivation.

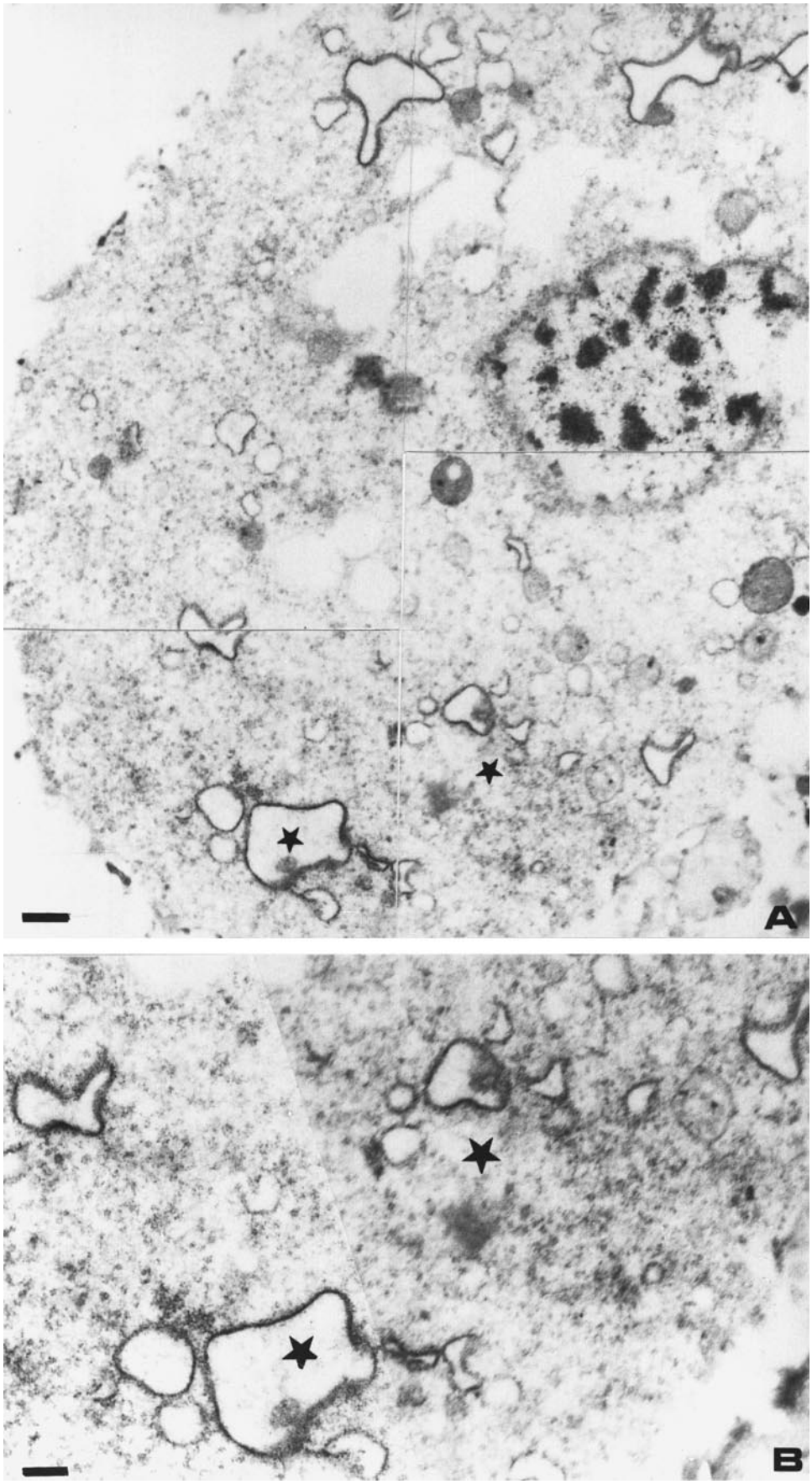
Light microscopy

Oxygen and glucose deprivation for 20 min produced the appearance of different immunoreactive structures at 180 min of the 'reperfusion' period. Immunoreactive cells with a neuronal morphology of pyramidal cells (Fig. 4A,C–E) and of associative neurones (Fig. 4B,F) were found in layers IV and V of the frontal cortex. The pattern of iNOS immunostaining was granular forming clusters. The granules were numerous, pervading cytoplasm and processes. Another immunoreactive group of cells presented a glial morphology, resembling astrocytes (Fig. 5A–C) or microglial cells (Fig. 5D–G,I) in all layers of the cortex and mainly in the corpus callosum. Additionally, other immunoreactive cells were swollen and might correspond to macrophages (Fig. 5H, J). The pattern of iNOS staining was homogeneously distributed throughout the cytoplasm conferring a dark background. No immunostaining was found in sections of control forebrain slices.

Double-fluorescence staining

To confirm the cellular types that present iNOS immunoreactivity, double staining was carried out. In sections from forebrain slices immediately after cutting (control 0), double staining for iNOS and NSE (a neuronal marker), for iNOS and GFAP (a marker of astrocytes) and for iNOS and tomato lectin (a microglial cell marker) were negative in all areas studied. In sections from control forebrain slices at 180 min (control 180), double staining for iNOS/GFAP and iNOS/tomato lectin were only occasionally positive, some astrocytes and microglial cells expressing iNOS immunoreactivity. In sections from forebrain slices exposed to oxygen and glucose deprivation, iNOS immunoreactive cells with morphology of pyramidal neurones (as those shown in Fig. 4) were found (Fig. 6). Moreover, double immunostaining for iNOS/NSE (Fig. 7A) and iNOS/GFAP (Fig. 7B) showed that some neurones (NSE-positive cells) and astrocytes (GFAP-positive cells) expressed iNOS immunoreactivity. In addition, some

FIG. 7. Double staining of sections from rat forebrain slices exposed to oxygen and glucose deprivation at 180 min of 'reperfusion' period ('ischaemic' 180; I 180). (A) Neurones stained with neurone-specific enolase (NSE) express inducible nitric oxide synthase (iNOS) immunoreactivity. (B) Astrocytes [glial-fibrillary acidic protein (GFAP)-positive cells] express iNOS immunoreactivity and (C) Microglial cells (tomato lectin-positive cells) also express iNOS immunoreactivity.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tomato lectin-positive cells (microglial cells) were also iNOS positive (Fig. 7C).

Electron microscopy

Cells with a neuronal morphology and necrotic aspect as a consequence of the ischaemia insult presenting a reactive product localized inside or surrounding the external membrane of dilated cisternae and swollen endoplasmic reticulum were found in cortex (Fig. 8A,B). Also, numerous immunoreactive cells showing a smaller diameter and dark nucleus with dense heterochromatin pervaded cortex and corpus callosum; these cells might correspond to microglial cells or astrocytes. Its immunoreactive product was found throughout the cytoplasm conferring a dark, finely granulated background. (Fig. 9A–D) No immunostaining was found in sections of control forebrain slices.

Discussion

The reported results demonstrate that neurones and glial cells express iNOS after oxygen and glucose deprivation in rat forebrain slices, as early as 120 min after the hypoxic insult. This conclusion is supported by the following data. (i) Direct assay of the enzyme activity indicated the presence of a calcium-independent NOS activity in rat forebrain slices after oxygen and glucose deprivation. (ii) Dexamethasone, cycloheximide and PDTC suppressed or attenuated the induction of this activity, suggesting that it might arise from the expression of iNOS in this preparation. (iii) Moreover, the detection and quantification of iNOS mRNA and protein as well as the cellular localization of iNOS by immunohistochemistry confirmed that this isoform was being expressed in rat forebrain slices exposed to oxygen and glucose deprivation.

The calcium-independent enzymatic activity could be significantly detected as early as 60 min after the end of the 'ischaemic' period. Consistently, the expression of iNOS was established after 2 h. Different authors have found iNOS expression in *in vivo* models of ischaemia-reperfusion but this expression took place not before 1–3 days (Endoh *et al.*, 1994; Wallace & Bisland, 1994; Iadecola *et al.*, 1995b). Because it is known that a large amount of the tissue damage, following cerebral ischaemia, occurs within the first few hours (Dereski *et al.*, 1993; Garcia *et al.*, 1993), our data suggest that NO can play an important pathogenic role in this process. In addition, there is an important role played by delayed cell death after ischaemia in which NO from this source might also participate.

It has been shown that iNOS activity is mainly regulated at the transcriptional level and the promoter region of the iNOS gene has been described in detail (Xie *et al.*, 1993). In rodents, a 2 kb 5′-flanking region of the start site of transcription has been identified as the main regulatory domain for the expression of iNOS. This region contains at least 24 consensus sequences for the binding of several nuclear factors involved in iNOS expression (Geller *et al.*, 1993; Xie *et al.*, 1993). Among them, activation of nuclear factor κB (NF-κB) has been recognized as an essential requirement for the expression of this gene (Xie *et al.*, 1994). In agreement with this, our data using PDTC, an inhibitor of NF-κB activation that interferes with the degradation of the inhibitory subunit of the complex (Sherman *et al.*, 1993; Xie *et al.*, 1994), or dexamethasone, which up-regulates the synthesis of this inhibitory subunit (Auphan *et al.*, 1995), both antagonized the expression of iNOS under ischaemia conditions.

We have shown that iNOS expression may occur in several types of CNS cells after the ischaemic insult, since iNOS immunoreactivity appeared not only in glial cells such as astrocytes and microglial cells, but also in neurones. Although previous work using *in vivo* ischaemia models has shown iNOS immunoreactivity located either in polymorphonuclear neutrophils (Iadecola *et al.*, 1995b) or in astrocytes (Endoh *et al.*, 1994; Wallace & Bisland, 1994), to our knowledge this is the first report of expression of iNOS in neurones after ischaemia. The neurones were found in the frontal cortex, showing pyramidal and multipolar morphology which was confirmed by double immunostaining as NSE-positive cells and GFAP- and tomato lectin-negative cells and also by their ultrastructural appearance. In all the neuronal types studied, iNOS immunoreactivity appeared mainly associated to the membranes of the endoplasmic reticulum or the cisternae system. Whether this implies any kind of association of iNOS with subcellular membranes remains to be elucidated. Although some immunoreactivity appeared located inside the nucleus, this is likely to be due to the cellular disintegration caused by the consequences of the ischaemic insult. Indeed the immunoreactivity of iNOS in all different positive cells was similar using both polyclonal antisera and the patterns of distribution of the reaction product were in agreement with those described in previous works (Evans *et al.*, 1996; Riveros-Moreno *et al.*, 1996).

The mechanisms of iNOS expression after cerebral ischaemia remain elusive. Pro-inflammatory cytokines are known to cause the induction of iNOS in several cell systems. Interestingly, interleukin-1β, tumour necrosis factor-α or interferon-γ are rapidly (within a few hours) induced in the brain following ischaemia (Minami *et al.*, 1992; Liu *et al.*, 1993, 1994). These cytokines have been involved in the expression of iNOS in astrocytes, microglia, polymorphonuclear cells and macrophages invading the area of infarction, as well as in neurones (Galea *et al.*, 1992; Simmons & Murphy, 1992, 1993; Peterson *et al.*, 1994; Minc-Golomb *et al.*, 1994, 1996). In our model, it is likely that these cytokines induce iNOS expression, thus mimicking the *in vivo* situation. Another possibility is that the oxidative stress produced in this situation might itself trigger the induction of iNOS. Indeed, a specific pathway for the induction of iNOS under anoxic conditions has been described, and an hypoxia-responsive enhancer has been characterized in the promoter region of iNOS from nucleotide – 227 to – 209, suggesting that iNOS is an hypoxia inducible gene (Melillo *et al.*, 1995). Interestingly, we could detect some basal iNOS expression in control rat forebrain slices. Such iNOS expression might be a consequence of the hypoxia that exists at the centre of the slice (Garthwaite & Garthwaite, 1988), because basal iNOS mRNA was not observed in freshly cut slices (data not shown) and suggests an important sensitivity to transient anoxia of this model in the transcriptional control of iNOS.

We have suggested that NO can play an important pathogenic role in the initiation of the tissue damage that occurs after cerebral ischaemia. The mechanisms of cell damage by NO include inhibition

FIG. 8. Electron micrographs of neurones in frontal cortex of rat forebrain slices exposed to oxygen and glucose deprivation at 180 min of 'reperfusion'. (A) Neurones with necrotic structural aspect from the cortical layer show a reactive product associated to the membrane of large cisternae and swollen endoplasmic reticulum (★). The nuclear heterochromatin is disaggregated forming clusters. (B) Large magnification showing the structure and distribution of the immunoreactive product in swollen cisternae (★). Scale bar = 500 nm (A) and 250 nm (B).

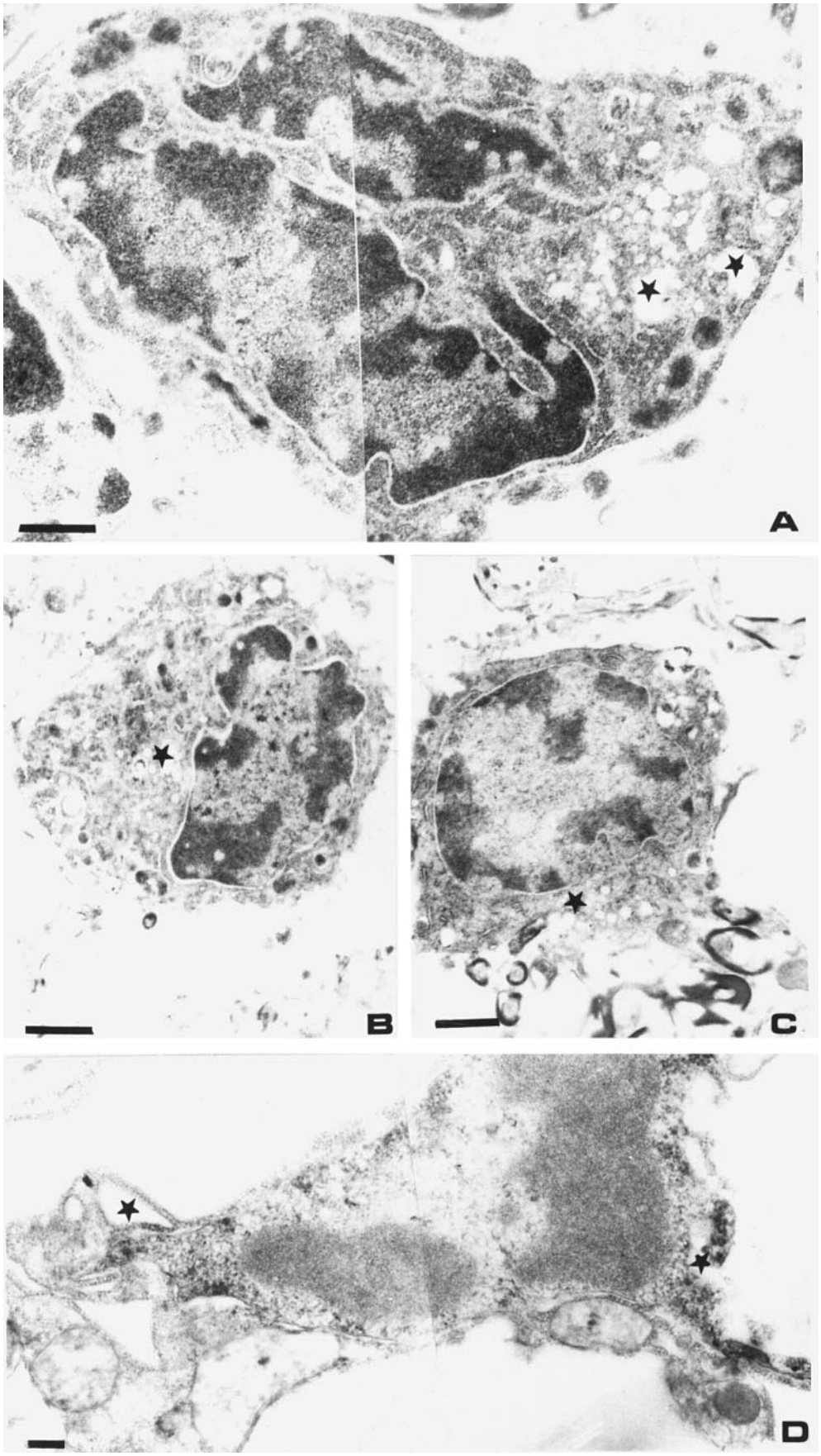

FIG. 9. Immunoelectron microscopy of astrocytes (A) and microglial cells (B–D) from sections of rat forebrain slices exposed to oxygen and glucose deprivation after 180 min of 'reperfusion'. The immunoreactive product is homogeneously distributed throughout the cytoplasm conferring a dark, finely granulated background (★). Scale bar = 500 nm (A,D) and 250 nm (B,C).

of a number of cellular processes, such as DNA synthesis and mitochondrial respiration. Some of these effects may be direct and others may arise from the reaction of NO with other oxygen radicals, such as superoxide, to form peroxynitrite (Beckman *et al.*, 1990), also implicated in neurotoxicity (Lipton *et al.*, 1993) and brain ischaemia (Nowicki *et al.*, 1991). In this context, we have recently suggested that normal cytosol concentrations of thiols and carbohydrates will block the inhibitory effects of peroxynitrite on mitochondrial respiration in intact cells and *in vivo*, unless this oxidant is formed in the close vicinity of a redox site within the respiratory chain (Lizasoain *et al.*, 1996). Our data showing iNOS expression in neurones suggests that high and sustained production of NO, together with the resulting formation of superoxide may, because of formation of peroxynitrite within the mitochondria, cause the irreversible inhibition of complexes I–III leading to neurotoxicity. Indeed, peroxynitrite can be formed in this model during the reperfusion period as a high synthesis of superoxide anion occurs in brain slices after hypoxia and during reoxygenation (Schreiber *et al.*, 1995).

In summary, we have demonstrated that iNOS is expressed in several cell types including neurones after oxygen and glucose deprivation. In addition, our model may be considered an useful *in vitro* method which is easily accessible, with functional glia and neurones, and is suitable for pharmacological studies trying to find new therapeutical approaches to management of cerebral ischaemia.

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Abbreviations

GFAP	glial-fibrillary acidic protein
LPS	lipopolysaccharide
L-NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
eNOS	endothelial NOS
iNOS	inducible NOS
NSE	neurone-specific enolase
PDTC	pyrrolidine dithiocarbamate.

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