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# Calcium-independent NO-synthase activity and nitrites/nitrates production in transient focal cerebral ischaemia in mice

[1]M. Grandati, C. Verrecchia, M.L. Revaud, M. Allix, R.G. Boulu & M. Plotkine

Laboratoire de Pharmacologie, Université René Descartes, 4 avenue de l'Observatoire, F-75270 Paris cedex 06, France

**1** The temporal changes in constitutive NO-synthase (cNOS) and in calcium-independent NO-synthase activities were studied in mice subjected to 2 h of transient focal cerebral ischaemia. The changes in brain nitrites/nitrates ($NO_x$) content were also studied.

**2** NOS activities were measured by the conversion of L-[$^{14}$C]-arginine to L-[$^{14}$C]-citrulline. Brain $NO_x$ contents were investigated by the Griess colourimetric method.

**3** cNOS activity in the infarcted cortical area was significantly reduced after 6 h of reperfusion and this activity remained attenuated for up to 10 days after ischaemia. A calcium-independent NOS activity began to increase 48 h after reperfusion, reached a maximum at 7 days and returned to baseline at 10 days.

**4** There was a significant increase of brain $NO_x$ content beginning after 3 days of reperfusion. This increase was maximal at 7 days and returned to baseline at 10 days.

**5** Thus, ischaemia followed by recirculation leads to a rapid, prolonged drop in cNOS activity in the infarcted cortex. There is also a substantial appearance of calcium-independent NOS activity in the later phase of transient ischaemia, leading to an important increase of $NO_x$ production.

**Keywords:** Focal cerebral ischaemia; reperfusion; constitutive nitric oxide synthase; calcium-independent nitric oxide synthase; nitrite; nitrate

## Introduction

Nitric oxide (NO) is synthesized from the amino acid L-arginine by the enzyme NO-synthase (NOS). Two distinct families of NOS have been identified, one constitutive and the other inducible. The constitutive isoforms are calcium/calmodulin-dependent and release NO for short periods in response to physiological stimuli. The inducible isoforms are induced by endotoxins and cytokines by a calcium-independent mechanism and, once expressed, release NO for long periods. Genes for three enzymes have been encoded: the constitutive neuronal (NOS 1) and endothelial (NOS 3) isoforms, and the inducible (NOS 2) isoform.

As NO is produced in response to glutamatergic activation (Garthwaite *et al*., 1989), it has been suggested that NO is involved in excitatory amino acid-induced neuronal death (Dawson *et al*., 1991). Because of the potential role of NO in glutamate-elicited neurotoxicity, the involvement of NO in focal cerebral ischaemia has been extensively investigated in models of both permanent and transient focal cerebral ischaemia. The role of NO has been assessed by use of NOS inhibitors and NO donors, but the results have been somewhat contradictory (for reviews see Dawson, 1994; Verrecchia *et al*., 1995). This apparent discrepancy and the effect of putative selective NOS inhibitors, like 7-nitro-indazole or aminoguanidine, suggest that NO has both deleterious and beneficial effects on the neuronal death induced by cerebral ischaemia (for reviews see Dawson, 1994; Verrecchia *et al*., 1995).

In our laboratory, we have shown that administration of a low dose of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), a non selective NOS inhibitor, is neuroprotective in both models of definitive and transient focal cerebral ischaemia (Buisson *et al*., 1992; Margaill *et al*., 1997). Moreover, we have shown a neuroprotective effect of a single 1 mg kg$^{-1}$ dose of L-NAME, even when given 9 h after transient ischaemia induction (Margaill *et al*., 1997). However, the actual NOS isoform(s) that is (are) involved in the neuroprotective effect of L-NAME remains to be established.

This led us to investigate the changes in NOS activities in cerebral ischaemia in order to elucidate the role of these enzymes in cerebral ischaemic damage. Little information is available concerning the activity of NOS in permanent (Iadecola *et al*., 1995a; Yoshida *et al*., 1995) or transient (Iadecola *et al*., 1996a) focal ischaemia models. The present study was therefore carried out to investigate the temporal changes in constitutive calcium-dependent NOS (cNOS) and in calcium-independent NOS activities in a model of transient focal cerebral ischaemia in mice. Moreover, nitrites/nitrates ($NO_x$), the end products of NO degradation, were evaluated to reflect NO production.

## Methods

### *Transient focal cerebral ischaemia*

All experiments were performed according to the NIH guidelines and French Department of Agriculture regulations (Licence N° 01352). Male Swiss mice (Charles River France, 28–32 g) were anaesthetized with chloral hydrate (400 mg kg$^{-1}$, i.p.) and maintained in normothermia until they had recovered from the anaesthesia by means of a heating blanket. The left middle cerebral artery (MCA) was exposed via a temporal craniotomy. The MCA was occluded at a site distal to the lenticulostriate artery with ligatures of nylon thread (10/0, Ethilon, Ethicon) for 2 h. The ligatures were then removed to allow recirculation. This model of MCA occlusion led to a cortical infarct.

### *NOS activities assay*

The catalytic activities of NOS were measured by the conversion of L-[$^{14}$C]-arginine to L-[$^{14}$C]-citrulline by a modified technique of Bredt & Snyder (1989). Brain samples were homogenized in ice-cold buffer (20 mM HEPES, 1 mM EGTA, 1 mM dithiothreitol, 0.32 M sucrose, 10 mg l$^{-1}$ leupeptin and 10 mg l$^{-1}$ pepstatin A, pH = 7.4) and centrifuged at 20000 *g*, for 15 min, at 0°C. The supernatants were used for assays. For

[1] Author for correspondence.

determination of total NOS activity, samples (25 $\mu$l) were incubated for 30 min at 37°C in a reaction mixture containing: 20 mM HEPES, 1 mM EGTA, 1 mM dithiothreitol, 0.32 M sucrose, 10 mg $l^{-1}$ leupeptin, 10 mg $l^{-1}$ pepstatin A, 200 $\mu$M NADPH, 50 $\mu$M tetrahydro-L-biopterin, 1 mM $CaCl_2$ and 1 $\mu$Ci $ml^{-1}$ L-[$^{14}$C]-arginine. Calcium-independent NOS activity was measured by omitting calcium from the reaction mixture. The concentrations of added cofactors were identical for total NOS and calcium-independent NOS assays, and exceeded the requirements for enzyme assay (Yoshida *et al*., 1995). The reaction was stopped by adding 1 ml ice-cold 30 mM HEPES containing 3 mM EDTA (pH 5.5). Samples were run through anion-exchange Dowex AG50W-X8 ($Na^+$ form prepared from the $H^+$ form) columns to remove L-[$^{14}$C]-arginine. L-[$^{14}$C]-citrulline was eluted from the columns with 2 volumes of 0.5 ml water and quantified by liquid scintillation. The L-[$^{14}$C]-citrullin concentration was computed after the blank values had been subtracted, which gave the non-specific radioactivity in the absence of enzyme. cNOS activity was computed by subtracting the calcium-independent NOS activity from the total NOS activity. Proteins in the supernatants were assayed by the method of Bradford (1976), with human serum albumin as standard. Data are expressed as pmol of L-[$^{14}$C]-citrulline $mg^{-1}$ protein $min^{-1}$.

### *Brain $NO_x$ assay*

Tissue $NO_x$ contents were determined as described by Tracey *et al*. (1995). Brain samples were weighed and homogenized in 400 $\mu$l deionized water and centrifuged at 20000 *g*, for 10 min, at 4°C. Fifty microlitres of supernatant was mixed with 20 $\mu$l of 0.31 M potassium phosphate buffer (pH 7.5), 10 $\mu$l of 0.86 mM $\beta$-NADPH, 10 $\mu$l of 0.11 mM FAD and 20 mu of nitrate reductase. Samples were allowed to incubate for 1 h at room temperature in the dark. Then, 5 $\mu$l of 1 M $ZnSO_4$ were added to the samples in order to precipitate the proteins. Samples were centrifuged at 20000 *g*, for 5 min, at 4°C and the supernatants were removed. One hundred microlitres of Griess reagent (1:1 mixture of 1% sulphanilamide in 5% $H_3PO_4$ and 0.1% N-(1-naphytyl)ethylenediamine) were added to 50 $\mu$l of supernatant and the mixture incubated for 10 min at room temperature. Absorbances were measured at 540 nm by a spectrophotometer (Milton Roy) and converted to $NO_x$ content by using a nitrate standard curve. Data are expressed as pmol $NO_x$ $mg^{-1}$ wet weight.

### *Plasma $NO_x$ assay*

The blood samples were added to microcentrifuge tubes containing 4 u of heparin sodium. Heparin-treated blood samples were centrifuged at 5000 *g*, for 10 min, at 4°C and the plasma was collected. Plasma $NO_x$ concentration was determined by mixing 6 $\mu$l of plasma with 44$\mu$l deionized water, then the protocol used was the same as brain $NO_x$ assays. Data are expressed as $\mu$M of $NO_x$.

### *Experimental protocols*

Mice were killed 6 h, 18 h, 24 h, 48 h, 3 days, 5 days, 7 days and 10 days after reperfusion to evaluate NOS activities ($n=4-6$). The brains were rapidly removed and the infarcted cortical area and the contralateral cortex were dissected out and stored frozen (−40°C) for assay of NOS activities. For brain $NO_x$ content assay, mice were killed after 24 h, 48 h, 3 days, 5 days, 7 days and 10 days of reperfusion ($n=5-6$). Infarcted area and contralateral cortex were immediately used for $NO_x$ assay. In the same mice, a 100 $\mu$l blood sample was obtained by retro-orbital sinus puncture one hour before the brain was removed. The plasma was collected and stored frozen (−40°C) in order to evaluate $NO_x$ concentration. Mice in which the MCA was exposed but not occluded served as sham-operated controls ($n=3-4$). Tissue samples were also taken from non-ischaemic mice for control assays ($n=7$ for NOS assay, $n=18$ for $NO_x$ assay).

### *Materials and drugs*

L-[$^{14}$C]-arginine (specific activity: 317 mCi $mmol^{-1}$) was obtained from Amersham, France and AG50W-X8 ($H^+$ form, 100–200 mesh) was purchased from Sigma Chemicals (France). Ready safe liquid scintillation (Aquasafe 300+) was from Zinsser Analytic. Nitrate reductase was obtained from Boehringer. All other chemicals were purchased from Sigma Chemicals (France).

### *Data expression and statistical analysis*

Data are expressed as means±s.e.mean. Statistical analysis was performed by one-way analysis of variance (ANOVA) with subsequent individual comparisons by a PLSD Fisher test. Differences were considered significant at a value of $P<0.05$.

## Results

The cNOS activity in control mice was 21.2± 4.8 pmol $mg^{-1}$ $min^{-1}$. The time course of cNOS activity in mice subjected to transient focal ischaemia is illustrated in Figure 1. In the infarcted area cNOS activity was dramatically reduced after 6 h of reperfusion (4.8±3.2 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice) and remained attenuated up to 10 days after MCA occlusion (7.4±2.9 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice). There was no significant change in cNOS activity in the contralateral cortex throughout the 10 days (Figure 1).

The calcium-independent NOS activity in control mice was 0.3±0.4 pmol $mg^{-1}$ $min^{-1}$. There was no significant calcium-independent NOS activity increase in the infarcted cortical area after 6–24 h of reperfusion (Figure 2). However, the calcium-independent NOS activity increased markedly 48 h (4.9±1.4 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice), 3 days (7.0±2.9 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice) and 4 days (6.5±4.1 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice) after reperfusion. Calcium-independent activity was maximal 7 days after reperfusion (10.6±3.9 pmol $mg^{-1}$ $min^{-1}$, $P<0.001$ versus control mice), and returned to baseline (0.7±0.4 pmol $mg^{-1}$ $min^{-1}$) after 10 days of reperfusion. The calcium-independent NOS activity in the contralateral cortex of ischaemic mice was not significantly different from control mice (Figure 2).

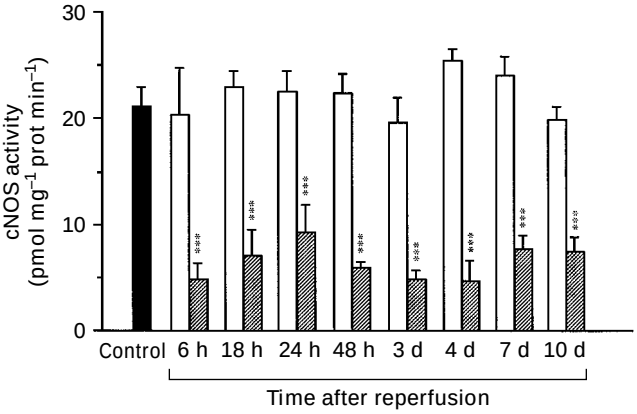


**Figure 1** Time course of constitutive NOS activity in the infarcted cortical area and the contralateral cortex during reperfusion after transient focal ischaemia in mice. Solid column: control mice ($n=7$), open columns: contralateral cortex ($n=4-6$), hatched columns: infarcted cortical area ($n=4-6$). Data are expressed as means ±s.e.mean. ***$P<0.001$ versus control mice.

NOS activities in sham-operated mice killed at 48 h and 7 days were not significantly different from control values (Table 1).

The brain $NO_x$ content in control mice was $44.9 \pm 2.3$ pmol $mg^{-1}$ wet weight. The post-ischaemic changes in brain $NO_x$ content are illustrated in Figure 3. In the infarcted area, no significant change in $NO_x$ content was observed 24 h and 48 h after reperfusion. In contrast, a significant increase of $NO_x$ content appeared 3 days after reperfusion ($115.4 \pm 21.6$ pmol $mg^{-1}$ wet weight, $P<0.001$ versus control mice). The $NO_x$ content was maximal 7 days after reperfusion ($243.6 \pm 23.1$ pmol $mg^{-1}$ wet weight, $P<0.001$ versus control mice) and returned to baseline ($72.4 \pm 8.1$ pmol $mg^{-1}$ wet weight) after 10 days of reperfusion. The $NO_x$ content in the contralateral cortex is ischaemic mice at different times of reperfusion, was not different from control mice (Figure 3). There was no change in brain $NO_x$ content in sham-operated mice killed at 48 h and 7 days (Table 1).

Plasma $NO_x$ concentration in control mice was $14.6 \pm 1.3$ $\mu$M. There was no significant change in plasma $NO_x$ concentration in either mice subjected to transient focal ischaemia or in sham-operated mice at any time of reperfusion (data not shown).

## Discussion

The present data show that there is a rapid and prolonged reduction in cNOS activity in the infarcted cortex after transient MCA occlusion in mice. cNOS activity was dramatically reduced after 6 h of reperfusion and remained attenuated for up to 10 days after MCA occlusion. The cNOS activity in the contralateral cortex was unchanged. This is in agreement with previous studies, in which permanent focal ischaemia models were used, showing that total NOS activity was lowered 1 to 3 days after permanent MCA occlusion in mice, and 6 h to 3 days after MCA occlusion in rats (Yoshida *et al*., 1995). A large reduction in cNOS activity was also described after permanent focal ischaemia in rats and this activity remained attenuated for up to 7 days after MCA occlusion (Iadecola *et al*., 1995a). Therefore, MCA occlusion followed by recirculation leads to a marked decrease in cNOS activity as in permanent focal ischaemia. The reduction in cNOS activity probably reflects the loss of NOS-containing neurones. Previous studies in our laboratory have shown that the number of NADPH diaphorase-containing neurones in infarcted area of rats subjected to transient focal ischaemia gradually decreases. This loss starts 6 h after ischaemia and is maximal at 24 h (Margaill *et al*., 1995). Similar data have been obtained by Iadecola *et al*. (1995a) for permanent focal ischaemia. These histochemical changes suggest that the reduction of cNOS activity in the area of infarction reflects loss of NOS-containing neurones.

In contrast to cNOS, there was a substantial increase in calcium-independent NOS activity within the infarcted area. A marked increased occurred 48 h after reperfusion with enzyme activity peaking at 7 days after reperfusion and returning to baseline by 10 days after onset of ischaemia. This calcium-independent activity only occurred in the infarcted area, not in the contralateral region. Calcium-independent NOS activity has also been described in rats subjected to both permanent (Iadecola *et al*., 1995a; Yoshida *et al*., 1995) and transient (Iadecola *et al*., 1996a) focal ischaemia. However, calcium-independent activity has not been detected in mice up to 4 days after permanent MCA occlusion (Yoshida *et al*., 1995). The authors concluded that NOS induction in rats and mice is very different. This is supported by the finding that there is a marked induction of NOS in the rat spleen 6 h after lipopolysaccharide injection but only a small increase of inducible NOS (iNOS) activity in mice spleen (Yoshida *et al*., 1995). However, it has been found that endotoxin stimulates the production of calcium-independent NOS with different time courses in various tissues. In particular, inducible NOS (iNOS)

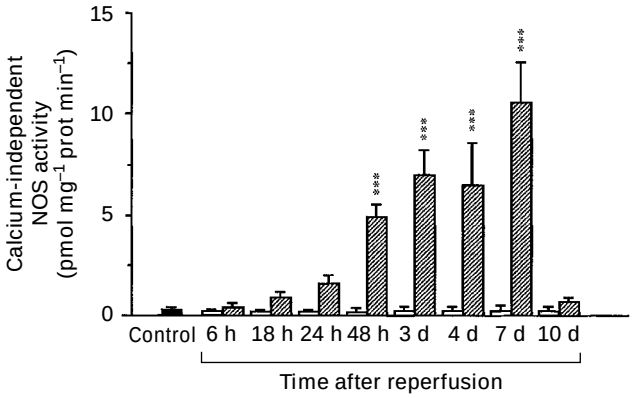


**Figure 2** Time course of calcium-independent NOS activity in the infarcted and contralateral cortex during reperfusion after MCA occlusion. Solid column: control mice ($n=7$), open columns: contralateral cortex ($n=4-6$), hatched columns: infarcted cortical area ($n=4-6$). Data are expressed as means ± s.e.mean. ***$P<0.001$ versus control mice.

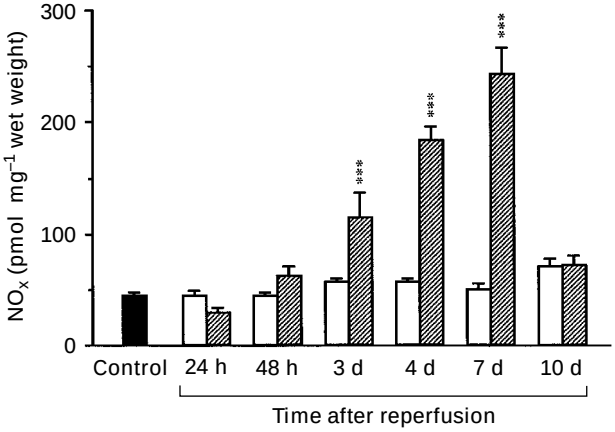


**Figure 3** Changes in brain $NO_x$ content in the cortical area and the contralateral cortex. Solid column: control mice ($n=18$), open columns: contralateral cortex ($n=5-6$), hatched columns: infarcted cortical area ($n=5-6$). Data are expressed as means ± s.e.mean. ***$P<0.001$ versus control mice.

**Table 1** NOS activities and brain $NO_x$ content in control mice and sham-operated mice killed at 48 h and 7 days

| | *cNOS activity* (pmol $mg^{-1}$ prot $min^{-1}$) | *Ca-independent NOS activity* (pmol $mg^{-1}$ prot $min^{-1}$) | n | *$NO_x$* (pmol $mg^{-1}$ wet weight) | n |
|---|---|---|---|---|---|
| Control | 21.2 ± 4.8 | 0.3 ± 0.4 | 7 | 44.9 ± 2.3 | 18 |
| 48 h | 20.7 ± 3.8 | ND | 4 | 41.4 ± 5.8 | 3 |
| 7 days | 23.0 ± 0.7 | ND | 4 | 62.0 ± 2.9 | 3 |

Data are expressed as mean ± s.e.mean. ND, not detectable.

activity in the spleen of mice subjected to septic shock is delayed and very small (Rees *et al*., 1995).

Our data show that the evolution of $NO_x$ content in brain parallels the time course of calcium-independent NOS activity. Indeed, there is a substantial increase in the $NO_x$ content in the infarcted cortical area beginning 3 days after reperfusion, peaking at 7 days after reperfusion and returning to baseline by 10 days after ischaemia onset. Interestingly, at 7 days of reperfusion, when calcium-independent NOS activation was maximal, this activity represents only 60% of cNOS activity measured in control mice. By contrast, $NO_x$ brain content was 6 fold more important than in control mice. These results suggest that this substantial production of $NO_x$ is probably due to the involvement of calcium-independent NOS. This is in agreement with *in vitro* results on the capacity of iNOS to release sustained (over several days) and high (pmol) levels of NO (Nathan, 1992). This is, to our knowledge, the first study demonstrating an increased $NO_x$ content in brain during reperfusion in a model of transient ischaemia. In a model of definitive ischaemia, Kader *et al*. (1993) showed that brain nitrites increase in the infarcted cortex compared to the contralateral cortex 5, 10 and 20 min after ischaemia and then normalize at 60 min. This burst in $NO_x$ content occurs after a sharp transient increase in NOS activity during the first hour of occlusion. However, in this study, later times were not studied.

Our data show that plasma $NO_x$ concentration is not modified during the early or late phase of reperfusion in our model. An increase of nitric oxide end products in the plasma during acute focal cerebral ischaemia and reperfusion has already been described (Kumura *et al*., 1994). This elevation in plasma $NO_x$ content has been shown immediately (30 min and 2 h) after a 2 hour-occlusion. The same group found an increase in plasma $NO_x$ in rats, attributable to iNOS activation, 3 days after a traumatic cortical injury (Yamanaka *et al*., 1995). This suggests that, in our model, plasma $NO_x$ concentration does not reflect the brain enzymatic activity, perhaps because the changes are too slight to induce effects in plasma.

It has been suggested that neurosurgical manipulations, like craniotomy and opening of the dura to expose the MCA, could induce NOS activity even in the absence of cerebral ischaemia (Zhang *et al*., 1996). However, in the model used in our study, the procedure for MCA exposure could not account for the appearance of calcium-independent NOS activity, because neither a calcium-independent NOS signal nor a change in $NO_x$ content were detected on either side of the cerebral cortex of sham-operated mice, in which the artery was exposed and manipulated but not occluded. Therefore the calcium-independent NOS activity and $NO_x$ production measured in our model is the consequence of cerebral ischaemia.

The present findings indicate that there is substantial calcium-independent NOS activity in the late phase of focal cerebral ischaemia, with a concomitant accumulation of $NO_x$ in the infarcted cortical area. The calcium-independent activity that we observed could be related to an inducible isoform of NOS. However, this needs to be confirmed in our experiments by measuring iNOS mRNA. Accordingly. Iadecola *et al*. (1995c; 1996a), in rat models of focal cerebral ischaemia, described the expression of iNOS mRNA the time course of which parallels the temporal profil of calcium-independent NOS activity.

The factors involved in the activation of iNOS following cerebral ischaemia are unknown. Reperfusion of previously ischaemic tissue could trigger the release of inflammatory cytokines. Indeed, cytokine production has been demonstrated after cerebral ischaemia and a cytokine receptor antagonist found to inhibit ischaemic and excitotoxic brain damage (Rothwell & Relton, 1993). As cytokines are known to induce NOS expression, they may activate iNOS in the area of infarction. The type of cell that produces iNOS in cerebral ischaemia has still to be identified, although iNOS activity has been demonstrated in cerebral microvessels after focal ischaemia (Nagafuji *et al*., 1994). Leukocytes, invading from the periphery, could be induced by NOS to produce NO, as leukocytes infiltration occurs in focal cerebral ischaemia (Barone *et al*., 1991). It may be significant that the size of the infarct elicited by focal ischaemia can be reduced by preventing the infiltration of polymorphonuclear leukocytes (Chopp *et al*., 1994). Moreover, a difference in the cellular localization of iNOS expression has been observed between transient and permanent ischaemia. Indeed, iNOS immunoreactivity was observed in polymorphonuclear cells infiltrating the infarcted area in a model of definitive cerebral ischaemia (Iadecola *et al*., 1995b) and in vessels throughout the ischaemia region after transient cerebral ischaemia (Iadecola *et al*., 1996a).

The increase in calcium-independent NOS activity could reflect the appearance of microglia in the infarcted area. Indeed, time-dependent morphological changes of microglial cells following cerebral ischaemia have been described by means of immunohistochemistry. Interestingly, a significant increase in immunoreactive cells was detected after a few hours of reperfusion and persisted for up to 7 days of reperfusion (Kato *et al*., 1996; Zhang *et al*., 1997). Microglial activation during ischaemia results in the expression of a lot of mediators with neurotoxic potential, including cytokines, free radicals and inducible NOS. This activation of intrinsic brain cells is known to contribute to the progression of neuronal damage (see for reviews Wood, 1994; Murphy *et al*., 1995). So, the calcium-independent NOS activation observed in our model of ischaemia could be related to gliosis, as has been described after excitotoxic lesions (Calka *et al*., 1996) or in the brain of stroke-prone spontaneously hypertensive rats (Gotoh *et al*., 1996).

NO production by iNOS is involved in inflammation (Moncada *et al*., 1991). As there is a marked inflammatory reaction after cerebral ischaemia (Barone *et al*., 1991; Kim *et al*., 1995), it is conceivable that NOS induction may participate in the inflammatory response after cerebral ischaemia. It has been found that treatment with aminoguanidine, a relative selective inhibitor of iNOS, 24 h after ischaemia, has a neuroprotective effect in both permanent and transient models of cerebral ischaemia (Iadecola *et al*., 1995b; Zhang *et al*., 1996). The same group has also used knockout mice with a null mutation of iNOS gene to test the hypothesis that iNOS expression contributes to cerebral ischaemic damage. Four days after MCA occlusion, the infarct was 31% smaller in knockout mice than in wild-type mice (Iadecola *et al*., 1996b). Therefore, NO produced by iNOS activation could play an important role in the late development of infarction. Accordingly, it has been found that neuronal damage in focal cerebral ischaemia progresses with time (Dereski *et al*., 1993). These findings suggest that the extent of brain damage is not set a few hours after the ischaemic event, but that the processes that ultimately lead to the tissue damage can be influenced long after the induction of the ischaemia. NO-mediated cytotoxicity can occur via free radical formation. Toxic radicals contribute to the brain injury induced by ischaemia (Cao & Phillis, 1994). NO could react with the oxygen superoxide ($O^{2-}$) to form peroxinitrite anions ($ONOO^-$), which are unstable at physiological pH and protonated to form peroxinitrous acid (ONOOH). Peroxinitrous acid spontaneously decomposes to nitrogen dioxide ($NO_2$) and the hydroxyl (·OH) radical (Beckman *et al*., 1990; Hogg *et al*, 1992). NO can directly damage DNA (Nguyen *et al*., 1992) and inhibit DNA replication by inactivating ribonucleotide reductase (Kwon *et al*., 1991). NO can also inhibit the glycolytic enzyme, glyceraldehyde-3-phosphate deshydrogenase by ADP ribosylation (Zhang & Snyder, 1992). Inhibition of the mitochondrial respiratory chain may also contribute to NO-mediated neurotoxicity. NO has been shown to inactivate iron-sulphur-containing enzymes, such as aconitase, succinate oxidoreductase and complexes I and II of the mitochondrial electron chain transport (Drapier & Hibbs, 1986; Lancaster & Hibbs, 1990).

In conclusion, our data demonstrate that there is a marked reduction in cNOS within the area of infarction in a model of transient focal cerebral ischaemia in mice. By contrast, there is a substantial activation of calcium-independent NOS and a production of brain $NO_x$ content. However, this calcium-independent isoform, the type of cells that can produce it and whether such isoform contributes to tissue damage in our model remain to be identified.

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