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iNOS contribution to the NMDA-induced excitotoxic lesion in the rat striatum.

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1 The aim of this study was to assess whether an excitotoxic insult induced by NMDA may induce an iNOS activity which contributes to the lesion in the rat striatum.

2 For this purpose, rats were perfused with 10 mM NMDA through a microdialysis probe implanted in the left striatum. Microdialysate nitrite content, striatal Ca-independent nitric oxide synthase activity and lesion volume were measured 48 h after NMDA exposure in rats treated with dexamethasone (DXM) (3 mg $kg^{-1} \times 4$) or aminoguanidine (AG) (100 mg $kg^{-1} \times 4$).

3 A significant increase in microdialysate nitrite content and in the Ca-independent NOS activity was observed 48 h after NMDA infusion. Both these increases were reduced by DXM and AG. The NMDA-induced striatal lesion was also reduced by both treatments.

4 Our results demonstrate that NMDA excitotoxic injury induces a delayed, sustained activation of a Ca-independent NOS activity. This activity is blocked by DXM and AG, strongly suggesting the involvement of iNOS. The fact that AG and DXM reduce the NMDA-elicited lesion suggests that iNOS contributes to the brain damage induced by excitotoxic insult.

Keywords: NMDA excitotoxicity; iNOS; nitrite; striatal microdialysis; dexamethasone; aminoguanidine

Introduction

Nitric oxide (NO) is a messenger involved in several biological processes including vasodilation, platelet aggregation, macrophage-induced cytotoxicity and neuronal signalling (for review, see Moncada *et al*., 1991). NO is synthesized from the amino acid, L-arginine, by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been encoded (for reviews, see Nathan, 1992; Wang & Marsden, 1995). The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutive. Both are Ca/calmodulin dependent and release NO for short periods in response to physiological stimulation. The inducible isoform (iNOS) is induced by agents such as endotoxins or cytokines, is calcium-independent and releases NO for long periods. Induction can be prevented by glucocorticoids like dexamethasone (Dudek *et al*., 1994; Geller *et al*., 1993).

NO is involved in excitotoxic and ischaemic neurotoxicity (for reviews, see Dawson, 1994; Verrecchia *et al*., 1995). It has been shown that NO derived from neuronal NOS has a deleterious effect during the early phase of ischaemic brain injury (Yoshida *et al*., 1994). In addition, NOS is induced in a later stage of ischaemia. Indeed, mRNA expression and activity of iNOS peak 24–48 h after the ischaemic insult (Grandati *et al*., 1997; Iadecola *et al*., 1995a). Hence, the large amount of NO produced by iNOS could contribute to tissue damage. Aminoguanidine, a relatively selective inhibitor of iNOS (Corbett *et al*., 1992; Wolff & Lubeskie, 1995), decreases infarct size when given 24 h after the ischaemic insult (Iadecola *et al*., 1995b).

Thus, iNOS induction appears to be one of the mechanisms leading to cerebral ischaemic damage. An important increase in synaptic glutamate concentration has been shown in the earlier stage of cerebral ischaemia (Butcher *et al*., 1990) which participates in the neuronal death *via* a mechanism defined as excitotoxicity (Olney *et al*., 1971). Schmidt *et al*. (1995) have demonstrated that iNOS is involved in cerebral damage induced by an excitotoxic insult triggered by quinolinic acid, an N-methyl-D-aspartate (NMDA)-receptor agonist. In this context, the present study was carried out to assess whether an NMDA-induced excitotoxic insult results in iNOS activity, assessed as Ca-independent, and to investigate the involvement of iNOS in the striatal lesion induced by NMDA. For this purpose, NMDA was directly infused into the striatum *via* a microdialysis probe. The nitrite content, indicating NO production, was determined in dialysates, the NOS activity was assessed in striatum homogenates and striatal lesion measured 48 h after NMDA exposure. In order to assess the triggering role of the NMDA receptor overstimulation, the effect of MK 801, a non competitive NMDA antagonist, and AP5, a competitive NMDA antagonist, was evaluated on NOS activity, nitrite production and striatal lesion formation. The effects of dexamethasone (known to inhibit iNOS expression) and of aminoguanidine (an iNOS inhibitor) on nitrite production, striatal iNOS activity and on the volume of NMDA-induced striatal lesion were evaluated.

Methods

All experiments were conducted strictly according to NIH recommendations and French Department of Agriculture guidelines (licence no. 01352).

Surgical procedure

Adult male Sprague Dawley rats (280–350 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic frame. A concentric microdialysis probe, prepared as described by Robinson & Whishaw (1988), was vertically implanted in the left striatum (0 mm anterior to the bregma, 3.5 mm lateral to the bregma and 7 mm ventral to the skull), according to the atlas of Paxinos & Watson (1986). The

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probe was secured to two anchoring screws with dental cement.

Microdialysis protocol–excitotoxic insult

The microdialysis probe was perfused with Ringer solution (composition in mM): NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26 at a flow rate of 1.5μl min^{-1} for 2 h. After this equilibration period, control animals were perfused with Ringer solution for 320 min. In the NMDA-treated group, Ringer solution was perfused for 80 min, then switched for 40 min to a Ringer solution containing 10 mM NMDA (pH 7.3–7.5). Thereafter, probes were perfused with normal Ringer solution until the end of the 320 min period.

Nitrite determination

In a first series of experiments, in order to evaluate nitrite production 48 h after NMDA exposure, animals were re-submitted to a 320 min period dialysis with Ringer solution.

The nitrite content in the 40 min dialysate samples was determined by the Griess colorimetric method (Green *et al*., 1982). Dialysate was incubated with an equal volume of Griess reagent (0.5% w v^{-1} sulfanilamide in 5% v v^{-1} phosphoric acid and 0.05% w v^{-1} n-1-naphthylethylenediamine dihydrochloride). The absorbance of the purple azo compound formed was measured at 540 nm (Milton Roy Spectronic 401). Total nitrite production was calculated by adding the amounts of nitrite in each 40 min dialysate sample collected during the 320 min period and expressed as picomoles per min (pmol min^{-1}).

NOS activity assay

In a second experiment, animals perfused with NMDA or Ringer solution, as previously described, were killed at 48 h and the striata were removed in order to determine Ca-independent NOS activity by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline using a modified technique of Bredt & Snyder (1989) (Grandati *et al*., 1997). NOS activity was measured in striata of naive animals (rats undergoing no surgery) and in sham-operated rats to assess whether probe implantation caused an increase in a Ca-independent NOS activity. Striata 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 20,000 g, for 15 min, at 0°C. The supernatants were used for assays. iNOS activity, assessed as Ca-independent NOS activity, was measured by incubating samples (25 μl) for 30 min at 37°C in a calcium-free 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 μM NADPH, 50 μM tetrahydro-L-biopterin and 1 μCi ml^{-1} L-[^{14}C]-arginine (final concentration, 3.3 μM). 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) columns to remove L-[^{14}C]-arginine. Columns were eluted with 2 volumes of 0.5 ml water and L-[^{14}C]-citrulline quantified by liquid scintillation. The L-[^{14}C]-citrulline concentration was computed after subtracting the blank value, which gave the non-specific radioactivity in the absence of enzyme. Protein in the supernatants was assayed by the method of Bradford (1976) using human serum albumin as standard. Data are expressed as pmol of L-[^{14}C]-citrulline per mg protein per min (pmol mg^{-1} min^{-1}).

Histology

In a third experiment, the volume of striatal lesion was assessed 48 h after infusion of 10 mM NMDA. Rats were killed by an overdose of pentobarbitone, the brains were removed, frozen in isopentane and stored at −40°C. Serial coronal forebrain sections (50 μm thick) were cut in a cryostat at 500 μm intervals, beginning at the level 11.2 mm anterior to the interaural line which corresponds to the beginning of the striatum. After staining with cresyl violet the infarction appeared well demarcated: lesioned areas were unstained (white tissue) and easily contrasted with areas of viable tissue, which stained violet. Lesioned areas were determined using an image analyser (IMSTAR, France) and the distances between respective coronal sections were used to calculate a linear integration for the lesion volume.

Treatments

In order to assess the triggering role of NMDA receptor overstimulation in our model, the effect of MK-801, a non competitive NMDA antagonist, and AP5, a competitive NMDA antagonist, was evaluated on NOS activity, nitrite production and on the lesion formation. MK-801 (3 mg kg^{-1}, i.p.) was given 40 min before the beginning of NMDA exposure. As AP5 does not cross the blood-brain barrier, it was given at 300 μM concomitantly with NMDA for 40 min *via* the microdialysis probe.

The role of iNOS in NMDA-induced nitrite production and striatal lesion at 48 h was assessed by giving dexamethasone (DXM) or aminoguanidine (AG). DXM (3 mg kg^{-1} i.p.) and AG (100 mg kg^{-1} i.p.) were administered in control and NMDA-treated rats: one hour before and 8, 24 and 36 h after NMDA exposure. Vehicle-treated rats received NaCl 0.9% (w v^{-1}) i.p. at the same time.

Drugs

NMDA, AP5 (2-amino-5-phosphonopentanoic acid) and aminoguanidine hemisulfate were purchased from Sigma (France). MK-801 (dizolcipine maleate) was purchased from RBI. Dexamethasone (Soludecadron 4 mg) was purchased from Merck Sharp & Dohme-Chibret.

Statistical analysis

Statistical comparison between two groups was evaluated by unpaired Student's *t*-test. Comparisons between multiple groups were evaluated by one way analysis of variance followed by the Scheffe's F test. Differences were considered significant at a value of $P<0.05$.

Results

Nitrite assay

The nitrite production in NMDA-infused rats, 48 h after exposure to NMDA, was 3.5 fold greater than that in control rats (Figure 1). In control rats, neither MK 801 (3 mg kg^{-1}) nor AP5 (300μM) modified the nitrite production. However, both these treatments significantly reduced nitrite production in NMDA-infused rats respectively by 68% and 50% (Figure 1).

In control rats, neither DXM (3 mg kg^{-1}, 4 times per 48 h) nor AG (100 mg kg^{-1}, 4 times per 48 h) altered the amount of

nitrite produced at 48 h. By contrast, in NMDA-infused rats, DXM and AG totally blocked the increase of nitrite production (Figure 2).

Striatal NOS assay

In control rats, 48 h after probe implantation, no significant Ca-independent NOS activity was observed compared to naive rats (Figure 3). By contrast, in the NMDA-infused group, Ca-independent NOS activity was markedly increased compared to the control group (Figure 3).

In control rats, Ca-independent NOS activity was modified neither by MK 801 (3 mg kg^{-1}) nor by AP5 (300μM) (Figure 4A). In contrast, in NMDA-infused rats, MK 801 and AP5 decreased Ca-independent NOS activity by 87% and 60% respectively compared to vehicle-treated rats (Figure 4B).

Neither DXM nor AG exerted any effect on the NOS activity measured in control rats (Figure 5A). By contrast, both drugs reduced by 62% the Ca-independent NOS activity observed 48 h after NMDA exposure compared to vehicle-treated rats (Figure 5B).

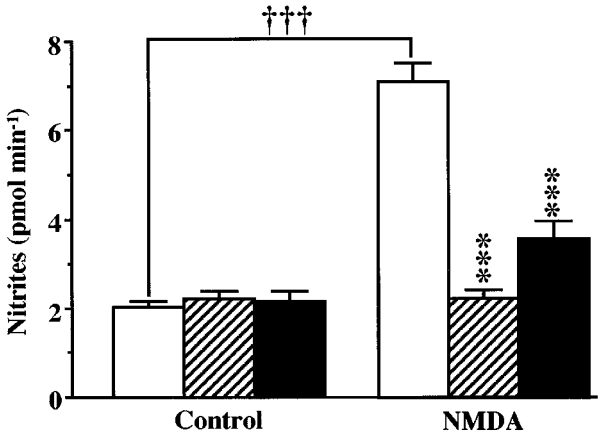

Figure 1 Effect of MK 801 and AP5 on nitrite production at 48 h in control rats and in NMDA-treated rats. Rats were given vehicle (open columns, $n=6$), MK 801 (3 mg kg^{-1}, $n=6-8$, hatched columns) or AP5 (300 μM, $n=6$, solid columns). Results are expressed as mean$\pm$s.e.mean. ***$P<0.001$ versus vehicle-treated rats. †††$P<0.001$.

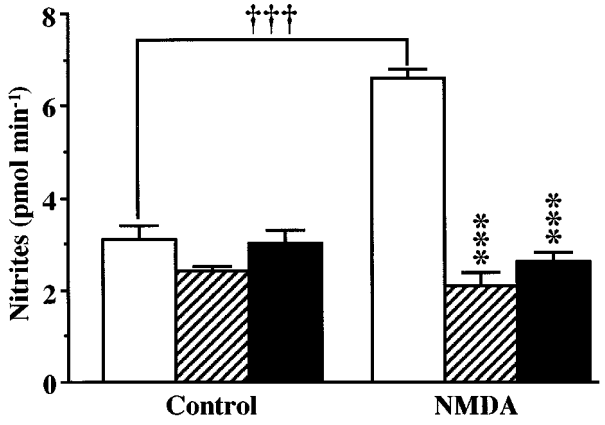

Figure 2 Effect of DXM and AG on nitrite production at 48 h in control rats and in NMDA-treated rats. Vehicle (open columns, $n=11$), DXM (3 mg kg^{-1}, $n=4-7$, hatched columns) and AG (100 mg kg^{-1}, $n=6-10$, solid columns) were administered 1 h before and 8, 24, 36 h after 10 mM NMDA infusion. Results are expressed as mean$\pm$s.e.mean. ***$P<0.001$ versus vehicle-treated rats. †††$P<0.001$.

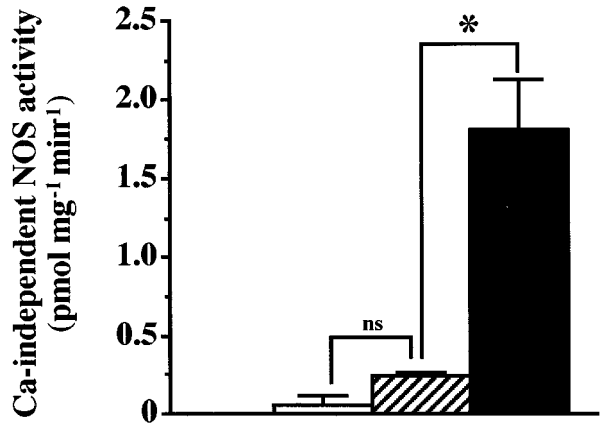

Figure 3 Effect of 10 mM NMDA on the striatal Ca-independent NOS activity expressed 48 h after NMDA dissolved in Ringer solution was perfused in the left striatum through the microdialysis probe. Control rats were given Ringer solution alone. Ca-independent NOS activity was assessed in naive (open columns, $n=4$), control (hatched columns, $n=13$) and NMDA-treated (solid columns, $n=13$) rats. Results are expressed as mean$\pm$s.e.mean. ns: non significant, *$P<0.05$.

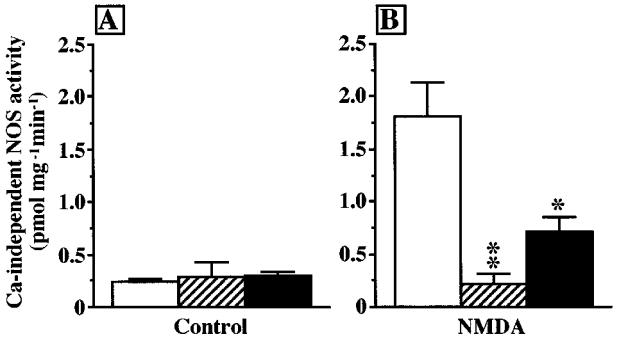

Figure 4 Effect of MK 801 and AP5 on Ca-independent activity expressed at 48 h in control rats (A) and in NMDA-treated rats (B). Rats were given vehicle (open columns, $n=13$), MK 801 (3 mg kg^{-1}, $n=6$, hatched columns) or AP5 (300 μM, $n=6$, solid columns). Results are expressed as mean$\pm$s.e.mean. *$P<0.05$ **$P<0.01$ versus vehicle-treated rats.

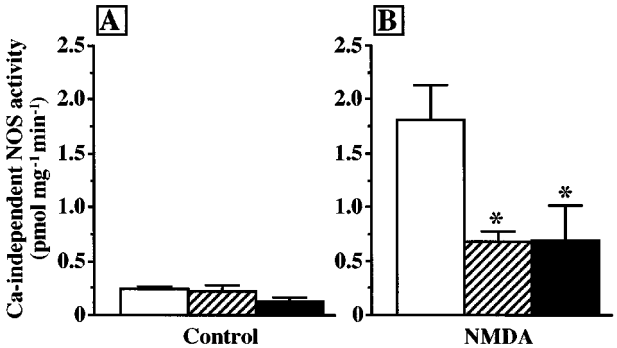

Figure 5 Effect of DXM and AG on Ca-independent NOS activity at 48 h in control rats (A) and in NMDA-treated rats (B). Vehicle (open columns, $n=13$), DXM (3 mg kg^{-1}, $n=6$, hatched columns) and AG (100 mg kg^{-1}, $n=6$, solid columns) were administered 1 h before and 8, 24, 36 h after 10 mM NMDA infusion. Results are expressed as mean$\pm$s.e.mean. *$P<0.05$ versus vehicle-treated rats.

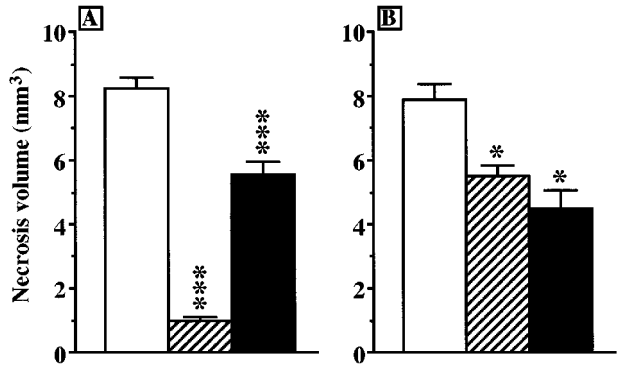

Figure 6 Effect of MK 801, AP5, DXM and AG on the volume lesion at 48 h induced by 10 mM NMDA. (A) Rats were given vehicle (open columns, $n=7$), MK 801 (3 mg kg^{-1}, $n=6$, hatched columns) or AP5 (300 μM, $n=6$, solid columns). (B) Vehicle (open columns, $n=11$), DXM (3 mg kg^{-1}, $n=7$, hatched columns), and AG (100 mg kg^{-1}, $n=10$, solid columns) were administered 1 h before and 8, 24, 36 h after 10 mM NMDA infusion. Results are expressed as means$\pm$s.e.mean. $^{*}P<0.05$ $^{***}P<0.001$ versus vehicle-treated rats.

Histology

MK 801 (3 mg kg^{-1}) and AP5 (300μM) decreased by 88% and 33% respectively the volume of NMDA-induced striatal lesion compared to the vehicle-treated rats (Figure 6A). Similarly, DXM (3 mg kg^{-1}, 4 times per 48 h) and AG (100 mg kg^{-1}, 4 times per 48 h) decreased by 30% and 40% respectively the volume of the NMDA-induced striatal lesion (Figure 6B).

Discussion

Our data show that perfusion of NMDA induces a striatal lesion which is related to the overactivation of NMDA receptor since a neuroprotective effect is observed with the NMDA antagonists, MK 801 and AP5. In these experiments, microdialysis has been used in conjunction with measurement of nitrite, a marker for NO production (Bredt & Snyder, 1989), to assess if NO is released by striatum of rats submitted to excitotoxic injury. In the present experiments, we did not measure nitrite/nitrate (NOx) concentration since preliminary experiments revealed that the nitrite/nitrate ratio does not change before and after NMDA challenge. Indeed, in both situations, nitrite proportion (30–40% of total NOx) remained constant (data not shown). Our data show that 48 h after NMDA perfusion, there is a dramatic enhancement in nitrite production associated with an increase in Ca-independent NOS activity. Both are totally blocked by MK 801 and AP5, suggesting that NMDA receptor overstimulation is involved in this phenomenon.

In order to assess if Ca-independent NOS activity is related to iNOS, we used DXM and AG. DXM has been demonstrated to be a potent inhibitor of iNOS gene transcription (Dudek *et al*., 1994; Geller *et al*., 1993; Kleinert *et al*.., 1996; Kunz *et al*., 1996; Radomski *et al*., 1990). Our results show that the chronic administration of this drug decreases by 70% the enhancement of nitrite production and by 62% the Ca-independent striatal NOS activity observed 48 h after NMDA infusion. Although the pharmacological mechanism of action of corticosteroids is not specific to the iNOS gene, these results seem likely to reflect an inhibition of iNOS induction. Moreover, chronic treatment with AG, a relatively selective iNOS inhibitor (Corbett *et al*., 1992; Corbett & McDaniel, 1996; Wolff & Lubeskie, 1995; Wolff *et al*., 1997) results in a similar decrease of nitrite production and Ca-independent NOS activity suggesting that iNOS is expressed in our model of excitotoxic injury. Moreover, it has been reported that AG, used at the same dose, does not affect either Ca-dependent NOS activity or the arterial pressure or cerebral blood flow, suggesting that endothelial and neuronal NOS are not influenced by AG (Iadecola *et al*., 1995b). It is conceivable that the protective effect of AG could be related to its other pharmacological properties. For example, AG has been shown to inhibit advanced glycation end (AGE) products formation which exert deleterious effects in a model of stroke in diabetic rat (Bucala *et al*., 1994). However, as AGE exist specifically in diabetes mellitus, it is most unlikely that this mechanism occurs in our model. AG has also been described to exert neuroprotective effect in the central nervous system of rat by inhibiting polyamine oxidase which metabolizes endogenous polyamines into cytotoxic aldehydic compounds (Brunton *et al*., 1994). The contribution of the inhibition of this pathway to the neuroprotective effect of aminoguanidine in our model remains to be established. Ca-independent NOS activity has been found in the striatum of rat given quinolinic acid (Schmidt *et al*., 1995). In addition, iNOS has been described in situations believed to involve the glutamatergic excitotoxic pathway like cerebral ischaemia (Iadecola *et al*., 1995a) and traumatic brain injury (Clark *et al*., 1996). Yamanaka *et al*. (1995) found an increase in nitrite/nitrate in rats, attributable to iNOS activation, 3 days after traumatic cortical injury. Until now no relationship has been established between excitotoxicity and iNOS induction during these brain injury models. Our results suggest that the excitotoxic phenomenon could be one of the pathways by which iNOS is induced after cerebral ischaemia or traumatic brain injury.

Inducible NOS has been demonstrated to play a key role in delayed neuronal death and in ischaemic lesion formation (Cockroft *et al*., 1996; Iadecola *et al*., 1995b; Zhang *et al*., 1996). Therefore, we assessed whether the Ca-independent NOS elicited by an excitotoxic injury was involved in the striatal lesion in our model. DXM and AG treatment also provides a significant neuroprotection and reduces the extent of NMDA-induced striatal lesion by 40% and 30% respectively. These results strongly suggest that the inducible isoform of NOS expressed in our model of excitotoxic injury contributes to the NMDA-elicited lesion. Furthermore, the protection we observed with AG in this study is similar to that obtained under ischaemic conditions (Iadecola *et al*., 1995b; Zhang *et al*., 1996; Cockroft *et al*., 1996). Thus, the delayed deleterious iNOS activation which occurs in ischaemia may, at least in part, also result from an initial excitotoxic insult.

The mechanisms by which iNOS is expressed following NMDA-induced excitotoxic injury remain unclear. Several groups have reported that iNOS expression is regulated by cytokines (Balligand *et al*., 1994; Chesrown *et al*., 1994; Hagan *et al*., 1996). Neurotoxic concentrations of excitatory amino acids or related agonists induce interleukin IL-1 expression (Hopkins & Rothwell, 1995) and IL-1 and IL-6 are produced in a model of mechanical brain injury (Woodroofe *et al*., 1991). Cytokines have also been implicated in the neurodegeneration elicited by cerebral ischaemia and over-activation of excitatory amino acid receptors (Rothwell & Relton, 1993; Feuerstein *et al*., 1994). The excitotoxic damage due to the striatal infusion of an NMDA-receptor agonist into the striatum is inhibited by IL-1 receptor antagonist (IL-1-ra) (Relton & Rothwell, 1992), suggesting that NMDA-induced iNOS activation may occur, at least in part, *via* an increase in IL-1 production. The nuclear

factor K_B (NF-K_B) is reported to regulate iNOS gene transcription (Barnes & Adcock, 1997). Physiologically, this factor is tightly bound to its cytosolic inhibitor, I-K_B. Free radicals have been shown to disrupt this binding, allowing NF-K_B to activate iNOS gene transcription (Milligan *et al*., 1996; Moormann *et al*., 1996). Brigelius-Flohé *et al*. (1997) demonstrated that NF-K_B is induced by IL-1 in human endothelial cells through the generation of free radicals. In addition, free radicals scavengers such as phenyl N-tert-butyl nitrone (Miyajima & Kotake, 1997) or 21-aminosteroids (Salahudeen *et al*., 1996) have been demonstrated to inhibit iNOS induction triggered by LPS or ischaemia. In addition, results published by our group (Lecanu *et al*., 1998) demonstrated that an oxidative stress triggered by inhibition of the mitochondrial respiratory chain results in the expression of a deleterious iNOS activity in the rat striatum. Taken together, these data suggest that the oxidative stress which occurs after the NMDA receptor overstimulation could be a pathway leading to iNOS induction.

Further studies are needed to identify the cell types involved in iNOS expression after NMDA exposure. Several types of cell in the brain are able to express iNOS activity, including endothelial cells (Gross *et al*., 1991), vascular smooth muscle (Nunokawa *et al*., 1993), neurones (Minc-Golomb *et al*., 1994; Sato *et al*., 1995), astrocytes and microglia (Murphy *et al*., 1993; Wood *et al*., 1994). NOS induction has been described in cerebral microvessels in cerebral ischemia (Nagafugi *et al*., 1994) and in polymorphonuclear cells infiltrating the infarcted area (Iadecola *et al*., 1995c). In a model of quinolinic acid lesioned striatum, a marked increase in iNOS in both astrocytes and glial cells was reported (Calka *et al*., 1996; Schmidt *et al*., 1995). These morphological data correlate well with an increase in Ca-independent NOS activity, suggesting that activated glia are an important source of NO (Schmidt *et al*., 1995). NO produced by iNOS has been reported to cause delayed neurotoxicity in mixed cultures of neurones and glia (Dawson *et al*., 1994) and NMDA-mediated neurone death is potentiated following the induction of NOS in astrocytes (Hewett *et al*., 1994). NO-mediated cytotoxicity can occur *via* free radical formation. Free radicals 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 dehydrogenase by ADP ribosylation (Zhang & Snyder, 1992). Inhibition of the mitochondrial respiratory chain may also contribute to NO-mediated neurotoxicity. NO has been reported 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 results demonstrate that NMDA-induced excitotoxic injury causes a delayed, sustained activation of the L-arginine-NO pathway. This pathway involves Ca-independent NOS activity which is blocked by DXM and AG, suggesting the involvement of iNOS. The fact that AG and DXM reduce the NMDA-elicited lesion suggests that iNOS contributes to the brain damage induced by excitotoxic insult. Our results suggest a role for such a mechanism in cerebral ischaemia and other neuropathologies in which glutamatergic excitotoxicity and iNOS activity have been shown.

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