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Implication of Glutamate in the Expression of Inducible Nitric Oxide Synthase After Oxygen and Glucose Deprivation in Rat Forebrain Slices

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Abstract: Nitric oxide synthesis by inducible nitric oxide synthase (iNOS) has been postulated to contribute to ischemia–reperfusion neurotoxicity. The expression of this enzyme has been demonstrated in cells present in the postischemic brain. The mechanisms of iNOS expression after cerebral ischemia are a subject of current research. We therefore decided to investigate whether glutamate, which is released in ischemia and is implicated in neurotoxicity, might be involved in the mechanisms by which oxygen and glucose deprivation (OGD) leads to the expression of iNOS in rat forebrain slices. In this model, we have shown previously that 20 min of OGD causes the expression of iNOS. We have now found that the NMDA receptor antagonist MK-801 blocks the expression of iNOS, suggesting that the activation of the NMDA subtype of glutamate receptor is implicated in the mechanisms that lead to the expression of this isoform. Moreover, we have found that glutamate alone could trigger the induction process, as shown by the appearance of a Ca^{2+}-independent NOS activity and by the detection of iNOS mRNA and protein in slices exposed to glutamate. Glutamate-dependent iNOS expression was concentration-dependent and was blocked by EGTA and by the inhibitors of nuclear factor κB (NF-κB) activation pyrrolidine dithiocarbamate and MG132. In addition, glutamate induced NF-κB translocation to the nucleus, an effect that was inhibited by MG132. Taken together, our data suggest that activation of NMDA receptors by glutamate released in ischemia is involved in the expression of iNOS in rat forebrain slices via a Ca^{2+}-dependent activation of the transcription factor NF-κB. To our knowledge, this is the first report showing an implication of excitatory amino acids in the expression of iNOS caused by ischemia. **Key Words:** Cerebral ischemia—NMDA—Neurotoxicity—Nuclear factor κB.
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Inducible nitric oxide (NO) synthase (iNOS) is a high-output isoform of NO synthase (NOS), Ca^{2+}- and calmodulin-independent, which is expressed after exposure of cells to cytokines and/or lipopolysaccharide (for review, see Nathan and Xie, 1994). This NOS isoenzyme mediates cytotoxicity in many cell systems (Moncada et al., 1991; Gross and Wolin, 1995). In this context, iNOS might contribute to the tissue damage that occurs after cerebral ischemia because its expression has been demonstrated not only in blood and glial cells present in the postischemic brain of in vivo models of cerebral ischemia–reperfusion (Endoh et al., 1994; Wallace and Bisland, 1994; Iadecola et al., 1995*a*), but also in neurons when using rat forebrain slices exposed to oxygen and glucose deprivation (OGD) (Moro et al., 1998). In support of this idea, aminoguanidine, a selective inhibitor of iNOS, has been shown to decrease damage caused by focal cerebral ischemia (Iadecola et al., 1995*b*), and we have found recently that 1400W, a more selective inhibitor of iNOS (Garvey et al., 1997), ameliorates the necrotic tissue damage produced by OGD in rat forebrain slices (Cárdenas et al., 1998).

The mechanisms of iNOS expression after cerebral ischemia are a subject of current research. Cytokines expressed after ischemia are known to activate the expression of inflammation-related genes such as iNOS in CNS cells (Galea et al., 1992; Simmons and Murphy, 1992, 1993; Peterson et al., 1994; Minc-Golomb et al., 1994, 1996). In addition, oxidative stress produced in this situation might itself trigger the expression of iNOS (Melillo et al., 1995).

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Abbreviations used: BH_4, 5,6,7,8-tetrahydrobiopterin; DTT, dithiothreitol; EAA, excitatory amino acids; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MK-801, dizocilpine; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; OGD, oxygen and glucose deprivation; PDTC, pyrrolidine dithiocarbamate.

After the first report showing that extracellular concentrations of glutamate and aspartate increased in the rat hippocampus during ischemia (Benveniste et al., 1984), numerous studies have shown that the neurotoxic actions of these excitatory amino acids (EAA) are implicated in the pathogenesis of brain injury (Choi, 1988) and in a wide range of neurological disorders (Olney, 1990; Zorumski and Olney, 1993). Following the initial observation that the activation of the NMDA subtype of glutamate receptor activates neuronal NOS (Garthwaite et al., 1988), it was postulated that an overproduction of NO derived from the excessive stimulation of neuronal NOS, a constitutive and Ca^{2+}-dependent NOS isoform, was the link between the actions of EAA and the subsequent cell damage (Dawson et al., 1991; Nowicki et al., 1991). However, activation of glutamate receptors has also been linked to a diversity of lasting changes in the CNS, such as the rapid induction of a number of immediate-early genes (for reviews, see Ghosh et al., 1994; Lerea, 1997). Moreover, stimulation of glutamate receptors can activate the nuclear transcription factor NF-κB in neurons (Guerrini et al., 1995; Kaltschmidt et al., 1995). In this context, iNOS is regulated mainly at the transcriptional level, and activation of NF-κB has been recognized as an essential requirement for the expression of this gene (Xie et al., 1994). Recently, it has been suggested that iNOS contributes to the NMDA-induced excitotoxic lesion in the rat striatum (Lecanu et al., 1998).

We investigated whether glutamate might also be involved in the mechanisms by which OGD leads to the expression of iNOS in rat forebrain slices.

MATERIALS AND METHODS

Preparation and incubation of slices

Male Sprague–Dawley rats (200–250 g) were killed by decapitation (according to procedures approved by the Committee of Animal Care at the Universidad Complutense of Madrid), and the forebrain slices were prepared as described (Moro et al., 1998). In brief, slices were preincubated in modified Krebs–Henseleit solution (preincubation solution) containing the following: 120 m*M* NaCl, 2 m*M* KCl, 0.5 m*M* $CaCl_2$, 26 m*M* $NaHCO_3$, 10 m*M* $MgSO_4$, 1.18 m*M* KH_2PO_4, and 11 m*M* glucose 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 another modified Krebs–Henseleit solution (incubation solution) containing the following: 120 m*M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2$, 26 m*M* $NaHCO_3$, 1.19 m*M* $MgSO_4$, 1.18 m*M* KH_2PO_4, 11 m*M* glucose, and 10 μM 5,6,7,8-tetrahydrobiopterin (BH_4) bubbled with 95% O_2/5% CO_2. The slices corresponding to the control group were then incubated 20 min further in the same conditions. Another set of slices was incubated in the same conditions but with 0.1–100 μM glutamate added in the presence or absence of 100 μM pyrrolidine dithiocarbamate (PDTC; an inhibitor of the activation of NF-κB; Schreck et al., 1992), 75 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; a proteasome inhibitor), or 1 m*M* EGTA. When EGTA was used, no Ca^{2+} was added to the incubation solution. Slices corresponding to the "ischemic" group were incubated 20 min in incubation solution without glucose and equilibrated with 95% N_2/5% CO_2 to mimic an ischemic condition (OGD), in the presence or absence of the NMDA receptor antagonist dizocilpine (MK-801; 100 n*M*) or PDTC (100 μM). 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 180 min after the OGD period and frozen immediately with liquid nitrogen. Incubation solution was also collected at 0, 30, 60, and 120 min after the OGD period for lactate dehydrogenase (LDH) or EAA assay.

NOS activity

NOS activity was determined after sonication of the forebrain slice (Labsonic 2000, Barcelona, Spain) at 4°C in 5 volumes of buffer containing 320 m*M* sucrose, 1 m*M* EDTA, 1 m*M* DL-dithiothreitol (DTT), 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 50 m*M* Tris brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 4°C at 12,000 *g* for 20 min, and the pellet was discarded. NOS activity was then determined in the postmitochondrial supernatant by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline as described by Salter et al. (1991) with modifications by Rees et al. (1995), according to which the cofactors NADPH (100 μM), BH_4 (3 μM), FAD (3 μM), and FMN (3 μM) are included in the enzyme assay. The activity of the Ca^{2+}-dependent NOS was calculated from the difference between the [^{14}C]citrulline produced from control samples and samples containing 1 m*M* EGTA; the activity of the Ca^{2+}-independent isoform was determined from the difference between samples containing 1 m*M* EGTA and samples containing 1 m*M* EGTA and 1 m*M* N^G-monomethyl-L-arginine.

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

Determination of iNOS protein by western blot

Slices were homogenized in lysis buffer (10 m*M* Tris, pH 8.0, 0.2% Nonidet P-40, 1 m*M* dithioerythritol). After centrifugation in a microcentrifuge for 15 min, homogenate containing 10 μg of protein was loaded. The proteins were size-separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (50 mA). The proteins were blotted onto a polyvinylidene difluoride membrane (Millipore) and incubated with a specific polyclonal anti-iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.; 1:1,000 dilution) (Lowenstein et al., 1992). Proteins recognized by the antibody were revealed by an ECL kit following the manufacturer's instructions (Amersham).

Detection of mRNA by northern blot

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)propanesulfonic acid buffering system (Chomczynski and Sacchi, 1987). After transference of the RNA to Nytran membranes (NY 13-N, Schleicher and Schüell), the level of iNOS mRNA was determined by hybridization using as probe an *Eco*RI-*Hin*dII (Amersham) fragment from the murine iNOS cDNA (kindly donated by Dr. Q.-W. Xie and Dr. C. Nathan, Cornell University) labeled with [α-^{32}P]dCTP (Random Primed labeling kit; Amersham). Upon hybridization, the membranes were exposed to an x-ray film (Kodak X-Omat) and the bands were quantified by laser densitometry (Molecular Dynamics). Hybridization with a probe specific for β-actin was used to normalize the RNA lane charge of the blot.

HPLC determination of EAA concentration

Analysis of the EAA aspartate and glutamate in each sample was performed by HPLC with fluorimetric detection (Perkin–Elmer binary LC pump 250 and fluorescence detector LC 240) following precolumn derivatization with the *o*-phthaldialdehyde procedure (Lindroth and Mopper, 1979). EAA derivatives were separated isocratically on a reverse-phase column (4.6 × 150 mm, 5-μm particle diameter, Nucleosil 100-C18) using a mobile phase consisting of sodium acetate buffer (0.05 *M,* pH 6.5), 20% methanol, and 2% tetrahydrofuran. The area of each peak was determined with a Perkin–Elmer–Nelson model 1020 integrator (Phoenix 8088 ROM BIOS version 2.52 software) and compared with the peak area of the corresponding external standard to determine the EAA concentration.

Preparation of nuclear extracts

A modified procedure based on the method of Schreiber et al. (1989) was used. Slices were homogenized with 100 μl of buffer A (10 m*M* HEPES, pH 7.9, 1 m*M* EDTA, 1 m*M* EGTA, 10 m*M* KCl, 1 m*M* DTT, 0.5 m*M* phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 5 m*M* NaF, 1 m*M* $NaVO_4$, 0.5 *M* sucrose, and 10 m*M* Na_2MoO_4). After 10 min at 4°C, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortex-mixed for 15 s, and nuclei were collected by centrifugation at 8,000 *g* for 15 min. The pellets were resuspended in 50 μl of buffer A supplemented with 20% glycerol, 0.4 *M* KCl, and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 *g* for 15 min, and aliquots of the supernatant were stored at −80°C. All steps of the fractionation were carried out at 4°C. To dephosphorylate proteins, extracts were treated for 1 h at 40°C with 1 unit of agarose-immobilized alkaline phosphatase/μg of protein. Appropriate controls of heat-inactivated alkaline phosphatase were used to ensure the specificity of the reaction.

Electrophoretic mobility shift assay (EMSA) for NF-κB

Oligonucleotides were synthesized in an oligonucleotide synthesizer (Pharmacia). The oligonucleotide sequence corresponding to the consensus NF-κB binding site (nucleotides −978 to −952) of the murine iNOS promoter was 5′-TGCTAGGGGGATTTTCCCTCTCTCTGT-3′ (Xie et al., 1994). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 m*M* Tris-HCl, pH 8.0, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme (Amersham) fragment in the presence of 50 μCi of [α-^{32}P]dCTP (Amersham) and the other unlabeled dNTPs in a final volume of 50 μl. The DNA probe (5×10^4 dpm) was used for each binding assay of nuclear extracts as follows: 3 μg of protein was incubated for 15 min at 4°C with the DNA and 2 μg of poly[dI–dC], 5% glycerol, 1 m*M* EDTA, 100 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 10 m*M* Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris borate–EDTA buffer (Díaz-Guerra et al., 1996). Supershift assays were carried out after incubation of the nuclear extract with the antibody (0.5 μg) for 1 h at 4°C, followed by EMSA.

LDH assay

As a marker of tissue necrosis, LDH released from damaged cells in the slices was determined in the incubation solution. LDH activity was measured spectrophotometrically at 340 nm by following the oxidation of NADH (decrease in absorbance) in the presence of pyruvate (Koh and Choi, 1988) using a Beckman DU-7500 spectrophotometer or a Thermomax microplate reader (Molecular Devices). Data are expressed as mOD/min and reflect the total LDH release.

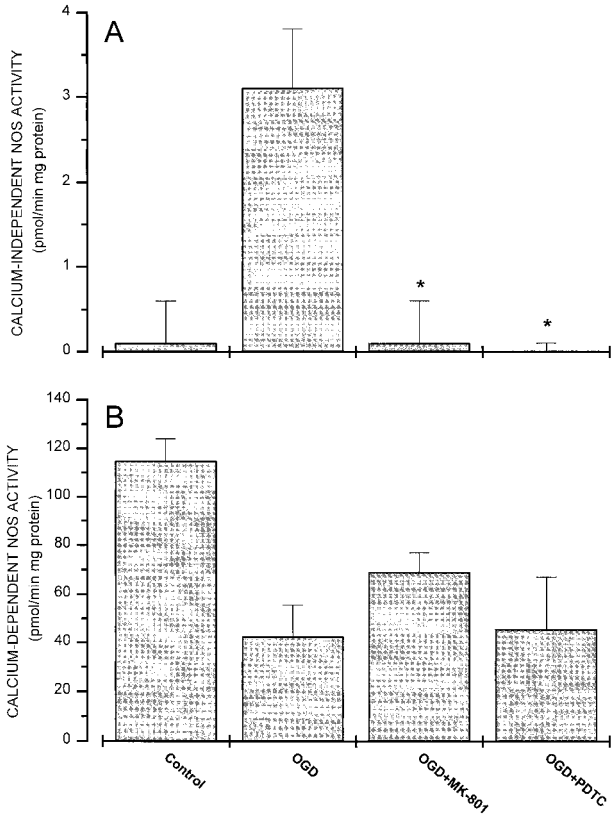

FIG. 1. NOS activities in control and OGD rat forebrain slices: effect of MK-801 (100 n*M*) and PDTC (100 μ*M*). NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline (see Materials and Methods). **A:** Ca^{2+}-independent NOS activity. **B:** Ca^{2+}-dependent NOS activity. Data are means ± SEM (n = 16). *$p < 0.05$ vs. OGD.

Chemicals and statistical analyses

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

RESULTS

NOS activity in rat forebrain slices exposed to OGD: effect of MK-801 and PDTC

As we have shown previously (Moro et al., 1998; De Alba et al., 1999), OGD for 20 min caused the appearance of a Ca^{2+}-independent NOS activity (Fig. 1A) and a decrease in Ca^{2+}-dependent NOS activity (Fig. 1B).

The addition of the NMDA receptor antagonist MK-801 (100 n*M*) or the inhibitor of the activation of tran-

TABLE 1. *Concentrations of glutamate and aspartate in incubation solution of rat forebrain slices*

Time of reperfusion (min)	Glutamate (ng/ml)		Aspartate (ng/ml)	
	Control	OGD	Control	OGD
0	72 ± 8	1,870 ± 25[a]	44 ± 1	556 ± 25[a]
30	67 ± 10	217 ± 24[a]	53 ± 7	175 ± 20[a]
60	44 ± 3	33 ± 3	42 ± 2	21 ± 3

EAA concentrations in incubation solution of control and OGD slices 0, 30, and 60 min after the end of the OGD period are presented. Results are means ± SEM (n = 4).

[a] $p < 0.05$.

scription factor NF-κB PDTC (100 μ*M*) inhibited the induction of the Ca^{2+}-independent NOS activity found in the rat forebrain slices exposed to OGD (Fig. 1A).

EAA efflux from rat forebrain slices exposed to OGD

OGD caused an increase in the concentrations of aspartate and glutamate present in the incubation solution of rat forebrain slices when measured at 0 and 30 min after the OGD period ($p < 0.05$, n = 4; Table 1). However, at later times, no significant differences were found between control and OGD-exposed slices ($p > 0.05$, n = 4; Table 1).

NOS activity in rat forebrain slices exposed to glutamate: effect of PDTC and EGTA

The incubation of rat forebrain slices with glutamate (0.1–100 μ*M*) for 20 min caused the induction of a Ca^{2+}-independent NOS activity (Fig. 2A), the magnitude of which was dependent on the concentration of glutamate. The addition of PDTC (100 μ*M*) inhibited glutamate (100 μ*M*)-induced Ca^{2+}-independent NOS activity (Fig. 2A). In addition, EGTA (1 m*M*), an extracellular Ca^{2+} chelator, also blocked glutamate (100 μ*M*)-induced Ca^{2+}-independent NOS activity (Fig. 2A).

In contrast, glutamate did not significantly affect Ca^{2+}-dependent NOS activity (Fig. 2B). In the presence of glutamate (100 μ*M*) plus PDTC (100 μ*M*), Ca^{2+}-dependent NOS activity decreased partially (Fig. 2B). The presence of EGTA in glutamate plus EGTA-treated samples hindered Ca^{2+}-dependent NOS activity measurement due to Ca^{2+} chelation (Fig. 2B).

Quantification of iNOS protein in rat forebrain slices exposed to OGD or to glutamate

OGD caused the expression of iNOS protein in rat forebrain slices as assessed by its detection in these samples (Fig. 3). The addition of the NMDA receptor antagonist MK-801 (100 n*M*) produced a decrease (40% of inhibition) in the levels of iNOS protein in rat forebrain slices exposed to OGD (Fig. 3). The exposure to glutamate (30–100 μ*M*) also caused the expression of iNOS protein (Fig. 3). The inhibitor of the activation of NF-κB MG132 (75 μ*M*) reduced the levels of iNOS protein induced by glutamate (Fig. 3).

Quantification of iNOS mRNA in rat forebrain slices exposed to OGD or to glutamate

OGD caused the expression of the gene encoding iNOS in rat forebrain slices as assessed by the detection of iNOS message in these samples (Fig. 4). In addition, the exposure to 100 μ*M* glutamate also caused the expression of iNOS message (Fig. 4).

Effects of glutamate on NF-κB activation

The exposure to glutamate (30–100 μ*M*) for 20 min caused the translocation of NF-κB to the nucleus in rat forebrain slices when measured 40 min after the exposure period (Fig. 5). MG132 (75 μ*M*) inhibited NF-κB translocation to the nucleus in slices (Fig. 5).

LDH efflux

LDH levels in the incubation solution of OGD slices were significantly higher than those found in control slices during the whole period of reperfusion (71 ± 8 vs. 19 ± 2 mOD/min for OGD and control slices, respectively; $p < 0.05$, n = 8). Similarly, the incubation of slices with 100 μ*M* glutamate caused the release of LDH (62 ± 3 vs. 19 ± 2 mOD/min for glutamate and control slices, respectively; $p < 0.05$, n = 8). However, when

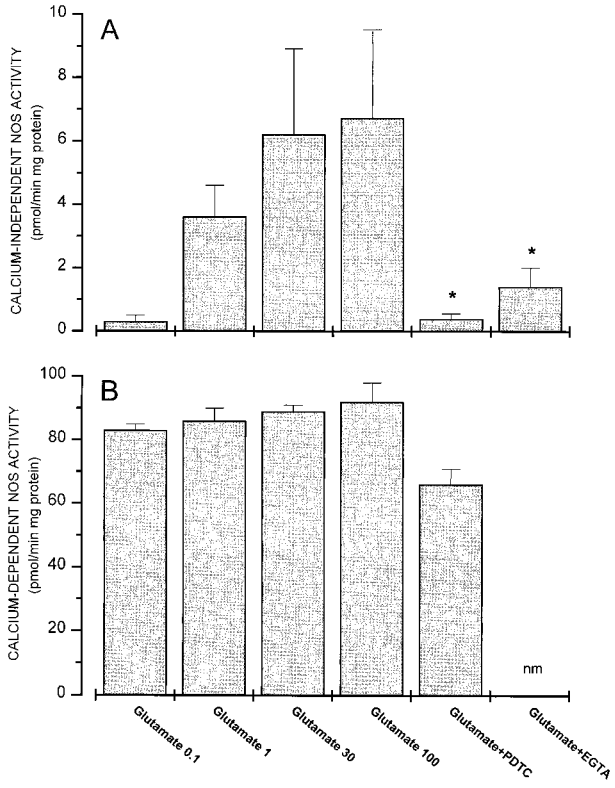

FIG. 2. A: Glutamate (0.1–100 μ*M*)-induced Ca^{2+}-independent NOS in rat forebrain slices: effect of PDTC (100 μ*M*) and EGTA (1 m*M*) on glutamate (100 μ*M*)-induced Ca^{2+}-independent NOS activity. **B:** Ca^{2+}-dependent NOS activity in samples described in A. Enzymatic activity was determined as described in the legend to Fig. 1. Data are means ± SEM (n = 6–16). *$p < 0.05$ vs. glutamate (100 μ*M*). nm, not measurable, due to Ca^{2+} chelation by EGTA.

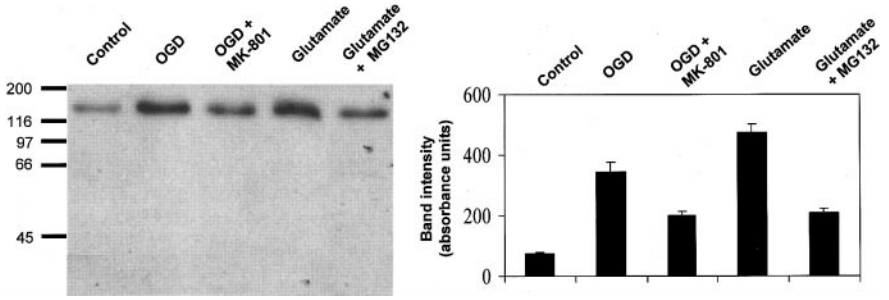

FIG. 3. Expression of iNOS protein after OGD and glutamate treatment in rat forebrain slices. **Left:** Western blot analysis of iNOS in soluble extracts from control, OGD, OGD plus MK-801 (100 n*M*)-, glutamate (30 μM)-, and glutamate plus MG132 (75 μM)-treated slices. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the levels of iNOS measured by western blotting. Commercial markers (Bio-Rad prestained) were used as molecular mass standards. **Right:** Laser densitometric analysis of iNOS expression.

slices were exposed to 1 μM glutamate, LDH release to the medium was not significantly different from that from control slices in the absence of this compound (22 $\pm$ 2 vs. 19 $\pm$ 2 mOD/min for glutamate and control slices, respectively; $p > 0.05$, n = 8).

DISCUSSION

We have shown previously that OGD causes the expression of iNOS in rat forebrain slices (Moro et al., 1998; De Alba et al., 1999). We now report that glutamate released from the "ischemic" tissue is implicated in the mechanisms that lead to the expression of this NOS isoform.

Our results showing direct measurements of enzyme activity indicate the presence of a Ca^{2+}-independent NOS activity in rat forebrain slices after 20 min of OGD. We have shown previously that this enzymatic activity is due to the expression of the inducible isoform of NOS, as assessed by the detection and quantification of both iNOS message and protein levels, as well as the cellular localization of iNOS by immunohistochemistry (Moro et al., 1998). We have now found that the NMDA receptor antagonist MK-801 blocks the expression of iNOS in rat forebrain slices deprived of oxygen and glucose, suggesting that the activation of the NMDA subtype of glutamate receptor is implicated in the mechanisms that lead to the expression of iNOS. Indeed, glutamate and aspartate are increased in the bathing solution of rat forebrain slices that have been exposed to OGD. Moreover, we observed that glutamate alone could trigger the induction process, resulting in the appearance of iNOS message, protein, and a Ca^{2+}-independent NOS activity in slices exposed to glutamate. This phenomenon was concentration-dependent and was blocked when extracellular Ca^{2+} was removed with EGTA, suggesting that the induction of iNOS is a Ca^{2+}-dependent process. This is consistent with our findings showing that OGD-induced expression of Ca^{2+}-independent NOS activity is mediated via the NMDA subtype of glutamate receptor, which is associated with the influx of Ca^{2+} (for review, see Collingridge and Lester, 1990). Measurements of Ca^{2+}-dependent NOS activities in these samples show that Ca^{2+}-independent NOS activity is ~5–10% of total

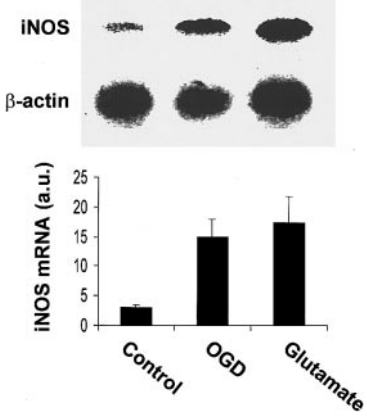

Fig. 4. **Top:** Northern blot analysis of the expression of iNOS mRNA in OGD-exposed and glutamate (100 μM)-treated slices. **Bottom:** Laser densitometric analysis of iNOS mRNA. a.u., absorbance units.

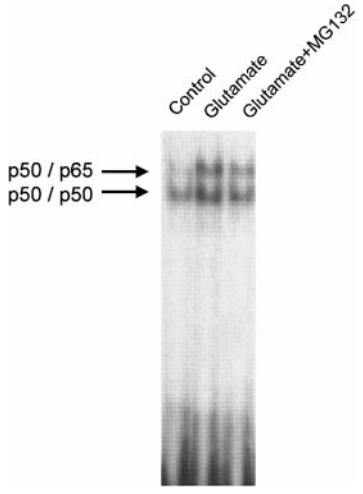

Fig. 5. NF-κB translocation to the nucleus caused by glutamate and its inhibition by MG132. NF-κB translocation was measured by EMSA (see Materials and Methods) in control, glutamate (100 μM)-, and glutamate (100 μM) + MG132 (75 μM)-treated brain slices at 40 min after the end of the exposure period. Results are representative of three individual experiments.

NOS activity 180 min after the end of the challenging period (OGD or glutamate). However, these values reflect the activity under optimal conditions in the tissue homogenate, which are well above the basal intracellular Ca^{2+} levels, at which Ca^{2+}-dependent isoforms are inactive.

The mechanisms of iNOS expression in the postischemic brain are not clear yet. In this context, proinflammatory cytokines are known to cause the induction of iNOS in several cell systems. It is interesting that cytokines such as interleukin-1β, tumor necrosis factor-α, and interferon-γ, which are rapidly induced in the brain following ischemia (Minami et al., 1992; Liu et al., 1993, 1994), have been involved in the expression of iNOS in astrocytes, microglia, polymorphonuclear cells, and macrophages invading the area of infarction, as well as in neurons (Galea et al., 1992; Simmons and Murphy, 1992, 1993; Minc-Golomb et al., 1994, 1996; Peterson et al., 1994). In addition, the oxidative stress produced in the ischemia–reperfusion process might itself trigger the induction of iNOS. Indeed, a specific pathway for the induction of iNOS under anoxic conditions has been described, and a hypoxia-responsive enhancer has been characterized in the promoter region of iNOS from nucleotide -227 to -209, suggesting that iNOS is a hypoxia-inducible gene (Melillo et al., 1995).

The precise mechanism of glutamate-dependent iNOS expression is not clear, but it should be mentioned that NMDA-receptor activation may lead to oxidative stress because it stimulates the formation of oxygen radicals, such as superoxide anion (Lafon-Cazal et al., 1993). In addition, proinflammatory cytokines might be released or expressed after the exposure to cytotoxic concentrations of EAA (De Bock et al., 1996). A third possibility is that glutamate leads to the expression of iNOS, by directly activating the transcription factor NF-κB (Guerrini et al., 1995; Kaltschmidt et al., 1995). Our data show that concentrations of glutamate that are not associated with cytotoxicity in our model (1 μM) are linked, however, to the expression of iNOS, thus arguing against the hypothesis that induction is caused by glutamate toxic indirect effects. Regardless of the initial stimulus responsible for iNOS expression in our preparation, NF-κB has been recognized as an essential requirement for the expression of this gene (Xie et al., 1994). Indeed, this pathway seems to be involved in our experimental model as suggested by the blockade of glutamate-induced iNOS expression caused by the proteasome inhibitor MG132, which inhibits activation of NF-κB by inhibiting the degradation of IκB (Tsubuki et al., 1993; Traenckner et al., 1994), and by PDTC, an inhibitor of the activation of this transcription factor, which does not interfere with proteasome activities (Schreck et al., 1992). Further support for the involvement of NF-κB was provided by the experiments showing that glutamate induces the translocation of NF-κB to the nucleus, an effect that was again inhibited by MG132. These findings, together with the data showing that the Ca^{2+} chelator EGTA blocks glutamate-induced expression of iNOS, suggest that Ca^{2+}-activated NF-κB activation is the mechanism responsible for the expression of iNOS in this model, consistent with the suggestion that NF-κB is a Ca^{2+}-sensitive transcriptional regulator (for review, see Baeuerle and Henkel, 1994) and that its activation by glutamate in neuronal cells is dependent on Ca^{2+} influx (Guerrini et al., 1995).

As we have shown previously (Moro et al., 1998), we could detect some basal iNOS expression in control slices, which might be a consequence of the hypoxia that exists at the center of the slice (Garthwaite and Garthwaite, 1988). In addition, our new data suggest that a basal release of glutamate is also likely to account, either directly or indirectly, for iNOS expression in control slices.

We have shown previously that OGD-induced iNOS expression in this model occurs in neurons and glial cells that exist in cortex and corpus callosum (Moro et al., 1998). As NMDA receptors in these regions are present not only in neurons, but also in astrocytes (for review, see Conti et al., 1997), glutamate-induced iNOS expression is also likely to occur in both cell types. Moreover, hippocampal astrocytes have been shown to respond to glutamatergic neuroligands, including NMDA, with increases in intracellular $[Ca^{2+}]$ (Porter and McCarthy, 1995), data in agreement with our results showing inhibition of glutamate-induced iNOS expression by Ca^{2+} chelators.

To our knowledge, this is the first report showing an implication of EAA in the expression of iNOS caused by an ischemic insult. In contrast with our findings, a suppressive role for glutamate in cytokine-induced iNOS expression in rat cultured astrocytes has been described (Lin and Murphy, 1997). This apparent controversy is likely due to the differences in both models and inducing stimuli. Very likely, our model using forebrain slices offered important advantages over other preparations for this study (Cohen et al., 1984; Newman et al., 1989; Pellmar, 1995; Taylor et al., 1995) because it is specific as far as cell composition and local effector release are concerned. Indeed, functional neurons, inflammatory competent cells, and intercellular connections are preserved that may help to elucidate important mechanisms, which are more difficult to approach in other preparations.

In summary, these data suggest that activation of NMDA receptors by glutamate released after an ischemic insult is involved in the expression of iNOS in rat forebrain slices via a Ca^{2+}-dependent activation of the transcription factor NF-κB. Our findings raise the possibility that the neuroprotective effects of antiexcitotoxic strategies may have additional beneficial effects in a wide range of neuropathologic disorders, which include degenerative diseases in addition to stroke, where NO and products of its further oxidation are implicated.

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