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Biphasic enhacement of nitric oxide synthase activity and cGMP level following brain ischemia in gerbils

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Abstract. This study was aimed to examine properties and changes in nitric oxide synthase (NOS) activity and cGMP level during reperfusion after 5 min of brain ischemia in gerbils. Animals were treated 5 min before ischemia with NOS inhibitors: N-Nitro-L-arginine (NNLA), or 7-Nitroindazole (7-NI), or with the inhibitor of guanylate cyclase, LY 83583, or with hydrocortisone for 7 days before ischemia. Northern blot analysis was performed using specific cDNA for inducible NOS. It was observed that ischemia significantly enhances NOS activity and cGMP level. During reperfusion, biphasic increase in NOS activity and cGMP level took place with two peaks 15 min and 2 h after ischemia. NNLA, 7-NI, and LY 83583 eliminated enhancements of NOS activity and cGMP level, whereas glucocorticoid remained without effect. There was no activation of gene encoding inducible NOS (iNOS). Our results indicate that ischemia-reperfusion activates constitutive NOS. It is suggested that nitric oxide (NO) production during reperfusion is related to neuronal degeneration and that inhibitor of NOS offers a new therapeutical strategies.

Key words: brain ischemia-reperfusion, nitric oxide, cGMP, inhibitors, glucocorticoid

INTRODUCTION

Nitric oxide (NO) is a highly unorthodox messenger which can readily diffuse through cell membranes to exert its biological action in intracellular and intercellular signalling. The formation of NO is catalyzed by a family of isoenzymes the called NO synthases (EC 1.14.13.39) from the semiessential amino acid L-arginine in mammalian cells (Forstermann et al. 1991, 1994, Moncada et al. 1991, Radomski et al. 1991, Nathan 1992, Stuehr and Griffith 1992). These enzymes are NADPH-dependent oxygenases and cytochrome P-450-like hemoproteins, they contain both the reductase and oxygenase catalytic domain and they are regulated by FAD and FMN at the same time (Klatt et al. 1992, McMillan et al. 1992, White and Marletta, 1992). The calcium-calmodulin dependent, constitutive isoforms of the enzyme (cNOS) that are expressed in neuronal cells are active as a monomer and can be activated by agonists that elevate the intracellular concentration of free Ca^{2+}. Calcium independent NO synthase, the inducible form of the enzyme (iNOS), appears to be active as a dimer and is expressed mainly in cell types such as macrophages and vascular smooth muscle cells, resulting from exposure to cytokines or bacterial lipopolysaccharide. On the basis of purification data at least six isoforms of NO synthase have been recognized (Mc Call and Vallance 1992). Recent studies indicate the presence of iNOS also in glial cells (Simmons and Murphy 1992). It was found that CNS damage of either viral or immune origin is associated with induction of iNOS (Karupiah et al. 1993, Koprowski et al. 1993, Weber et al. 1994). It is known that NO plays a significant role in brain in processes of neurotransmission (Moncada et al. 1991, Snyder 1992, Garthwaite and Santham, 1993). Nitric oxide produced tonically by the constitutive form of NOS has been implicated specifically in some forms of synaptic plasticity including both long term potentiation (LTP) and long term depression (LTD) (Bohme et al. 1991, Shibuki and Okada 1991). However, NO produced in excessive amounts is toxic for nervous tissue. A key role for NO has also been postulated in the pathogenesis of nerve cell damage resulting from acute insults, such as hypoxia-ischemia. Encouraging results have been reported using competitive NOS inhibitors in some animal models of stroke (Nowicki et al. 1991, Buisson et al. 1992, 1993, Nagafuji et al. 1992, Strosznajder et al. 1994). Not all available data support a role of neuronal NO formation in neuronal death. Wink et al. (1993) and Lipton et al. (1993) in an *in vitro* study demonstrate a cytoprotective effect of NO against cell death. Also, Sancesario et al. (1994) in an *in vivo* study observed that inhibition of nitric oxide production aggravates ischemic damage of hippocampal neurones. However, the recent study of Huang at el. (1994) indicated that in mutant mice deficient in neuronal NO synthase, infarct volumes after middle cerebral artery occlusion were significantly lower than in neuronal NOS containing mice. Moreover, it was observed that local cerebral ischemia is associated with activation of inducible form of NOS in the brain (Iadecola et al. 1995). Nitric oxide formation induces cGMP elevation in neighbouring cell. The resulting increase in cGMP levels is the major mechanism underlying many of the neuronal actions of NO. However, the mechanisms by which NO causes cells and tissue damage are not well understood and are currently receiving attention. Until now little has been known about NOS/cGMP during reperfusion time after global brain ischemia.

The aim of this study was to investigate the effect of ischemia-reperfusion injury on NO/cGMP level in brain hemispheres and to characterize the activity and the type of NOS isoenzyme(s) activated during different time of reperfusion after transient forebrain ischemia.

METHODS

Materials

Male Mongolian gerbils 60-70g were supplied by Animal Farm, Lomna, Poland. [^{3}H]L-arginine (68 Ci/mmol) and Cyclic GMP [^{3}H] assay kit were purchased from Amersham, Buckinghamshire, Eng-

land. 7-Nitroindazole was purchased from Calbiochem,New York,USA, LY 83583 was obtained from RBI,Natic, USA. N-nitro-L-arginine, TRIS, EDTA, EGTA, dithiothreitol (DTT), HEPES, pepstain A, tripsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), NADPH, calmodulin, trichloroacetic acid were obtained from Sigma(St.Louis, MO, USA). Dowex AG50W-X8 cation exchange was purchased from Serva,Heindenberg,Germany. Hydrocortisone was from Polfa, Jelenia Góra, Poland.

Ischemia-reperfusion injury

Mongolian gerbils were anaesthetized with 2% halotane for induction and 0.5% for maintenance of anaesthesia in 70% N_2O and 30% O_2. The body temperature was kept at 37°C by using a controlled heating pad. Brain ischemia was induced by ligation of both common carotid arteries using clips for 1, 2.5 and 5 min. The sham-operated animals serve as a control. The group were allowed to survive for 5, 15, or 30 min 1, 2, or 4 hours and 1, 4, or 7 days after ischemia. The animals then were quickly decapitated and the brains were removed and frozen in liquid nitrogen to inactivate the tissue.

Treatment with inhibitors and glucorticoid

The animals were divided into experimental groups, 4-6 animals in each group, and were treated with different compounds and different times of recovery. The animals were treated with N-nitroL-arginine (NNLA) in a dose of 30 mg/kg b.w., LY 83 583 was administered in a dose of 6 mg/kg b.w., and 7 nitroindazole (7NI) was given in a dose of 25 mg/kg b.w. These drugs are administered i.p. 5 min before induction of ischemia. Other groups of animals were injected subcutaneously with hydrocortisone in a dose of 40 mg/kg b.w. for 7 days before ischemia. The last injection was given 2 h before 5 min ischemia.

Determination of NO synthase activity

NOS activity was determined by the method of Bredt and Snyder (1990) modified by Hecker and co-workers (1994) based on conversion of [3H]L-arginine to radioactive L-citrulline. The homogenat (200 µg of protein) was incubated for 30 min at 37°C with 10 µM [^{3}H]L-arginine (1 µCi) 1 mM NADPH, 1 µM calmodulin in 50 mM Hepes buffer pH 7.4 containing 1 mM DTT, 1mM EDTA and 2 mM $CaCl_2$ in final volume of 300 µl . The reaction was terminated by addition of 1 ml of ice-cold 100 mM Hepes buffer pH 5.5 containing 10 mM EDTA and 500 mg of Dowex AG 50W-X8 (counterion Na^+) cation exchange resin (Serva), then incubated for 5 min at 0-4°C and finally centrifuged at 1,000 g for 5 min. Aliquots of 0.5 ml of supernatant fractions were mixed with 10 ml of Bray's fluid into scintillation vials. Radioactivity of samples was measured in LKB Wallac 1409 liquid scintillation counter.

Determination of cGMP levels

The brain was homogenized in cold 20% trichloroacetic acid (TCA). The homogenate was centrifuged at 3,000 rpm for 5 min. The supernatant was washed four times with water-saturated diethyl ether and neutralized with 1N NaOH to pH 7.4 before determination of cGMP. The content of cGMP was determined using the radioimmunoassay method (Amersham).

RNA isolation and Northern Blot analysis

Total RNA was extracted with 1 ml phenol, 1 ml phenol/chloroform (24:1), and 1ml chloroform. Then RNA was isolated by centrifugation over 2M LiCl. For Northern blot analysis about 10 µg RNA samples were separated electrophoretically in 1% agarose formaldehyde gels according to Sambrook et al. (1989). RNA was then capilary-transferred to nylon membrane in 20x SSC (3 M NaCl, 0.3 M Na citrate) as described by Thomas (1980). The blots were prehybridized and then hybridized at 68°C for 24 h in hybridization buffer to the ^{32}P-cCTP labelled cDNA specific for the mouse iNOS (a gift from Drs. Q-W Xie and C Nathan USA.) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH)

cDNA. The blots were washed intensively three times with 2x SSC, 0.1% SDS at 64°C for 20min. Then blots were placed at -70°C with XAR-5 film (Kodak, Rochester NY) to generate autoradiograms.

RESULTS

Nitric oxide synthase activity and cGMP level in the gerbil brain hemispheres during ischemia and reperfusion time

The activity of NOS was determined during different times of ischemia and reperfusion in the presence of Ca^{2+} and calmodulin. Ischemia induced by ligation of both common carotid arteries for 1 min activates NOS activity by about 100% respectively as compared to control values. During the next 2.5 and 5 min of ischemia the activity of NOS further increases as compared to control values. Concomitantly the level of cGMP enhanced by 50% during the first minute of ischemia remains unchanged during the prolonged time of ischemic insult (Fig. 1).

The activity of NOS and the level of cGMP were subsequently investigated during reperfusion lasting 15, 60, 120, 240 min, 1, 4 and 7 days after 5 min of global ischemia. A biphasic increase of NOS activity was observed 15 min and 2 h and 4 h after

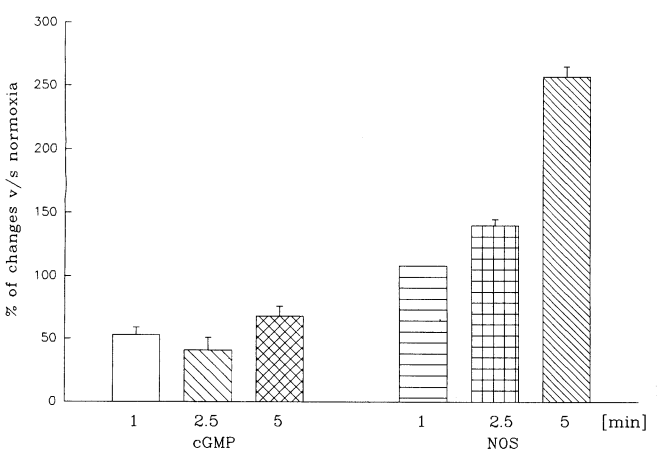

Fig. 1. Ca^{2+} dependent nitric oxide synthase activity and cGMP level in brain hemispheres during different time of brain ischemia. The value represent the means ± SD from 3 experiments carried out in triplicates. All values are statistically significant with $*P<0.05$ *versus* the control value.

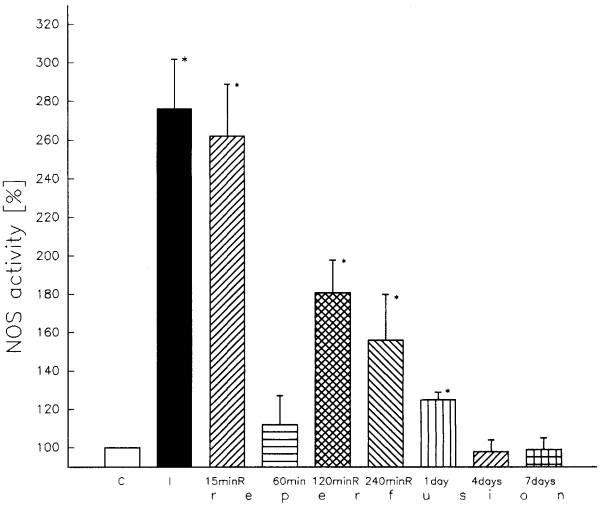

Fig. 2. Ca^{2+}-dependent nitric oxide synthase activity during different reperfusion time after short 5 min global brain ischemia. The activity of enzyme was determined in presence of 2 mM Ca^{2+} and 1 μM calmodulin and other cofactors as described in Methods. The values represent the means ± SD from 5-3 experiments carried out in triplicates. $*P<0.05$ *versus* the control value.

ischemia. This enzyme activity was enhanced by about 260%, 180% and 160% respectively as compared to control values. One day after ischemia the activity of NOS was slightly but significantly higher as compared to control. Four days after ischemia the activity of NOS was similar to control (Fig. 2). At the same reperfusion time a similar biphasic increase of cGMP level was found. The level of cGMP increases by about 100% 15 min after ischemia and by about 60% over control value 2 h after ischemia. Four days and seven days after ischemia the level of cGMP was slightly enhanced, but this change was statistically insignificant as compared to control values (Fig. 3).

Effect of N-nitro-L-arginine on NOS activity and cGMP level in brain subjected to ischemic-reperfusion injury

The specific inhibitor of NOS, N-nitro-L-arginine (NNLA), administered i.p. in a dose of 30 mg /kg b.w. 5 min before ischemia protects the brain

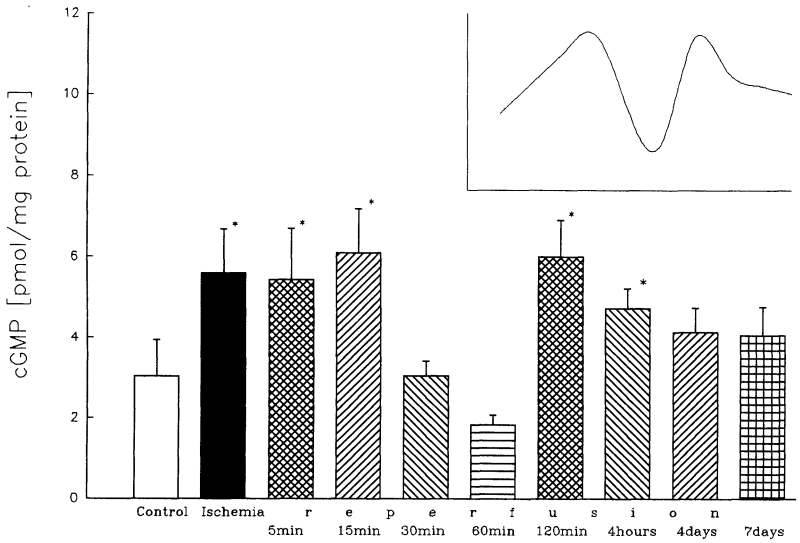

Fig. 3. Cyclic GMP level in brain hemispheres during different reperfusion period after short term of global ischemia. The values represent the means ± SD from 5-3 experiments carried out in triplicates. *P<0.05 *versus* the control value.

against NO liberation not only during ischemia but also during reperfusion time (Fig. 4). Moreover, NNLA reduces significantly the level of cGMP during ischemia and short reperfusion time (Fig. 5).

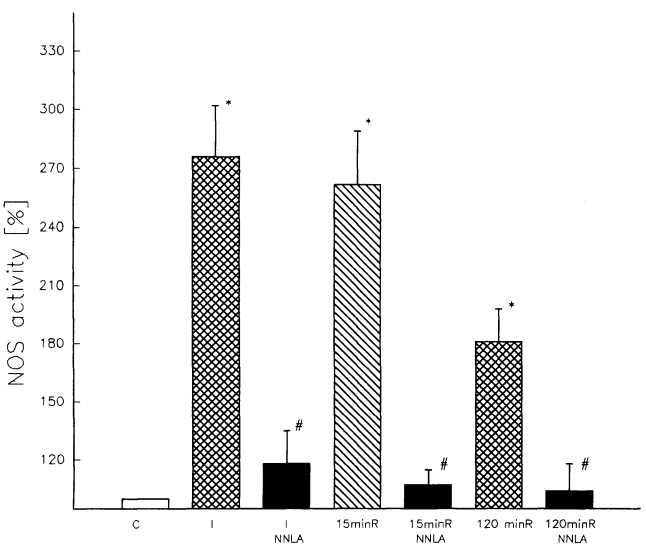

Fig. 4. Effect of N-nitro-L-arginine (NNLA) on ischemia-reperfusion evoked stimulation of NOS activity in gerbils brain. NNLA was applied i.p. in a dose of 30 mg/kg b.w. 5 min before ischemia. C, control; I, ischemia; R, reperfusion. The value represent the mean ± SD from 5-3 experiments carried out in triplicates. *P<0.05 *versus* the control value. #P<0.05 *versus* the ischemia value.

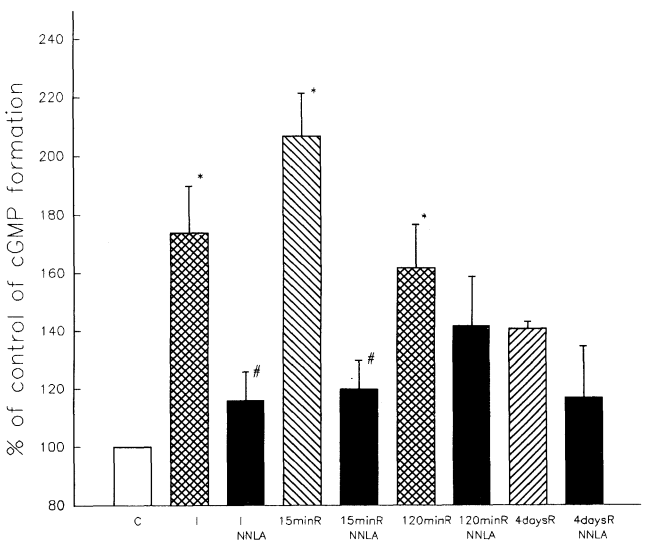

Fig. 5. Effect of N-nitro-L-arginine (NNLA) on ischemia-reperfusion evoked cGMP accumulation in gerbils brain. NNLA was applied i.p. in a dose of 30 mg/kg b.w. 5 min before ischemia. C-control, I-ischemia, R-reperfusion. The values represent the means ± SD from 3 experiments carried out in triplicates. *P<0.05 *versus* the control value. #P<0.05 *versus* the ischemia value.

Action of the neuronal NOS inhibitor 7-NI and the inhibitor of guanyl cyclase LY 83 583 on NOS activity and cGMP level in brain subjected to ischemia-reperfusion injury

The specific inhibitor of the neuronal form of NOS 7-NI administered i.p. 5 min before ischemia

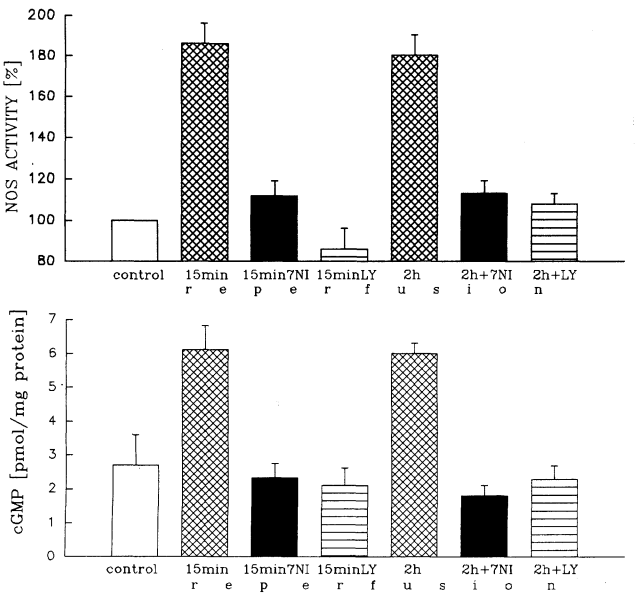

Fig. 6. Effect of neuronal NOS inhibitor 7-Nitroindazole (7NI) and guanyl cyclase inhibitor LY 83 583 (LY) on reperfusion evoked NOS activation and cGMP accumulation. 7 NI in a dose of 25 mg/kg b.w. and LY 83 583 in a dose of 6 mg/kg b.w. were administered i.p. 5 min before induction of ischemia. The values represent the means ± SD from 3 experiments carried out in triplicates. *$P<0.05$ *versus* value of 15 min and 2 h reperfusion without inhibitor.

in a dose of 25 mg/kg b.w. eliminates the effect of ischemia on NOS activity and cGMP level observed after 15 min and 2 h of reperfusion (Fig. 6).

The inhibitor of guanyl cyclase LY83 583 applied i.p. in a dose of 6 mg/kg b.w. 5 min before ischemia decreases not only the level of cGMP in the brain but also the activity of NOS investigated 15 min and 2 h after ischemic insult (Fig. 6).

Effect of hydrocortisone on NOS activity and cGMP level in brain subjected to ischemia-reperfusion and Northern blot analysis of iNOS mRNA

Hydrocortisone was administered subcutaneously in a dose of 40 mg/kg b.w. for 7 days before 5 min ischemia. This glucocorticoid has no effect on cGMP level in normoxic and ischemic brain (Fig. 7). For the better evaluation of the type of NOS isoenzyme activated during the reperfusion time after ischemia mRNA was isolated and Northern blot with cDNA for iNOS was performed. The results of this study presented on Fig. 8 indicated the lack of signal for iNOS in brain hemispheres during the reperfusion. Observations were conducted starting 15 min until 7 days after ischemia.

DISCUSSION

The results of these studies indicate:

- The increase of nitric oxide synthase activity during ischemia and elevation of NO-dependent cGMP formation in brain hemispheres.
- The biphasic increase of the neuronal Ca^{2+}/calmodulin dependent form of NOS and concomitant elevation of cGMP 15 min and 2h-4h after ischemia in brain hemispheres.
-That the specific inhibitors of NOS, NNLA, 7-NI, eliminate enhancements of NO/cGMP level in brain hemispheres evoked by ischemia.

Moreover, the lack of glucocorticoid action on NOS activity and cGMP and the lack of gene induc-

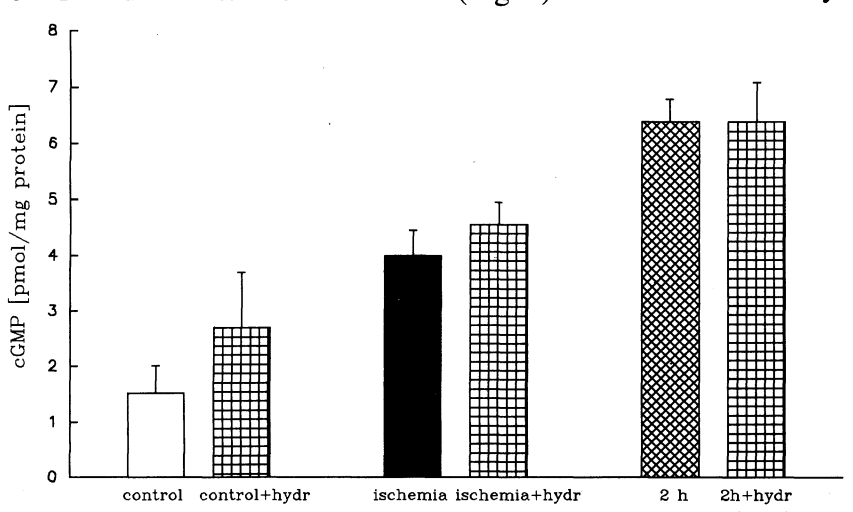

Fig. 7. Effect of hydrocortisone on ischemia-reperfusion evoked cGMP formation in brain hemispheres. Hydrocortisone was applied subcutaneously 40 mg/kg b.w. for 7 days before ischemia. On the last day the animals were treated 2 h before ischemia. The values represent the means ± SD from 6-3 experiments carried out in triplicates.

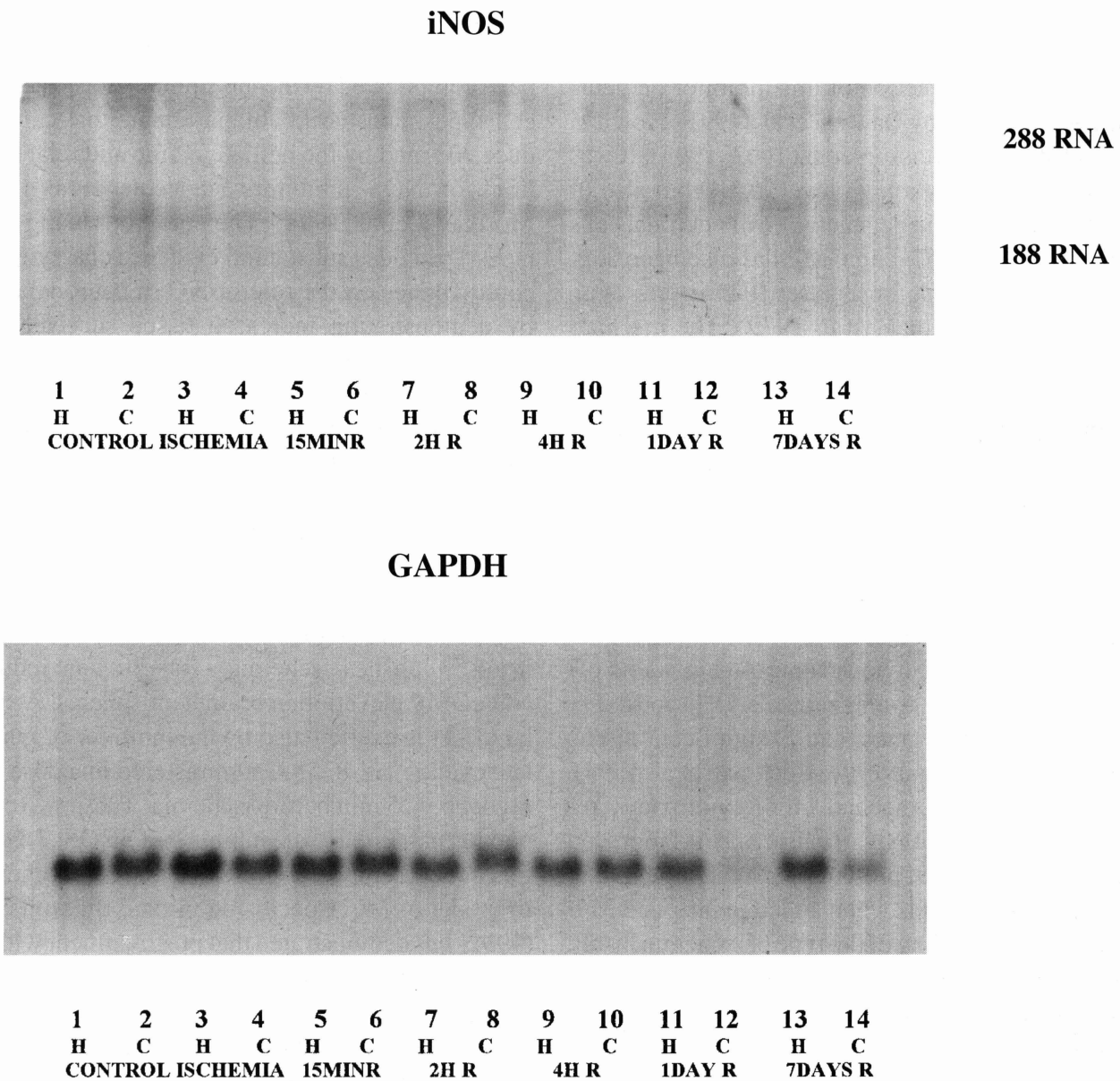

Fig. 8. Northern analysis of iNOS mRNA of brain hemispheres and cerebellum subjected to ischemia-reperfusion injury. H, brain hemispheres; C, cerebellum; R, reperfusion; 1,2 control; 3,4 ischemia; 5,6 15 min R; 7,8 2 h R; 9,10 4 h R; 11,12 1 day R; 13,14 7 days R.

tion for the inducible form of NOS activity during reperfusion time was found.

These results indicate that the level of NO/cGMP messengers is changing during brain ischemia and reperfusion injury mainly as a consequence of cNOS action. However, on the basis of our results we are not able to exclude the involvment of iNOS in some areas of the brain. Nitric oxide is now established as a biological mediator of clinical relevance. It seems that NO/cGMP elevation occurring 2-4 hours after short global ischemia may be mainly responsible for the neurotoxicity. The excessive production of NO and higher level of cyclic GMP may cause disfunction, degeneration and death of neurones. Our previous studies with the specific inhibitor of NOS, NNLA, demonstrated an ameliora-

ting effect of NNLA on ischemia-induced neuronal death in CA_1 layer of hippocampus (Strosznajder et al. 1994). Positive action of this inhibitor on neurones was observed by Dawson et al. (1991), Nowicki et al. (1991),and Buisson et al.(1992, 1993). It was found that not only a high level of NO but also of cGMP plays a role in degeneration of neuronal cells (Roos et al. 1990) Cyclic GMP has also been suggested to have a role in seizures (Ferrendelli et al. 1974, 1980, Freedman et al. 1979). The mechanisms by which NO causes cell death and tissue damage are currently receiving attention. It seems that during reperfusion and also in physiological and pathological brain aging, the interaction of NO with superoxide (O_2-) occurs. This interaction is no longer considered to be simply a means of neutralizing these radicals. This reaction results in generation of a potent oxidant, the peroxynitrite $ONOO^-$ radicals. This compound is stable enough to diffuse to other cells or it can be decomposed in acidic pH into the other two potent radicals OH^- and NO_2, which all together may cause significant tissue damage (Stemler et al. 1992, Beckman et al. 1994). A second potential mechanism of cytotoxicity relates to the ability of NO to cause nitrosylation and ADP-rybosylation of proteins and nucleic acids and to break DNA strands when added to intact cells. It seems that, depending of the type of ischemic insult, its severity and duration, NO may lead to more or less vividly expressed biochemical and morphological abnormalities. In some forms of local ischemia the activation of iNOS was reported in brain vessel wall (Zhang et al. 1993) and glial cells. The recent studies of Simmons and Murphy (1992) Minc Golomb and Schwartz (1994) show that astrocytes and microglia express the inducible form of NOS. Also some other studies present evidence for neuronal inducible NOS (Weber et al. 1994). The cytotoxic effects of NO in brain subjected to ischemia-reperfusion injury were counterbalanced by the proposed cytoprotective effect of NO observed, however exclusively or mainly in *in vitro* studies (Lipton et al. 1993, Wink et al. 1993). Today the therapeutic challenge is to manipulate these NO pathways specifically and selectively.

Previous demonstrations of the beneficial effects of NOS inhibition in *in vivo* ischemia models have been complicated by the multiplicity of NO actions, cell types, and tissue compartments that can produce NO, and by the hemodynamic and related actions of NOS inhibitors (Iadecola et al. 1994, Morikawa et al. 1994). The study of Huang et al. (1994) provides substantial evidence clarifying the controversies on the role of NO in neuronal death by demonstrating increased tissue survival after cerebral ischemia in mutants lacking NO production in neurones.

The former finding is consistent with the data showing that 7-NI, an inhibitor of the neuronal isoform of NOS *in vivo*, decreases infarct size after middle cerebral artery (MCA) occlusion in rats (Yoshida et al. 1994).

Our results also indicate that 7-NI, administered in a dose of 25 mg/kg b.w. 5 min before ischemic insult, abolishes ischemia - reperfusion induced NO/cGMP elevation in brain hemispheres. A similar effect is exerted also by the inhibitor of guanylate cyclase LY 83583, administered in a dose of 6 mg/kg b.w. 5 min before ischemia. These two compounds particularly the inhibitor of nNOS, 7-NI offers novel therapeutic strategies for brain ischemia. In model of MCA occlusion in rats, Buisson et al. (1992) has demonstrated that posttreatment with N-nitro-L-arginine methyl ester (L-NAME) at 3 mg/kg i.p. induced an important neuroprotection at the cortical and striatal levels. In further studies on the mechanisms involved in this neuroprotection, Buisson et al. (1993) suggest the inhibition of NO induced glutamate overflow occurring in ischemic brain. It has been suggested for a long time that the overstimulation of NMDA receptor by excitatory amino acid initiates several phenomena which lead to neuronal death during cerebral ischemia (Choi 1990). Activation of excessive nitric oxide synthesis is one of the major events leading to neuronal damage. In the gerbil model of global brain ischemia, activation of Ca^{2+}/calmodulin dependent NOS seems to be responsible for NO synthesis. An increase in intracellular Ca^{2+} resulting from the activation of voltage-gated Ca^{2+} channels or ligand

gated Ca^{2+} channels or from the mobilization of intracellular Ca^{2+} may activate the enzyme. In our study we were not able to find the activation of iNOS in brain during reperfusion time. On the other hand it is possible that cDNA for mouse iNOS used in our study is not specific enough for gerbil brain iNOS. In spite of that, our studies indicated the lack of NOS activity in the absence of Ca^{2+} and the lack of hydrocortisone effect on NO/cGMP level in brain. The above facts or data bring us to the final conclusion that during ischemia and also during reperfusion period mainly the constitutive neuronal form of NOS is activated.

It seems that biphasic activation of NOS and cGMP production during reperfusion in brain hemispheres may be related to tissue damage. NO production during ischemia may be a physiological response. It is possible that calcineurin/cGMP mediated dephosphorylation-phosphorylation processes may be involved in modulation of NOS activity and NO production during ischemia/reperfusion. NOS is a highly regulated enzyme. It is known that neuronal NOS can be phosphorylated by protein kinase C (PKC), cAMP dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase and probably also by cGMP-dependent protein kinase (Brune et al. 1991, Bredt et al. 1992, Dawson et al. 1994). Phosphorylation by all these kinases decrease enzyme catalytic activity (Bredt et al. 1992, Dawson et al. 1994).

In summary, a significant increase of NOS activity during ischemia and then biphasically during reperfusion time results in a burst of NO production and release of cGMP. The precise biochemical mechanism underlying the activation (inactivation) of NOS as well as the pathophysiological consequences of excessive NO and cGMP formation in postischemic tissue are under the investigation.

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