Inhibitor of Nitric Oxide Synthesis Reduces Hypoxic-Ischemic Brain Damage in the Neonatal Rat

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ABSTRACT. We evaluated the neuroprotective effect of the nitric oxide synthesis inhibitor, *N*^G-nitro-L-arginine in a neonatal hypoxic-ischemic rat model. Unilateral hypoxic-ischemic injury was produced in the brain of 7-d-old rats using a combination of a common carotid artery ligation and a hypoxic (8% oxygen) exposure for 2.5 h. In our experimental condition, rectal temperatures did not differ between *N*^G-nitro-L-arginine-treated and saline-injected pups. We killed the animals 72 h later and assessed the hypoxic-ischemic brain damage histologically. *N*^G-nitro-L-arginine (2 mg/kg) administered intraperitoneally 1.5 h before hypoxia resulted in 77% reduction of the infarcted hemispheric volume and 87% reduction of the infarcted striatal volume compared to saline injected controls. *N*^G-nitro-L-arginine given 1.5 h before the insult also significantly prevented hypoxic-ischemic damage in the five hippocampal structures examined, dentate gyrus, CA4, CA3, CA1, and subiculum. *N*^G-nitro-L-arginine administered immediately after hypoxia did not prevent hypoxic-ischemic brain damage. These results indicate that nitric oxide plays a key role in producing neonatal hypoxic-ischemic brain damage. (*Pediatr Res* 35: 10–14, 1994)

Abbreviations

NMDA, N-methyl-D-aspartate
NO, nitric oxide
NOARG, *N*^G-nitro-L-arginine

Hypoxia-ischemia increases the extracellular concentration of endogenous excitatory amino acids, which trigger a cascade of biochemical events, such as protease, lipase, and protein kinase C activation; free radical formation; and uncoupling of oxidative phosphorylation within mitochondria via an increase in free cytosolic Ca²⁺; this cascade ultimately leads to neuronal death (1). NMDA, a glutamate receptor agonist, plays a major role in the production of hypoxic-ischemic brain damage in the immature rat. Among the excitatory amino acid agonists, intracerebral injection of NMDA produces the greatest degree of brain injury in 7-d-old rats [for review see (2)]. The hypoxic-ischemic brain damage in immature rats is identical in appearance and time course to the cytotoxic reaction to NMDA (3). MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate], a noncompetitive NMDA antagonist, protects against hypoxic-ischemic brain damage in neonatal rats (4).

NO, a free radical gas, is synthesized by the vascular endothe-

lium in the conversion of L-arginine to L-citrulline by the enzyme NO synthase (5), whose activity has also been reported in fore-brain homogenates (6). NMDA stimulates NO synthesis (7), and NO mediates glutamate neurotoxicity *in vitro* (8). NOARG, an analog of L-arginine, competitively inhibits the enzymatic formation of NO both in cultured endothelial cells (9) and cerebellar homogenates (10), also prevents neurotoxicity induced by NMDA in cortical cultures (8), and easily crosses the blood-brain barrier (10).

Contradictory reports concerning the possible neuroprotective effect of NO synthesis inhibitors in adult models (11–16) prompted us to investigate whether NO was related to the genesis of cerebral hypoxic-ischemic brain damage in neonates. Seven-day-old rats were subjected to unilateral carotid artery ligation followed by hypoxic exposure. This neonatal rat model shows infarction of the cerebral hemisphere ipsilateral to the carotid artery ligation (17), and has been frequently used to investigate the pathogenesis of cerebral hypoxia ischemia (for review see Refs. 1 and 18). We investigated the neuroprotective effect of NOARG administered before and after a hypoxic insult.

MATERIALS AND METHODS

We used the method of Rice *et al.* (17) to produce hypoxic-ischemic brain damage in neonatal rats. On the day of surgery, 7-d-old Std:Wister rat (Japan SLC, Inc.) littermates (the day of birth is defined as d 1) were paired according to sex and body weight (± 0.5 g), each pair consisting of a pup assigned to NOARG treatment and a saline-injected control. If one member of a pair died, the other member was discarded. Under ether anesthesia, the left carotid artery was exposed through a midline neck incision, doubly ligated, and severed between the two ligatures. After surgery, the rat pups were allowed to recover for 2.5 to 3.5 h, and at the same time were fasted for 3.5 h before hypoxic exposure by separating them from their dams.

Hypoxic exposure was achieved by placing littermates (8–10 pups) in a 2.0-L air-tight plastic box submerged in a 37°C water bath and flushed for 2.5 h with a humidified 8% oxygen and 92% nitrogen mixture delivered at 1.1 L/min. After 1 h recovery in the 37°C water bath, they were returned to their mothers until killed at the age of 10 d.

The effect of NOARG was assessed in the two groups, each consisting of pairs from at least three litters. In the prehypoxic treatment group (group 1, $n = 12$ pairs), 2 mg/kg of NOARG (Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally 1.5 h before the hypoxic insult. The reason why we chose the dose of 2 mg/kg is that, in preliminary experiments, higher doses of NOARG significantly increased mortality during hypoxia (3 or 4 mg/kg, *ca.* 50%; 8 or 15 mg/kg, *ca.* 100%). Even if the pups survived the insult, they looked moribund and appeared to develop the same extent of brain damage compared to the saline-injected littermates. In the posthypoxic treatment group (group 2, $n = 12$ pairs), 2 mg/kg of NOARG was given

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Table 1. Percent volume of hypoxic-ischemic brain damage*

	<i>n</i>	Cerebral cortex	Striatum
Group 1 (1.5 h prehypoxia)			
Saline	8	59 ± 7	50 ± 9
NOARG	8	13 ± 8†	6 ± 6†
Group 2 (0 h posthypoxia)			
Saline	11	64 ± 5	67 ± 5
NOARG	11	58 ± 6	62 ± 6

* Percent volume = (sum of ipsilateral damaged area/sum of contralateral area) × 100. Data are mean ± SE.

† $p < 0.01$; significant versus corresponding saline-injected control (Mann-Whitney U test).

immediately after the cessation of hypoxia. Each experimental animal received NOARG dissolved in normal saline (5 ml/kg), and its paired littermate was administered the same volume of normal saline.

In rats administered NOARG ($n = 8$) or saline ($n = 8$) 1.5 h before the hypoxic insult, each rat pup underwent rectal temperature monitoring from 1.5 h before hypoxia until 24 h after the cessation of hypoxia at 10 time points (1.5 and 0 h before hypoxia, 1.5 h after the start of hypoxia, 0, 0.5, 1, 2, 3, 4, and 24 h after hypoxia).

The rat pups were perfusion-fixed with 4% formaldehyde buffered to pH 7.40 72 h after the hypoxic-ischemic insult at 10 d of age. The pups were anesthetized with pentobarbital (50 mg/

kg) intraperitoneally, and perfused with heparinized saline followed by neutral-buffered formaldehyde solution via the left ventricle. Each pump was decapitated after perfusion, and the head was stored in the same fixative at 4°C for at least 16 h. Coronal blocks of the brain were processed in a graded series of ethanol and xylene. After embedding in paraffin wax, 8-μm-thick coronal slices were sectioned every 200 μm and stained with hematoxylin-eosin.

Areas of hypoxic-ischemic damage were calculated at six coronal levels (4) selected according to a stereotaxic atlas of the 10-d-old rat brain (19). The coronal levels with longitudinal coordinate and anatomic structure were as follows: A 7.0 mm at the nucleus accumbens, A 5.6 mm at the caudate-putamen and the anterior commissure, A 4.4 mm at the globus pallidus, A 3.2 mm at the anterior tip of the dorsal hippocampus, A 2.0 mm at the middle of the dorsal hippocampus, and A 0.8 mm at the ventral hippocampus. All measurements of area were performed on maps of these six coronal levels, using an image analyzer (LUZEX-2D, Nikon Corporation, Japan) with a magnification factor of about 2200 pixels/mm². The measurements included the cerebral cortex in all six coronal planes and the striatum in two coronal planes at A 5.6 mm and A 4.4 mm, and areas showing hypoxic-ischemic damage in the same region. The percent volume occupied by hypoxic-ischemic necrosis was obtained in the cortex and the striatum by dividing the sum of damaged areas ipsilateral to the carotid ligation by the sum of the contralateral areas of each structure.

The hypoxic-ischemic changes of five hippocampal structures,

Fig. 1. Coronal brain sections showing the middle of the dorsal hippocampus in the coronal plane A 2.0 mm according to an atlas of 10-d-old rat brain (13). *A* and *B* are saline-injected controls 1.5 h before and immediately after hypoxia, respectively. *C* and *D* are pups administered NOARG intraperitoneally 1.5 h before and immediately after hypoxia, respectively. A neuroprotective effect is observed in *C*. Hematoxylin-eosin stain; original magnification ×14.5.

Fig. 2. Amount (mm^2) of hypoxic-ischemic necrosis in neonates in different coronal planes of the cerebral cortex and striatum in group 1 in which NOARG was administered 1.5 h before the insult. There are significant differences ($p < 0.01$; Mann-Whitney U test) between saline-injected controls and NOARG-treated pups in all coronal planes examined in both cerebral cortex and striatum. Data represent mean $\pm$ SE ($n = 8$ pairs).

dentate gyrus, CA4, CA3, CA1, and subiculum, in the dorsal hippocampus in the coronal plane at A 2.0 mm were assessed according to a semiquantitative 4-point scale (4): 0, no damage; 1, rare to occasional eosinophilic neurons (approximately less than 10%); 2, moderate number of eosinophilic neurons (approximately 10 to 50%); and 3, frequent eosinophilic neurons (greater than 50%). Light microscopic examination was performed with the examiner blinded to the experimental protocol. The mean value from ipsilateral hippocampi evaluated independently was obtained from each animal.

Data are presented as mean $\pm$ SE. The mortality rate of the

animals was evaluated by two-tailed Fisher's exact probability test, and differences in body weight and rectal temperature were evaluated by two-tailed unpaired t test. The extent of the hypoxic-ischemic volume and area in the cortex and the striatum, and that of the neuronal damage score in the hippocampus were not distributed normally; therefore, they were tested by the two-tailed Mann-Whitney U test. A probability of less than 0.05 was considered significant.

RESULTS

The mortality rates during hypoxia did not differ significantly in the groups with prehypoxic treatment with NOARG (16.7%, 2 of 12) and saline (25.0%, 3 of 12), or in the groups with posthypoxic treatment with NOARG (7.1%, 1 of 12) and saline (0%, 0 of 12). There was no significant body weight difference between NOARG-treated pups and saline-injected controls at d 7 or d 10; from 10.5 ± 0.2 g on d 7 to 12.2 ± 0.5 g on d 10 in NOARG-treated pups *versus* from 10.5 ± 0.2 g on d 7 to 11.0 ± 0.3 g on d 10 in saline-injected controls in group 1 ($n = 8$ pairs), and from 10.3 ± 0.3 g on d 7 to 10.5 ± 0.5 g on d 10 in NOARG-treated pups *versus* from 10.3 ± 0.2 g on d 7 to 9.8 ± 0.3 g on d 10 in saline-injected controls in group 2 ($n = 11$ pairs). Rectal temperatures did not differ between NOARG- and saline-pre-treated pups at any points examined.

Cortical and striatal lesions with hypoxic-ischemic changes were sharply demarcated and were easily delineated using the image analyzer. The percent volume of hypoxic-ischemic damage in the cerebral cortex and striatum ipsilateral to the carotid artery ligation relative to the contralateral cerebral cortex and striatum is shown in Table 1. The mean percent volume of hypoxic-ischemic brain damage of the NOARG-pretreated pups showed 77% reduction in the cerebral cortex and 87% reduction in the striatum relative to that of the saline-injected controls. However, there was no protective effect in the NOARG-post-treated pups, both in the cerebral cortex and the striatum (Table 1). The hypoxic-ischemic lesions in the ipsilateral hemisphere were distributed, in order of decreasing damage intensity, in the cerebrum with preservation of a small rim of cortex dorsomedially, the hippocampus, the striatum, the corpus callosum, and the thalamus (Fig. 1), and when NOARG was effective, the protection was seen in all coronal sections examined both in the cerebral cortex and the striatum (Fig. 2).

Hypoxic-ischemic damage of the hippocampus in this current study was so severe that in some of the control rats these neurons showed widespread necrosis and were replaced by vacuoles. Pretreatment with NOARG attenuated the hypoxic-ischemic neuronal damage in all five substructures of the ipsilateral hippocampus, especially in the dentate gyrus and the CA1 region. No protective effect was observed with posthypoxic treatment with NOARG in all five substructures (Table 2).

DISCUSSION

Our results demonstrate that, in the neonatal period, treatment with an inhibitor of NO synthesis before an episode of cerebral

Table 2. Semiquantitative ischemic neuronal scores of the ipsilateral hippocampal substructures in the neonatal hypoxic-ischemic model*

	<i>n</i>	DG	CA4	CA3	CA1	SUB
Group 1 (1.5 h prehypoxia)						
Saline	8	2.9 ± 0.1	2.5 ± 0.2	3.0 ± 0	2.8 ± 0.2	2.8 ± 0.2
NOARG	8	$1.3 \pm 0.4^\dagger$	$0.8 \pm 0.4^\ddagger$	$1.9 \pm 0.4^\ddagger$	$0.9 \pm 0.3^\dagger$	$1.3 \pm 0.4^\ddagger$
Group 2 (0 h posthypoxia)						
Saline	11	3.0 ± 0	2.9 ± 0.1	3.0 ± 0	2.9 ± 0.1	3.0 ± 0
NOARG	11	2.7 ± 0.1	2.7 ± 0.1	3.0 ± 0	2.8 ± 0.1	2.9 ± 0.1

* Abbreviations are: DG, dentate gyrus; SUB, subiculum; CA1-4, cornu ammonis fields 1 to 4. The number of eosinophilic neurons in each structure was assessed semiquantitatively as follows: 0, no damage; 1, <10%; 2, 10 to 50%; 3, >50% eosinophilic neurons. Data are mean $\pm$ SE.

$^\dagger p < 0.01$.

$^\ddagger p < 0.05$; significant *versus* corresponding saline-injected control (Mann-Whitney U test).

hypoxia-ischemia provides marked protection against brain damage. However, therapy started immediately after the cessation of hypoxia failed to protect against brain damage. These results indicate that NO synthesis in brain tissue plays a critical role in the development of neonatal hypoxic-ischemic brain damage.

Glutamatergic innervation of the striatum (20) and density of NMDA binding sites in the hippocampus (21) peak at around the 7th postnatal day. This transient overinnervation coincides with the enhanced neurotoxicity of microinjected NMDA and the enhancement of hypobaric-ischemic damage in the immature rat brain (2, 22). Activated NMDA receptors increase free cytosolic Ca^{2+} , which stimulates NO synthase (23) with binding to calmodulin (24). NO, which is a free radical gas in itself, rapidly reacts with superoxide to form the stable peroxynitrite anion. Once protonated, peroxynitrite decomposes and generates a strong oxidant with reactivity similar to that of the hydroxyl radical (25). In the present model, prevention of the neurotoxicity of the formed free radicals after hypoxic-ischemic damage is one of the neuroprotective effects of the inhibitor of NO synthesis. Allopurinol decreases superoxide radicals by inhibition of xanthine oxidase, and can thereby reduce perinatal hypoxic-ischemic brain damage (26).

Neuronal NADPH diaphorase-containing neurons, which are selectively preserved 1 wk after hypoxic-ischemic injury in neonatal pups (27), are identical to NO synthase-containing neurons (28). NADPH diaphorase-containing neurons are also relatively resistant to damage by low concentrations of NMDA agonists (29). Why these neurons are selectively resistant to neurotoxic insults has not been fully elucidated. The diaphorase activity of NO synthase may be associated with this neuroprotection. In addition, NO synthase-containing neurons are enriched in manganese superoxide dismutase (30) which could be protective by removing a superoxide anion that produces a strong oxidant with NO. Alternatively, the present evidence, which indicates possible effects of NO in both oxidizing a redox modulatory site consisting of thiol groups on the NMDA receptor-channel complex (31) and decreasing NMDA-mediated increase in intracellular Ca^{2+} (32), may represent a feedback inhibitory mechanism for NO production. In the presence of high concentrations of NMDA agonists, NADPH diaphorase-containing neurons, which are the source of neurotoxic NO (33), can release large amounts of NO (34) to kill nearby neurons, and may consequently produce widespread neuronal damage (29).

Glyceraldehyde-3-phosphate dehydrogenase, an important enzyme in the glycolytic pathway, is inactivated by ADP-ribosylation induced by NO (35). Because glucose supplementation is beneficial to the perinatal brain exposed to hypoxia-ischemia (36, 37), in contrast to adult animals, inhibition of NO synthesis may preserve the glycolytic pathway during the hypoxic-ischemic insult and may ameliorate the ultimate brain damage. Alternatively, NO inhibits both mitochondrial enzymes including complex I (NAD[P]H:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase) and aconitase leading to subsequent inhibition of mitochondrial respiration, and the rate-limiting enzyme in DNA replication, ribonucleotide reductase, leading to inhibition of DNA synthesis in the macrophage effector cells (38). All of these enzymes have a catalytically active nonheme iron-sulfur center which binds to NO leading to intracellular iron loss. Therefore, NO could mediate cytotoxicity by inhibiting mitochondrial functions in the hypoxic-ischemic brain (present study), as well as that of macrophages (39). There are at least two distinct NO synthases. One is the constitutive, Ca^{2+} /calmodulin-dependent enzyme, which releases NO for short periods in response to receptor activation in neurons, endothelial cells (40), and possibly in astrocytes (41). The other is the inducible form, which is induced by immunological stimuli and synthesizes NO Ca^{2+} independently over a period of hours to days in macrophages (42). Both NO synthases are soluble, require NADPH, form citrulline as a coproduct, and are competitively inhibited by L-arginine analogs. Recently, it was elucidated that

glial cells of the central nervous system could express an inducible form of NO synthase, which may contribute to hypoxic-ischemic brain damage (43). The reason why in several studies (13, 14, 16) it failed to protect the brain from the ischemic insult with L-arginine analogs administered for a short time may be that the inducible form of NO synthase could not be inhibited completely. However, the reason why prehypoxic treatment with one dose of NOARG could protect against hypoxic-ischemic brain damage in our study is unknown.

A possible explanation for the lack of effect of NOARG administered after the insult is that NO synthase activated by hypoxic stimuli could not generate NO because of the lack of molecular oxygen, which both constitutive and inducible NO synthases incorporate into NO. However, NO synthases are at once fully activated upon return of the rat to room air, and can immediately generate a burst of NO, which cannot be blocked by NOARG. Effective levels of NOARG would not be reached because the peritoneal vascular beds are vasoconstricted and absorption is slow. The lack of posthypoxic effect of the NO synthase inhibitor is not in accord with the neuroprotective effect of posthypoxic treatment with the NMDA receptor antagonist, MK-801, in another neonatal rat model of hypoxic-ischemic brain damage (4).

Hypothermia can reduce ischemic brain damage both in adult rats (44) and immature rats (45). Postischemic treatment with NOARG reduces the area of cerebral infarction in adult mice (11) and rats (12) subjected to middle cerebral artery occlusion. N^G -nitro-L-arginine methyl ester, an inhibitor of NO synthesis, could reduce body temperature. When the body temperature was maintained at 37°C, N^G -nitro-L-arginine methyl ester administered 30 min before and 30 min after middle cerebral artery occlusion could not protect against ischemic damage in the cerebral hemisphere (13). In our study, each pup administered either NOARG or saline was kept in an atmosphere maintained at 37°C from soon after carotid artery ligation to 1 h after the hypoxic insult. There were no significant differences in rectal temperature between NOARG- and saline-pretreated pups from the time of treatment until 24 h after the cessation of hypoxic exposure. Therefore, the possible induction of hypothermia by NOARG is not able to explain its protective effect in this model of neonatal hypoxic-ischemic brain damage.

NOARG is reported to cause a pressor effect and bradycardia (46), and to decrease cerebral blood flow (47). It is not feasible to cannulate vessels in these small animals; therefore, whether circulatory effects of NOARG contribute to the neuroprotection is not clear. The arginine analogs could also inhibit the mitochondrial electron transport system by inhibiting the reduction of ferric cytochrome C (48). However, if NOARG had decreased the cerebral blood flow and inhibited the mitochondrial electron transport, it could have worsened the brain damage in our model.

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