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L-NAME Reduces Infarct Volume in a Filament Model of Transient Middle Cerebral Artery Occlusion in the Rat Pup

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ABSTRACT

The importance of nitric oxide (NO) during focal cerebral ischemia remains controversial as studies have suggested both a neurotoxic and neuroprotective role. In the 7 d old rat pup, N^G-nitro-L-arginine, a nitric oxide synthase inhibitor, reduced infarct volume in a model of unilateral carotid ligation with 2.5 h exposure to 8% O_2. The current study examined whether NO is neurotoxic in a filament model of transient middle cerebral artery occlusion (MCAO) in the 14–18-d-old rat pup. We developed a reproducible filament model of transient MCAO in 14–18-d-old spontaneously hypertensive rats (35 g) by passing a no. 6-0 (0.07-mm) nylon filament via the carotid artery to occlude the middle cerebral artery for 4 h under normoxic conditions. After filament removal and reperfusion for 24 h, we determined infarct volume using the mitochondrial stain 2,3,5-triphenyltetrazolium chloride. NO synthesis was inhibited using N^G-nitro-L-arginine methyl ester (L-NAME) at a dose of 3 mg/kg, intraperitoneally, 1 h before MCAO. We measured infarct volume in control ($n = 7$) and L-NAME ($n = 7$) groups. L-NAME reduced infarct volume by 55% ($p < 0.01$). In the control group, infarct volume (180 ± 29 mm^3) averaged 49 ± 7% of the left hemisphere (359 ± 16 mm^3). In the L-NAME-treated group, infarct volume (77 ± 19 mm^3) was 22 ± 5% of the left hemispheric volume (344 ± 2 mm^3). These findings support earlier studies that used models of neonatal hypoxic-ischemic brain injury and suggest a neurotoxic role of NO. They extend these observations by demonstrating a significant reduction in infarct volume in a stroke model in the immature rat pup. (***Pediatr Res* 38: 652–656, 1995**)

Abbreviations

CBF, cerebral blood flow
L-NAME, N^G-nitro-L-arginine methyl ester
MCAO, middle cerebral artery occlusion
NO, nitric oxide
NOS, NO synthase
SHR, spontaneously hypertensive rat
TTC, 2,3,5-triphenyltetrazolium chloride
NMDA, *N*-methyl-D-aspartic acid

Hypoxic-ischemic injury of the nervous system has been attributed, in part, to the neuronal release of excitatory amino acids. Recent studies suggest that this type of injury involves glutamate-induced neurotoxicity which might, to some degree, be mediated by NO (1–3). However, the role of NO remains controversial as there are now more than a dozen studies since 1991 which suggest either a neurotoxic or neuroprotective role of NO during ischemia (4–7). Endothelial NO release during ischemia might be beneficial by increasing CBF; alternatively, neuronal NO release during ischemia or reperfusion might be neurotoxic.

The role of hypoxic-ischemic excitotoxic neonatal cerebral injury has also received increasing attention. Demonstration of the role of glutamate release and NMDA receptor activation during injury is now well established (8). Of interest is the observation that neonatal hypoxic-ischemic injury selectively spares NADPH-diaphorase-staining neurons that have been shown in adult models to synthesize NO (2, 9). In 7-d-old rat pups, inhibition of NOS with N^G-nitro-L-arginine (50–100 mg/kg) reduced hemispheric weight disparity in a model of left common carotid ligation with 8% O2 and suggests that NO was neurotoxic (10). More recently, Hamada *et al.* (12) found a 77% reduction in cortical and an 88% reduction in striatal infarct volume using a similar model (N^G-nitro-L-arginine, 2 mg/kg intraperitoneally) in 7-d-old rat pups with infarct volume determined by hemotoxylin and eosin staining.

We developed a reproducible transient MCAO stroke model in 14–18-d-old SHR pups and in the present study examined whether low dose NO inhibition with L-NAME (N^G-nitro-L-arginine methyl ester) affects cerebral infarct volume. We developed this model to reflect the physiologic events that occur during stroke due to cerebrovascular occlusion (11).

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With this filament model of MCAO there is minimal surgical trauma resulting in better preservation of cerebrovascular and endothelial function in contrast to doing a craniotomy that involves temporary surgical ligation of the middle cerebral artery, a procedure that is quite difficult. We used L-NAME, a nonspecific endothelial and neuronal NOS inhibitor administered intraperitoneally in low doses (3.0 mg/kg) 1 h before the onset of 4-h MCAO and 24-h reperfusion because our previous studies demonstrated that this dose reduced infarct volume in the adult SHR by 55% (13). Although the 14–18-d-old rat pup model of transient MCAO may not be considered a true neonatal model, it is a model involving the immature brain that reflects changes occurring with development.

METHODS

Surgical preparation. We used 14–18-d-old SHR pups (n = 14) whose weight averaged 35 g. In our previous studies we used adult SHR (weight = 400 g) because we have been able to create an extremely reproducible infarct using either a craniectomy (3 h MCAO and 2 h reperfusion) or a filament model (2 h MCAO and 24 h reperfusion) (13, 14). In preliminary studies, we were able to produce a reliable infarct in these pups but animal size limitations precluded doing these studies in younger animals (11, 15).

SHR pups were anesthetized with 2% isoflurane (Forane) by mask and allowed to breathe spontaneously. End tidal isoflurane concentration (1.2%) was determined with a Puritan-Bennett Datex Capnomac infrared anesthetic gas analyzer as previously reported (16, 17). Body temperature was maintained at 37°C throughout the experimental procedure, and the rat pups were maintained during the 24-h period of reperfusion in a temperature-controlled environment at 95–100°F. The temperatures were rechecked at 24 h and were similar in both control and experimental groups.

Establishment of reversible focal cerebral ischemia. SHR pups were placed in the supine position on the platform of a Zeiss stereoscopic microscope (model #OMP1), and the left common carotid artery was exposed. The left external carotid artery was isolated and ligated. A tie was loosely placed around the internal carotid artery to control backflow, and a vascular clamp was placed on the common carotid artery 1 cm proximal to the bifurcation. A small incision was made in the external carotid artery close to the ligature and a no. 6–0 (0.07 mm) nylon filament passed proximally into the internal carotid artery to transiently occlude the middle cerebral artery. The filament was then sutured in place, the clamp on the common carotid artery removed, and the skin closed with 3-0 silk. The duration of this procedure was 20 min. The pups were allowed to recover from anesthesia and were returned to their litters and maintained in a normoxic environment for 4 h. After this period of normoxic MCAO, the pups were reanesthetized, the filament carefully removed, the external carotid artery ligated, and the wound closed. The pups were then returned to their litters for 24 h after which time they were killed so that infarct volumes could be determined.

Effect of L-NAME on cerebral infarct volume. We examined whether L-NAME (3 mg/kg, intraperitoneally; n = 7) given 60 min before 4-h MCAO and 24-h reperfusion reduced infarct volume compared with control animals (n = 7) given an equal volume of Ringer's lactate solution. The dose of L-NAME and time period were chosen to ensure that the inhibitory effects of L-NAME on vascular and parenchymal NO synthesis were optimal during the period of occlusion and were based on previous pharmacokinetic studies that showed that the onset of the L-NAME effect begins within 30 min of infusion (18, 19).

Measurement of infarct volume. Neuropathologic evaluation of brain injury was accomplished using the histochemical stain TTC as previously reported by us and other investigators (13, 20–24). In normal brain, TTC is converted by mitochondrial oxidative enzymes to a red-formazan product, resulting in a deep red staining of brain parenchyma. Prolonged ischemia renders mitochondrial enzymes dysfunctional and causes failure of TTC conversion, resulting in a pale area in the brain. Several recent studies have shown good correlation in the measurement of infarct volume using TTC or conventional neuropathology at 24 h in the rat MCAO model (22, 23).

At the end of the 24-h reperfusion period, the pups were placed in an induction chamber, deeply anesthetized with 4.0–4.5% Forane, after which a 24 gauge catheter (Insyte, Becton Dickinson) was inserted into the left ventricle, and a 2% TTC solution prewarmed to 37°C in isotonic saline was infused (4 mL/min) for 8 min with effluent released through an incision in the right atrium. An additional 10 min for staining was allowed before fixation with a 10% buffered formalin/10% gluteraldehyde solution (1:1) using the same rate and volume of infusion. The brain was then removed, embedded in an egg albumin:gelatin medium, and mounted on a vibratome (Vibratome Series 1000; Technical Products International Inc., St. Louis, MO). Serial slices in 1.0-mm increments spanning the area of middle cerebral artery distribution were cut, and seven standardized coronal brain sections were selected and photographed with color slide film (Ektachrome, Tungsten 160 ASA). The photographs were analyzed using a Drexel/DUMAS Image Processing System and the area of deficient TTC staining determined manually by an observer blinded to treatment assignment. For each section, the volume of deficient staining and the volume of the entire hemisphere were determined, and a percent infarct volume was calculated. To estimate the percent infarct volume for the entire hemisphere, the infarct volumes and total hemisphere volumes were separately added, and the ratio was calculated. Because edema may alter the measurement of infarct volume, we corrected for this by using the previously published method of Swanson *et al.* (25).

Data analysis. The percent volume of infarction in control and L-NAME-treated animals was compared using a factorial one-way analysis of variance with two independent groups. Comparison of the seven sectional infarct volumes between control and L-NAME groups were analyzed with a repeated measures two-way analysis of variance. Throughout the text, results are given as the mean ± the SEM, and n refers to the number of animals. Unless otherwise specified, statistical significance was assumed at the $p < 0.05$ level.

RESULTS

L-NAME significantly reduced infarct volume by 55% ($p < 0.01$). In the control group ($n = 7$; weight = 35.6 ± 1.2 g), infarct volume (180 ± 29 mm^3) averaged 49 ± 7% of the left hemisphere (359 ± 16 mm^3). In the L-NAME-treated group ($n = 7$; weight = 35.5 ± 1.4 g), infarct volume (77 ± 19 mm^3) was 22 ± 5% of the left hemispheric volume (344 ± 2 mm^3). In addition, the left hemisphere volume in the control group was 4.6% greater than that observed in the L-NAME group and provides additional supportive data that L-NAME reduced neurotoxicity. In Figure 1 a representative section of a control and L-NAME section are shown, demonstrating the volume of mitochondrial pallor that reflects focal ischemic injury. Figure 2 depicts the seven serial cross-sections from the SHR pup brain with the black shaded area indicating the mean volume of infarction for that section. Significant differences were observed between control and L-NAME groups for sections two to seven ($0.009 < p > 0.03$; p value for section one = 0.12).

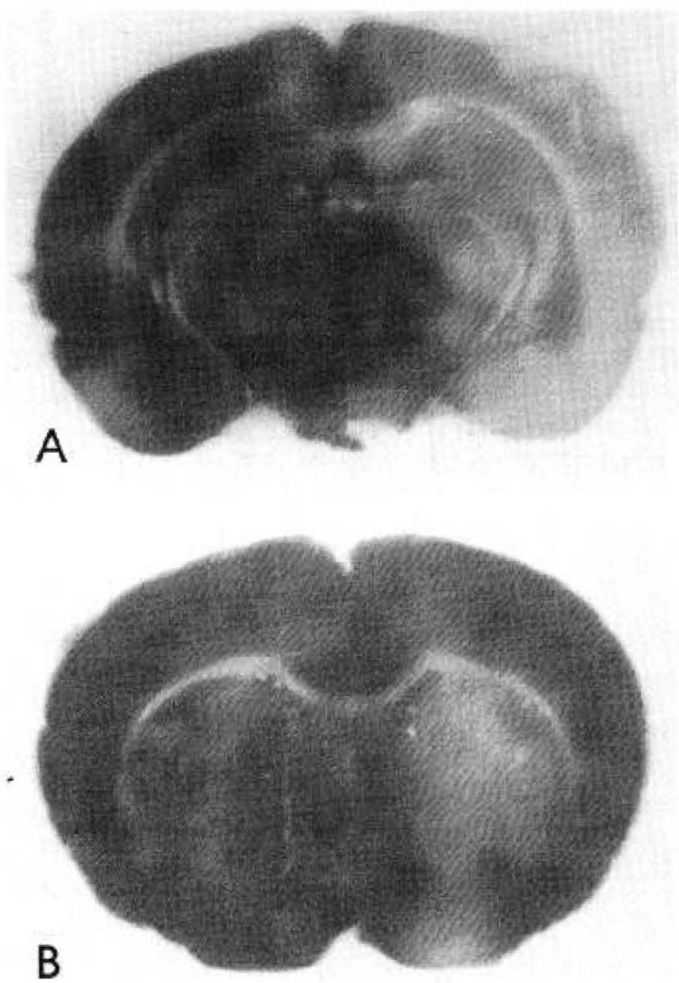

Figure 1. Coronal TTC-stained brain sections demonstrating an area of pallor indicating ischemic injury in the distribution of the middle cerebral artery from a control (*A*) and a L-NAME-treated (*B*) rat pup. The area of pallor includes cortical and caudoputamenal regions. The area of injury measured 68% of hemispheric area in the control section and 32% in the L-NAME section.

DISCUSSION

The present study demonstrates that inhibition of NOS using low dose L-NAME reduced infarct volume in a filament model of transient MCAO in the 14–18-d-old rat pup. These findings support previous studies that have suggested that NO may be neurotoxic in the developing nervous system (10, 12).

NO and models of focal cerebral ischemia. Studies examining the role of NO during focal cerebral ischemia have been difficult to interpret as the results have been conflicting (4–7). As yet, no unifying theory has been proposed that can reconcile these contradictory findings with the dual nature of NO as both a vasodilator and neurotoxin. Experimental variables such as the model used to create ischemia (permanent *versus* focal), the dose and specific NOS inhibitor administered, and the duration of MCAO and reperfusion have all affected the results and thus the interpretation of the role of NO. Studies by several investigators have suggested that NO release during ischemia might increase CBF (4, 5). Our recent studies (13) as well as those of Huang *et al.* (26) have provided convincing evidence that although NO release during focal ischemia is likely to increase CBF and potentially reduce infarct volume, the overall effect of NO is that of serious neurotoxicity.

Glutamate and NO neurotoxicity in the newborn. There is substantial evidence that glutamate toxicity may be important in the developing brain (8, 27, 28). Marked elevation of extracellular glutamate and aspartate occur in the striatum and cortex during fetal lamb asphyxia and parallel the severity of asphyxia in a neonatal rat model of focal cerebral ischemia (29, 30). In addition, the neuropathologic progression after hypoxic-ischemic injury in the neonate is indistinguishable from that due to excitotoxic damage due to glutamate administration (31). It has also been shown that MK-801, a NMDA receptor antagonist, can block hypoxic-ischemic injury in the neonatal rat (28).

As glutamate and other excitatory neurotransmitters are suspected of playing a role in the neurotoxicity associated with hypoxic-ischemic brain injury, it has been suggested that NO might be one of the agents that mediates this response (1, 2). NO is formed in response to NMDA receptor activation by excitatory neurotransmitters such as glutamate (32). NOS inhibitors decrease brain NO production and also prevent NMDA neuronal toxicity in rat cortex, caudate-putamen, and hippocampal cell culture studies as well as *in vitro* in the hippocampal slice preparation and *in vivo* in the hippocampus (2, 33–35). Finally, NOS inhibition with L-NAME, has been shown to reduce infarct volume produced by NMDA receptor activation by 35% (36).

The relation between glutamate neurotoxicity and NO during development remains complex. Each of the glutamate receptor subtypes possess unique developmental neurotoxicity profiles (37). In the 7-d-old rat pup glutamate neurotoxicity is mediated primarily by NMDA and quisqualate/α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor activation in contrast to the adult rat where kainate is more neurotoxic. Moreover, glutamate susceptibility in different brain regions and neuronal populations changes significantly with development. In addition, both cytosolic and particulate forms

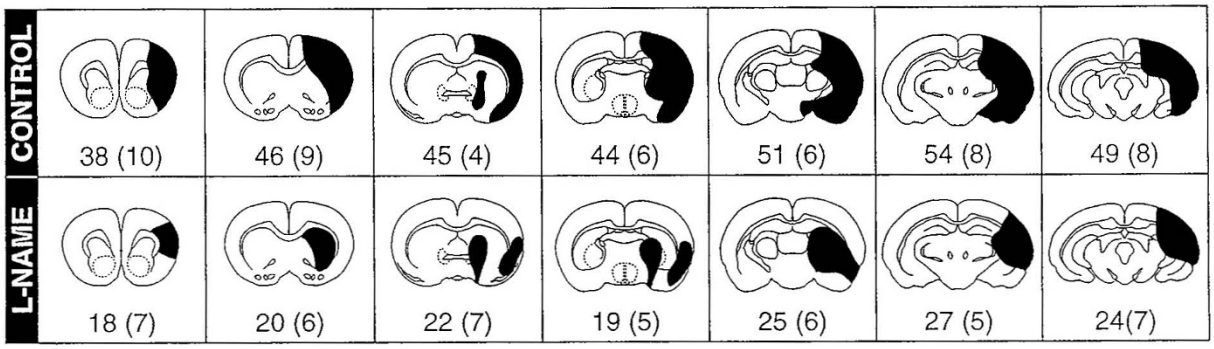

Figure 2. Seven representative cross-sections from the brain of control ($n = 7$) and L-NAME-treated ($n = 7$) groups are depicted from anterior (*left; section one*) to posterior (*right; section seven*). The darkened areas represent the mean percent volume of infarction for that group at that particular cross-sectional level. The distribution of infarct that is outlined was based on the results of our determination of the areas of infarction for each sectional level. The mean (± SEM) percent volume of infarction are given under each section. L-NAME significantly reduced infarct volume in *sections two* to *seven* ($0.009 < p > 0.03$; p value for *section one* = 0.12).

of NOS change with development (38, 39). In rat cerebrum, particulate NOS increases in the first postnatal week, then decreases and becomes almost undetectable in the adult. In contrast, cytosolic NOS increases somewhat in the rat cerebrum and about 8-fold in the cerebellum over the same time frame. Irrespective of these complex developmental changes, the fact that NOS inhibition reduced infarct volumes in 7- (12) and 14–18-d-old rat pups (current study) suggests a relation between focal ischemic injury, NO synthesis and neurotoxicity.

NO neurotoxicity during focal cerebral ischemia in the developing brain. Several mechanisms have been suggested to explain NO-mediated neurotoxicity including free radical induced damage to proteins, DNA, and membrane lipids; direct inactivation of enzymes involved in mitochondrial respiration; and energy depletion subsequent to activation of poly-ADP ribose synthase after NO-dependent damage (7). The relative contributions of any of these mechanisms in either focal or global neonatal hypoxic-ischemic injury have yet to be investigated. Of interest is the observation that NOS expression (and presumably NO production) may play an important role in the regulation of axonal projections during development (40). This may be of particular importance during focal cerebral ischemia as recent studies have shown not only an increase in NO immediately after occlusion but a delayed more marked increase in NO production that peaks at 24 h and lasts for several days (41). Thus, NO neurotoxicity could persist long after the insult and might have a subtle but definite impact on neuronal and glial development.

A new model of stroke in the immature rat pup. The present study demonstrates the feasibility of adapting an adult model of reversible MCAO without craniectomy to the rat pup. The desire to develop a reliable rat pup model of reversible focal ischemia was based on the limitations of currently available models of focal cerebral ischemia in the newborn animal. The Rice-Vannucci model of unilateral carotid ligation with 2–3 h of exposure to 8% O_2 has been used in the rat to examine a variety of questions related to the pathogenesis of neonatal hypoxic-ischemic injury (11, 42). Although, the lesion created with this model is unilateral, it is primarily a model of a diffuse hypoxic-ischemic hemispheric insult rather than one due to focal occlusive cerebrovascular disease (*e.g.* neonatal stroke) as is the filament MCAO model.

Since 1986, several studies have been published describing filament models of reversible MCAO without craniectomy in the adult rat (43–46). Infarct volume (TTC) averaged 37.6% with permanent MCAO and 21.9% after 2 h and 25.7% after 4 h of reversible MCAO (45). A more recent study found that CBF was reduced to near constant values at 20, 60, and 120 min of occlusion and averaged 10% of controls in the area of irreversible infarction and 15–20% in the penumbral region (46). With 24-h permanent MCAO, infarct volume measured 70% in cortex and caudoputamen; with 1-h reversible MCAO infarct volume at 7 d measured 30–35%.

The volume of infarct involving cortical as well as caudoputamenal regions that we observed in our 14–18-d-old rat pup model is similar to that noted in the above studies. Developing a newborn model of reversible MCAO without craniectomy was technically challenging. In contrast to adult rats that weighed 350–400 g, 14–18-d-old rat pups average 35 g. The filament diameter and length are substantially smaller than that used in adults. In these animals it is also difficult to cannulate the femoral artery for measurement of different physiologic variables or the femoral vein for drug infusions and intraperitoneal injections are usually necessary.

A limitation of this filament model is the age of the rat pups when a stroke can reliably be created. Unilateral carotid ligation with 8% O_2 can be accomplished in the rat pup after 7 d of age (42). In the filament model of MCAO without craniectomy, size limitations preclude performing this procedure before 14 d (35 g). Thus, there are limitations in considering this a model of neonatal stroke as many biochemical, physiologic, and anatomical changes occur in the rat pup between d 7 and d 14–18 (38, 39). These two models of cerebrovascular injury (*i.e.* unilateral carotid ligation with 8% O_2 *versus* normoxic filament MCAO), however, can be considered complementary as they examine two different types of cerebral insults (hypoxic-ischemic injury *versus* stroke) that occur in the neonatal period. The clinical relevance for developing a model of neonatal stroke has become apparent over the past decade as neuroimaging studies have convincingly demonstrated that

such lesions are more common than previously recognized and account for serious neurologic morbidity. Although the incidence is uncertain, recent studies have suggested that neonatal stroke accounts for up to 20% of patients with neonatal seizures, and other investigators have found a 5% incidence of neonatal stroke at autopsy (47).

CONCLUSIONS

L-NAME reduced infarct volume by 55% in a filament model of transient focal cerebral ischemia in the SHR pup. These findings are similar in magnitude to that which we observed in the adult SHR using the same dose of L-NAME (13). They also support earlier studies that used models of neonatal hypoxic-ischemic brain injury and suggest a neurotoxic role for NO in the developing nervous system.

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