The Selective Inhibitor of Neuronal Nitric Oxide Synthase, 7-Nitroindazole, Reduces the Delayed Neuronal Damage Due to Forebrain Ischemia in Rats

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Background and Purpose—The present study was designed to investigate whether neuronally derived nitric oxide (NO) plays a toxic role in the cascade of cellular events triggered by global cerebral ischemia in rats.

Methods—7-Nitroindazole (7-NI) was used as a selective inhibitor of neuronal NO synthase. Global ischemia was induced for 20 minutes in anesthetized rats following the four-vessel occlusion model. Electroencephalogram and brain and body temperatures were continuously monitored. All rats were thermoregulated for the entire duration of anesthesia. 7-NI (25 mg/kg) or its vehicle was given intraperitoneally just after the carotid clamping and again 1 hour later. Rats were randomly divided into four groups: (1) vehicle (n = 7); (2) 7-NI (n = 7); (3) L-arginine (300 mg/kg IP) + 7-NI (n = 7); and (4) 7-NI associated with warming to 37°C for 7 hours after disruption of anesthesia to compensate for the decrease in temperature induced by 7-NI (n = 9). Seven days after ischemia, hippocampal CA1 damage was evaluated by classic histology. The lesion was scored with the use of a point scale, and the surviving neurons were counted.

Results—Lesion scores were significantly lower and neuron counts higher in the two (warmed and unwarmed) groups of rats in which 7-NI was given alone than in vehicle- and L-arginine + 7-NI–treated rats.

Conclusions—The results indicate that 7-NI was neuroprotective in 20-minute global ischemia in rats and that the neuroprotective effect of 7-NI was mostly due to the blockade of NO synthesis, suggesting that NO released from neurons in ischemic conditions has a deleterious influence on hippocampal pyramidal neurons. (Stroke. 1998;29:1248-1254.)

Key Words: cerebral ischemia ■ hippocampus ■ neuroprotection ■ nitric oxide

Transient cerebral ischemia is associated with NO release, but the question of whether NO is beneficial or toxic in this pathology remains unanswered. It is suggested that the effect of NO depends on the stage of evolution of the ischemic process and on the cellular source of NO. The balance between the activation of two different (neuronal and endothelial) calcium-dependent isoforms of NOS in the acute stage of ischemia has been invoked to explain the contradictory results obtained with nonselective NOS inhibitors in rat focal ischemia and in rat and gerbil global ischemia. On the basis of studies in knockout mice lacking specific NOS isoforms, it has been proposed that, in cerebral ischemia, the activation of type 3 (endothelial) NO synthase (eNOS) is beneficial, whereas the activation of type 1 (neuronal) NO synthase (nNOS) is detrimental. NO produced by these enzymes is involved in the relaxation of cerebral blood vessels and in the neurotoxicity of glutamate, respectively. However, no direct validation of this hypothesis has been demonstrated in rats submitted to severe global cerebral ischemia, which is the most widely used model.

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The selective and delayed hippocampal damage induced in pyramidal neurons by transient global ischemia is believed to be triggered by excessive glutamate release associated with inhibition of glutamate reuptake mechanisms under conditions of energy failure. The overstimulation of NMDA receptors induces an overload of Ca²⁺, leading to a persistent activation of nNOS. This enzyme is present not only in hippocampal interneurons but also in the pyramidal neurons of the CA1/CA3 layers and in the dentate gyrus, as shown by immunocytochemistry and by in situ hybridization. Most in vitro studies in cultured neurons conclude that NO is involved in NMDA toxicity, either directly or indirectly, through its reaction with superoxide to form the powerful cyto-oxidant, peroxynitrite. In addition, in vitro experiments in hippocampal slices, which enable avoidance of direct effects on the vascular system, suggest that the activation of nNOS is involved in ischemic degeneration in the CA1 region. However, some discrepant results were obtained under similar experimental conditions.

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The development of a relatively selective inhibitor of nNOS, 7-nitroindazole (7-NI),\textsuperscript{5,28,29} has enabled investigators to explore in vivo the possible roles of NO released by neurons, as opposed to l-arginine derivatives, which nonselectively block all types of NOS, thus preventing any possible beneficial vasodilatory effect of NO released by vascular endothelial cells. 7-NI was found to produce significant neuroprotection against NMDA-mediated excitotoxic striatal lesions and also, and more effectively, against secondary excitotoxic lesions.\textsuperscript{30} In rat focal cerebral ischemia, a significant neuroprotection was found under 7-NI, which was reversible by t-arginine.\textsuperscript{31} In 5-minute global ischemia in gerbils, a neuroprotective effect of 7-NI was also demonstrated.\textsuperscript{32} However, it was not evaluated in this study whether the NO precursor t-arginine in excess can reverse the neuroprotective effect of 7-NI. Thus, it was not possible to be entirely conclusive on the role of neuronal NO generation in the detrimental consequences of global ischemia.

To explore this problem in rats, we have chosen to study the four-vessel occlusion model. We have attempted to determine whether 7-NI, given at the onset of ischemia, is neuroprotective in 20-minute global cerebral ischemia and whether its influence is due to nNOS inhibition or mediated by thermal changes. Actually, 7-NI does not modify temperature in gerbils, whereas it has been shown to depress temperature in rats,\textsuperscript{33} and we have found in preliminary experiments that 7-NI induced a significant decrease in body and brain temperatures in awake rats. We have thus evaluated the influence of hypothermia induced in a delayed fashion by 7-NI, since hypothermia is very efficient to ensure neuroprotection in rats submitted to global ischemia, even when its induction is delayed.\textsuperscript{33–36}

**Materials and Methods**

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed under permit No. 02934 from the French Ministry of Agriculture. The protocols received full review and approval by the CNRS Animal Care and Use Committee before we conducted the experiments.

Male Wistar rats weighing 280 to 330 g (Charles River) were used in this study. They had access to food and water ad libitum and were housed in individual cages.

**Protocol for Induction of Ischemia**

The rats were prepared for transient global ischemia according to the four-vessel occlusion model.\textsuperscript{37} On the day before the experiment, the rats were anesthetized with halothane (1.5% in 30% oxygen and 70% N\textsubscript{2}O) and placed in a stereotaxic frame (David Kopf). The skin and muscles over the first two cervical vertebrae were incised and separated from the midline. Both vertebral arteries were electrocoagulated after exposure of the alar foramina under a surgical microscope (Zeiss). In the same experimental session, three silver electrodes were cemented to the cranial bone in order to record the EEG on a polygraph (ECEM).

On the day of the experiment, anesthesia was induced with halothane (4% in 30% oxygen and 70% N\textsubscript{2}O) and maintained at 1%. Body temperature was kept at 37°C by a homeothermic blanket control unit (Harvard Apparatus) throughout the entire period of ischemia and postischemic recovery under anesthesia. A 33-gauge thermocouple was placed under the temporalis muscle to monitor brain temperature. The EEG was continuously recorded. The bilateral CCAs were isolated from surrounding tissues through a midline neck incision, and silk ligatures were placed loosely around them.

Transient forebrain ischemia was induced by occlusion of the CCA with the use of metal clips. The completeness of ischemia was confirmed by the observation of a flattened EEG. At that time halothane inhalation was discontinued. After 20 minutes of ischemia, reperfusion was accomplished by releasing the clips. Halothane inhalation was adjusted to 0.6% for 1 hour of recovery, and the success of reperfusion was attested to by the recovery of some EEG activity. The wounds were sutured and infiltrated with lidocaine, and anesthesia was then discontinued. EEG and brain temperature recordings were stopped just before anesthesia was discontinued.

When the rats began to recover motor activity, they were placed in a flexible hammock where they were free to move their head and limbs. Body temperature was monitored for 7 hours, and then the rats were returned to their cages. Seven days later, the brains were processed for histological analysis of the hippocampal damage.

**Evaluation of Hippocampal Damage**

The rats underwent profound halothane anesthesia. They were perfused through a transcardiac catheter with heparinized saline (100 mL) followed by paraformaldehyde 4% (400 mL) at a constant pressure of 110 to 120 mm Hg. The brains were carefully removed and incubated with phosphate-buffered 4% paraformaldehyde for 48 hours at 4°C. They were then soaked in 30% sucrose phosphate buffer for 48 hours. Twelve-micrometer sections were coronally cut with a cryomicrotome (Bright) every 500 μm at the level of the dorsal hippocampus (2.8, 3.3, 3.8 mm caudal to the bregma) and stained with cresyl violet. The middle zone of the dorsal hippocampal CA1 subsector was targeted for the assessment of the neuronal damage because it is the zone most affected by the delayed neuronal death\textsuperscript{38} and because its anatomic localization enables different investigators to perform reproducible evaluations on many slices. Decontamination of ischemic areas was performed on three sections per animal with the use of an ocular grid under an optical Leitz microscope at ×160 magnification by three observers blinded to the experimental protocols. They gave a score to the middle CA1 hippocampal subsector, graded from 0 (no visible damage) to 3 (extensive pyramidal cell loss), with 1 indicating scattered ischemic neurons and 2 indicating that approximately one half of the pyramidal cells were lesioned.

In addition, the intensity of ischemic injury within the hippocampus was quantified by counting numbers of normal-appearing neurons per high-power field (×250) in the middle CA1 subsector. All normal-appearing hippocampal neurons in a 490-μm length of stratum pyramidale were counted bilaterally and averaged. Three sections were examined per animal, and the counts were averaged. CA1 cell counts from three sham-operated animals provided an assessment of uninjured/normal CA1. Cell counts were conducted by an observer (V.S.) who was blinded to the experimental protocols.

**Experimental Groups**

All rats were given either 7-NI (25 mg/kg) or its vehicle (peanut oil) intraperitoneally twice at 1-hour intervals. The dose of 7-NI was selected on the basis of functional studies\textsuperscript{39} and because it is the most widely used dose in neuroprotection studies. The timing of 7-NI...
injection was motivated by the transient effect of 7-NI on NOS activity. The first injection was performed just after both CCAs were clamped, and the second injection was given 1 hour later, ie, after 40 minutes of reperfusion. The rats were randomly divided into four groups. Group 1 was given peanut oil (2 mL/kg). Group 2 was given 7-NI (25 mg/kg). Group 3 was given 7-NI with the same dose as group 2, but L-arginine (300 mg/kg IP) was additionally administered 30 minutes before each 7-NI injection. Group 4 was given 7-NI with the same dose and timing as group 2, but body temperature was maintained at 37.0±0.2°C for a 7-hour-period of postanesthesia recovery with the use of an external lamp.

**Materials**

7-NI (Research Biochemical International) was suspended in peanut oil by sonication. L-Arginine (Sigma Chemical Co) was dissolved in normal saline, and pH was adjusted at 7.0.

**Statistical Analysis**

Differences in temperature between groups were statistically evaluated with a one-way ANOVA followed by Scheffé’s test. Within each group, differences in temperature changes in relation to time were assessed with Dunnett’s test. Differences in lesion scores were analyzed by intergroup comparisons with a nonparametric test (ANOVA followed by Tukey’s test on ranks). Differences in normal-appearing neuron counts were assessed by ANOVA followed by Tukey’s test. Differences were regarded as statistically significant at P<0.05. All data are presented as mean±SEM.

**Results**

All rats presenting electrical activity during the ischemic period or seizure activity during the recovery were discarded from the study. Some rats died during the ischemic insult, and some did not recover from ischemia, indicating a no-reflow phenomenon. No significant difference between groups was measured in the percentage of occurrence of these phenomena.

**Temperature**

There was no significant difference in temporalis muscle temperature between groups in basal conditions, in ischemic conditions, or during reperfusion under anesthesia. The mean basal temporalis muscle temperatures were 36.4±0.1°C, 36.5±0.2°C, 36.7±0.1°C, and 36.5±0.1°C in groups 1, 2, 3, and 4, respectively. During ischemia, temporalis muscle temperature decreased significantly by approximately 1°C in all groups and remained significantly lowered until the end of CCA occlusion. When the clips were released, temporalis muscle temperature returned to its basal level within 10 minutes. No significant changes in temporalis muscle temperature were then measured during the entire reperfusion period under anesthesia.

There was no significant difference in body temperature between groups during the entire experimental period under anesthesia, ie, during surgery, ischemia, and 60-minute reperfusion (Figure 1). The mean body temperatures were 37.0±0.1°C, 36.9±0.3°C, 37.1±1.7°C, and 36.9±0.8°C in groups 1, 2, 3, and 4, respectively, under basal conditions. These values did not significantly change under anesthesia except in groups 1 and 4, in which a small significant increase was measured transiently at the end of the ischemic period. However, when anesthesia was discontinued, body temperature remained constant in groups 1 (oil) and 4 (thermoregulated), while it rapidly and significantly decreased in groups 2 (7-NI treatment) and 3 (L-arginine +7-NI treatment), attaining minimal values of 34.4±0.3°C and 34.7±0.9°C, respectively, 90 minutes later, ie, approximately 3 hours after the first 7-NI injection. Body temperature remained at this minimal level for approximately 2 hours and then started to progressively increase, reaching its basal level within 3 hours, ie, 8 hours after the first 7-NI injection.

**Hippocampal Damage**

The damage induced by ischemia in the CA1 layer of the hippocampus was significantly higher in the vehicle-treated rats than in the 7-NI–treated rats whether or not there was compensation for postanesthesia hypothermia. In addition, L-arginine reversed the neuroprotection afforded by 7-NI.

The statistical results of both lesion scores and neuron counts are presented in Figure 2. The lesion score in the middle CA1 hippocampal subsector was 2.9±0.1 in vehicle-treated rats, 1.3±0.4 in 7-NI–treated rats (P<0.05 versus...
Figure 2. Qualitative and quantitative assessments of neuronal damage in the middle CA1 subsector of hippocampus after 20-minute global ischemia in Wistar rats divided into four groups according to the legend of Figure 1. Top, Lesion scores (mean±SEM) according to a scale of 0 (no damage) to 3 (extensive damage). Bottom, Numbers (mean±SEM) of normal-appearing neurons in a 490-μm length of stratum pyramidale. A control group (n=3) has been added to determine the number of neurons in nonischemic conditions. L-arg indicates L-arginine; □, significant difference at P<0.05 with oil-treated rats; and *, significant difference at P<0.05 with control nonischemic rats.

The comparison of normal-appearing neuron counts between groups confirmed that the 7-NI–treated, whether warmed or not, had a significantly better histological outcome than both the vehicle and L-arginine+7-NI–treated rats. The number of normal-appearing neurons in the middle CA1 hippocampal subsector was significantly lower in each group of ischemic rats than in the control nonischemic group (109.8±2.4).

The prolonged decrease in temperature induced by 7-NI at the dose and timing used in the present study was determined to be 57% in a previous study in which the conversion of [14C]L-arginine to [14C]L-citrulline was measured with the use of the ex vivo Bredt and Snyder assay. It was also previously found in the conscious rat that 7-NI does not affect arterial blood pressure, indicating its lack of effect on eNOS. Another study has clearly shown that 7-NI does not inhibit eNOS present in vascular endothelial cells since it did not modify the cerebrovascular response to acetylcholine. It is thus clear that the inhibition of nNOS was rather selective in the present experimental conditions. Interestingly enough, some hippocampal pyramidal neurons contain eNOS, but it seems that 7-NI selectivity is mostly cellular rather than enzymatic so that 7-NI probably acts on both Ca2+-dependent forms of NOS possibly present in neurons.

The NO precursor, L-arginine, partly reversed the neuroprotective effect of 7-NI, clearly indicating that the inhibition of NOS activity is involved in the process of neuroprotection. Any deleterious influence of L-arginine itself through an enhancement of NO production can be discarded, since Kirsch et al have shown that it does not affect neurological outcome after global ischemia. The incomplete reversion by L-arginine is probably due to the fact that 7-NI competes with L-arginine for binding to the prosthetic heme group of NOS but also additionally affects the pyridine site of the enzyme, such an effect being reversed by tetrahydrobiopterin (BH4) only. The transient nature of the effect of 7-NI on NOS activity enables one to propose that the detrimental influence of NO released by neurons occurs in the early stages after cerebral ischemia.

The prolonged decrease in temperature induced by 7-NI does not seem to be related to NOS activity since it was not reversed by L-arginine and because such a hypothermic effect never occurred with Nγ-substituted arginine analogues. Neither is it due to an influence of 7-NI on brain metabolism since the cerebral metabolic rate of glucose was unchanged in most structures of conscious rats at 30 to 40 minutes after the injection. However, glucose consumption was not explored at longer delays after 7-NI injection. It is interesting to note that the relatively late hypothermia induced by 7-NI did not occur in gerbils, occurred irregularly in mice, and has usually not been detected in rats, except by Schulz et al. This is probably due to the thermostatically controlled conditions in which the experimental procedures under anesthesia were performed, associated with the absence of physiological measurements after discontinuation of anesthesia. Hypothermia, even when induced late (2 hours) into the reperfusion phase, has been shown to be neuroprotective in rats submitted to global ischemia. However, in this previous study the decrease in temperature was both higher and of longer duration.
duration than in the present study. More recently, Nurse and Corbett have shown that a protracted period of subnormal temperature during the posts ischemic period can obscure the interpretation of drug studies. Their demonstration was applied to the AMPA receptor antagonist NBQX, whose neuroprotective effect did not take place when the posts ischemic brain temperature of NBQX-treated gerbils was regulated in the long term. In contrast, our results show that the compensation for the prolonged 7-NI–induced hypothermia in the postanesia recovery period did not significantly modify the neuroprotective effect of 7-NI, although a tendency toward aggravation was measured. This reinforces our conclusion that the neuroprotection exerted by 7-NI in rat global ischemia was mainly due to nNOS inhibition.

Such a conclusion is in good agreement with the generally hypothesized sequence of events leading to neuronal death in transient ischemic conditions, ie, depolarization, increase in intracellular glutamate concentration, overstimulation of glutamate receptors (notably NMDA receptors), increase in extracellular Ca++ concentration, activation of enzymes (notably constitutively expressed NOS), release of NO (which rapidly reacts with superoxide produced in excess during reperfusion), formation of peroxynitrite, and nitrosylation of proteins. NO may also damage DNA through nucleotide base deamination and may trigger programmed cell death. However, this could be modulated by some possible protective effects of NO, such as inhibition of NOS activity, NMDA receptor function, and glutamate release. All these processes have been recently reviewed in detail.6,8

The neuroprotection that we found with 7-NI in rat transient global ischemia agrees well with the data from experiments in mice selectively deficient in NOS isoform15 and supports a detrimental role of NO released by neurons. However, neither study indicates whether the neuroprotection is permanently established when neuronally derived NO is depleted, or whether the damage is only delayed. It cannot be excluded that the neuroprotective effect of 7-NI was partly due to a cerebrovascular effect. Indeed, the possible involvement of neuronally derived NO in the local regulation of cerebral blood flow must be taken into account when addressing the question of NO neurotoxicity, especially when studying hippocampal damage, since a close association between NOS-positive neurons and blood vessels has been found in the hippocampus. Because 7-NI has been shown to decrease blood flow in all brain structures in basal conditions13 and to reduce the cerebrovascular responses to hypercapnia, somatosensory activation, and limbic seizures, it is likely that it may reduce the hyperemic phase of posts ischemic reperfusion. The injury due to reperfusion after global ischemia has been extensively studied, and free radicals, especially superoxide, were found to play a major role in this deleterious process. A reduction by 7-NI of the posts ischemic hyperemia would lead to a decrease in free radical production in a period of attenuated NO synthesis, thus limiting the formation of cytotoxic oxidants as peroxynitrite and being beneficial for the tissue.

In conclusion, this study provides evidence of the neuroprotective effect of 7-NI against the delayed hippocampal damage induced by 20-minute global ischemia in rats. The present results confirm and extend the findings obtained in rat focal ischemia and in gerbil global ischemia. They strengthen the hypothesis that neuronally derived NO is toxic in ischemic conditions, since they demonstrate that L-arginine reversed the neuroprotective effect of 7-NI, whereas normothermic conditions during postanesthesia reperfusion did not significantly modify the hippocampal damage.

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References

NO has been implicated in the pathobiology of various neurological problems, including cerebral ischemia. While NO produced by endothelial NOS may improve cerebral blood flow and outcome after cerebral thromboembolic events, NO produced by neuronal or inducible NOS may initiate and/or enhance various pathological processes felt to lead to ischemic cell death. This study determined whether the selective inhibition of nNOS by 7-NI would be neuroprotective in a model of transient global ischemia in rats. Specific attention was given to the consequences of 7-NI administration on body temperature because of the beneficial effects of mild hypothermia in this ischemia model.

The authors report that 7-NI treatment led to a reduction in body temperature after the discontinuation of anesthesia. However, significant neuroprotection was also reported with 7-NI treatment whether or not there was compensation for postanesthesia hypothermia. Finally, 1-arginine reversed the neuroprotective effects of 7-NI treatment.
This study is important because it provides novel data concerning the ability of 7-NI treatment to promote neuroprotection of the CA1 hippocampus after global ischemia. It should be stressed, however, that 7-NI treatment was initiated immediately after the carotid clamping. We therefore have no information concerning the “therapeutic window” for 7-NI treatment in this experimental setting. The findings do, however, implicate nNOS in the pathophysiology of the acute injury process.

Recent ischemia studies have shown that neuroprotective treatments that provide partial protection several days after the insult may not confer long-term improvements in behavioral or histopathological outcome. It will therefore be important in future studies to determine whether 7-NI treatment in this ischemia model protects against cognitive abnormalities and improves long-term hippocampal pathology. These data will be important for future considerations regarding the use of selective inhibitors of neuronal NOS in clinical stroke trials.

References
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