Subacute But Not Acute Generation of Nitric Oxide in Focal Cerebral Ischemia

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Background and Purpose—Excessive release of nitric oxide (NO) has been implicated in the pathophysiology of neurodegeneration in ischemic stroke. We compared intracerebral release of indicators of NO generation at the acute and subacute stages of transient focal cerebral ischemia.

Methods—In vivo microdialysis in the rat striatum was performed at the acute (first hours) and subacute (after 24 or 48 hours) stages of cerebral ischemia or sham operation to monitor intracerebral release of the stable NO metabolites nitrite and nitrate.

Results—Whereas only a nonsignificant trend toward increased release of these NO metabolites was evidenced in acute cerebral ischemia, a significant NO generation was observed subacutely, 48 hours after induction of cerebral ischemia. Aminoguanidine, a selective inhibitor of inducible NO synthase, suppressed this delayed release of nitrite and nitrate.

Conclusions—Whereas these observations do not support a major NO generation in acute cerebral ischemia, they indicate an inducible NO synthase–dependent NO generation predominantly at the subacute phase of ischemic neurodegeneration. Therefore, NO generation may play a pathophysiological role in delayed ischemic neurodegeneration. (Stroke. 2000;31:2208-2211.)

Key Words: cerebral ischemia, focal ■ nitric oxide ■ nitric oxide synthase ■ rats

Whereas in low concentrations (nanomolar range), nitric oxide (NO) plays a physiological role in neuronal signaling, in high concentrations (micromolar range) this molecule is enormously cytotoxic. The production of NO, itself a free radical, promotes tissue injury, eg, by reaction with superoxide anion to produce the extremely toxic peroxynitrite or by interaction with proteins, transition metals, and iron-sulfur–containing or heme-containing compounds. Excessive NO generation has been implicated in ischemic neurodegeneration and is currently intensely studied as a therapeutic target in ischemic stroke. In this study we comparatively studied the intracerebral generation of NO at the acute and subacute stages of transient focal cerebral ischemia.

Materials and Methods

Stroke Models

Male adult albino rats of a Wistar-derived strain (Charles River. Wiga, Germany) weighing 240 to 440 g, were housed under standard conditions with free access to food and water. Infarcts were induced as previously described. Briefly, as the rats were monitored for blood pressure, temperature, and oxygenation, the right carotid artery was exposed after median incision of the neck skin. The right middle cerebral artery (MCA) was occluded with a silicone rubber cylinder attached to a nylon surgical thread introduced from the common carotid artery immediately after ligation of the ipsilateral external and proximal common carotid artery. The cylinder was made of a 4-0 nylon surgical thread, coated with silicone and hardener to thicken the distal 5 mm to 0.25 to 0.30 mm. The thread was advanced for 16 to 20 mm into the lumen of the internal carotid artery until it blocked the origin of the MCA. Then the internal carotid artery was ligated just distal to the point of insertion. Recirculation was performed by

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pulled the thread out after 2 hours. The sham operation was performed as described above, except that the filament was not advanced to block the MCA.

In Vivo Microdialysis
Under conditions similar to those of the in vivo experiments (perfusion rate of 2 μL/min, 37°C), an in vitro recovery for nitrite dissolved in Ringer’s solution of 33±4% was obtained. In vivo microdialysis was performed as previously described. Rats were anesthetized with a mixture of 2% fentanyl (6 mg/kg body wt IP) plus medetomidine (0.3 mg/kg body wt IP) and placed in a stereotaxic apparatus. Thereafter, the dura was exposed through a 2-mm hole in the skull, and the microdialysis probe was inserted in the striatum. The coordinates to which the tip of the dialysis probe (100 000-Da cutoff, CMA 12) was lowered with respect to bregma and dural surfaces were as follows: anterior +1.7 mm, lateral −2.0 mm, and vertical 6.0 mm, with the incisor bar at −2.5 mm. After implantation, the probe was secured with dental cement to 2 small anchoring screws on the top of the skull. After surgery, the animals were housed with free access to food and water. A modified Ringer’s solution (consisting of mmol/L NaCl 121, KCl 3.5, MgCl2 1.2, CaCl2 1.2, NaH2PO4 1.0, NaHCO3 25 (pH 7.4) was constantly perfused through the implanted probe at a rate of 2 μL/min with the use of a microinjection pump. Dialysates were collected every 10 minutes and immediately frozen.

Experimental Conditions
In each experimental group 6 to 8 animals were studied. For analysis of NO generation in acute cerebral ischemia, microdialysis probes were implanted 24 hours before the induction of cerebral ischemia or sham operation. Microdialysis was started 3 hours before ischemia to equilibrate the system for the first hour and to obtain baseline values for the 2 subsequent hours. In this group, measurements were performed directly before and after induction of experimental ischemia and reperfusion (or sham operation).

For determination of NO generation in subacute cerebral ischemia, microdialysis was performed beginning either 24 or 48 hours after induction of ischemia. Under these conditions, microdialysis probes were implanted and perfused 3 hours before the first measurements to equilibrate the system. In an additional study group, the effects of the selective inhibitor of inducible NO synthase (iNOS), aminoguanidine, on subacute NO generation were tested, at a dosage previously described (400 mg/kg IP, twice daily, beginning on the day of experimental ischemia).

Analysis of Release of NO Metabolites
NO was quantified in microdialysates via the nitrite method based on the Griess reaction, with the use of a colorimetric assay from Boehringer. Dialysates collected within intervals of 1 hour were pooled to obtain 2 fractions for duplicate determinations. The nitrate present in the sample is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the nitrate reductase. The nitrite formed reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to yield a red-violet diazot dye, which is measured on the basis of its absorbance in the range of 550 nm. Known concentrations of sodium nitrite were included as standards. The lower limit of detection is 0.32 μmol/L for nitrite. The intratest variance is <10%, and the intertest variance is <20%.

Statistical Analysis
Results are expressed as mean (±SE). The Mann-Whitney test was used for the nonparametric statistical analysis. Differences were considered significant at P<0.05.

Results
Hemodynamic Variables, Changes in Body Weight, and Neurological Scores
Hemodynamic variables were within the same range in animals with ischemia and sham operation. Thus, mean arterial pressures during the operation were 115.52±16.50 and 101.43±11.54 mm Hg in the sham-operated groups, 113.15±6.79 mm Hg in the group with acute ischemia, and 127.26±21.01 and 101.72±10.97 mm Hg in the groups with subacute ischemia. Repeated measurements of body weight revealed a slight weight loss 2 days after surgery in both sham-operated (from 324.3±27.1 to 321.3±24.2 g) and ischemic (from 330.8±37.3 to 308.3±26.3 g) animals. This decrease in body weights did not significantly differ between both groups. Neurological scores according to Bederson et al10 tended to be slightly better in animals that had experimental ischemia 48 hours before (2.3±1.9) compared with those that had ischemia 24 hours before (3.5±1.0).

NO Generation in Acute Cerebral Ischemia
In sham-operated animals, extracellular release of NO metabolites was detectable that was stable in time (Figures 1 to 3). However, compared with these control animals, no significant changes in nitrate/nitrite release were observed in animals with acute cerebral ischemia (Figure 1). We observed only a minor, nonsignificant trend toward increased release of the NO metabolites peaking directly after induction of ischemia and, again, immediately after reperfusion (Figure 1).

NO Generation in Subacute Cerebral Ischemia and its Suppression by Aminoguanidine
Twenty-four hours after onset of cerebral ischemia, concentrations of nitrate/nitrite in dialysates tended to be transiently

Figure 1. Nitrite/nitrate release in acute focal cerebral ischemia and sham operation. (B indicates baseline; I, ischemia; and R, reperfusion).

Figure 2. Nitrite/nitrate release in subacute focal cerebral ischemia (after 24 hours) and sham operation.
increased, although this increase was not significant compared with sham-operated animals (Figure 2). However, 48 hours after induction of experimental cerebral ischemia, extracellular concentrations of NO metabolites were significantly increased (Figure 3). Levels peaked after 2 hours of measurement (6 hours after initiation of microdialysis) and declined thereafter. Administration of the iNOS inhibitor aminoguanidine completely inhibited this pronounced delayed NO generation (Figure 3).

### Histological Analyses

Histological examination (hematoxylin-eosin staining of coronary 10-μm brain sections) confirmed infarctions in the MCA territory, their lack in the sham-operated group, and the correct placement of the microdialysis probes. Percentages of the infarcted area compared with the total hemisphere were 77±5% (quantified at the coronary planes in the area of the tip of the dialysis probes with a computer-assisted planimetry device). Using perfusion with ink, we confirmed in additional animals that no reperfusion occurred in permanent MCA occlusion and declined thereafter. Administration of the iNOS inhibitor aminoguanidine completely inhibited this pronounced delayed NO generation (Figure 3).

### Discussion

Despite the immense current interest in the role of NO generation as a therapeutic target in ischemic stroke, in vivo data on release of this molecule in cerebral infarction are still scarce. In this study we report that NO metabolites are released predominantly in subacute cerebral ischemia and only in minute amounts in acute disease.

Our method was sensitive enough to detect a release of NO metabolites even in nonischemic brain (sham operation) and under basal conditions before induction of ischemia in quantities reported in earlier studies. These basal nitrite levels were extremely stable as a precondition to detect even minor changes in concentrations. Dialysis probes were intracerebrally placed at different intervals in animals with acute and subacute infarction to avoid prolonged insertion times of the microdialysis probes beyond limits that might have caused nonspecific local inflammation (and iNOS expression). We studied changes in relation to sham-operated control groups, controlling for such methodological problems.

Although we observed a nonsignificant trend toward transiently increased concentrations during ischemia and again immediately after reperfusion, these changes were minute compared with the NO generation observed in subacute disease. This delayed release of NO metabolites in the high, cytotoxic concentration range is the major finding of this study. Whereas a trend toward increased nitrite release was detected 24 hours after induction of cerebral ischemia, nitrite release was significantly increased at day 2. Interestingly, such predominantly delayed NO generation is in accordance with the recent detection of NO metabolites in high homogenates predominantly 3 days after transient cerebral ischemia and agrees exactly with the observation of subacute glial activation and intracerebral migration of macrophages after 2 days at sites of ischemic injury. These cells harbor the subacutely synthesized iNOS that mediates generation of much larger quantities of NO than the constitutive isoenzymes held responsible for NO production in acute stroke.

In such an environment, further cerebral injury associated with insertion of the dialysis probes could trigger a maximal iNOS-dependent NO generation. Indeed, the inhibitory effects of aminoguanidine delineate such a crucial role of iNOS expression in subacute cerebral ischemia. A beneficial effect of aminoguanidine on resulting infarct volumes has already been shown in earlier works and its confirmatory evaluation was beyond the aims of this longitudinal study.

The transient temporal profile of NO release in subacute cerebral ischemia is unclear. It is unlikely that dietary differences could explain increased NO generation in cerebral ischemia since postoperative changes in body weights did not significantly differ between ischemic and sham-operated groups.

This, to our knowledge, first study comparing NO generation at different stages of focal cerebral ischemia supports a pathophysiologic role of iNOS-dependent NO generation predominantly in the subacute phase of disease. It should be noted that the results of this study need to be considered in relation to the chosen animal model.

This predominantly subacute NO generation could explain the large number of negative studies investigating the therapeutic effects of nonspecific or constitutive NOS inhibitors in acute cerebral ischemia. At the same time, this study reveals a high susceptibility of ischemically injured brain tissue to respond to further noxious stimuli (eg, infection, blood pressure variations, or dehydration) by excessive NO generation. This raises the possibility that interventions in NO-mediated toxicity could be neuroprotective even 1 to 2 days after onset of cerebral ischemia, as suggested in a recent preliminary report.

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Figure 3. Nitrite/nitrate release in subacute focal cerebral ischemia (after 48 hours), sham operation, or subacute cerebral ischemia treated with the iNOS inhibitor aminoguanidine (AG). *P<0.05 compared with sham operation.
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