Inhibitors of Protein Synthesis Preserve the N-Methyl-D-Aspartate–Induced Cerebral Arteriolar Dilation After Ischemia in Piglets

Roland Veltkamp, MD; Ferenc Domoki, MD; Ferenc Bari, PhD; Thomas M. Louis, PhD; David W. Busija, PhD

Background and Purpose—Cerebral arteriolar dilation to N-methyl-D-aspartate (NMDA) is a neuronally mediated process that is sensitive to cerebral ischemia. We tested the hypothesis that pretreatment with transcription or translation inhibitors preserves the vascular response to NMDA after global cerebral ischemia.

Methods—Pial arteriolar diameters were measured in anesthetized piglets by use of a closed cranial window and intravital microscopy. Arteriolar responses to NMDA (10^{-5} and 10^{-6} mol/L) were measured before and 1, 2, and 4 hours after 10 minutes of ischemia. Ischemia was induced by increasing intracranial pressure. Subgroups were pretreated with vehicle, topical actinomycin D (Act-D) 10^{-5} or 10^{-6} mol/L, or intravenous cycloheximide (CHX) 1.0 or 0.3 mg/kg 15 minutes before ischemia. The effects of Act-D and CHX on vascular responses to NMDA without preceding ischemia were also examined.

Results—In the vehicle group, arteriolar responses to NMDA were clearly attenuated 1 hour after ischemia but returned to baseline at 2 to 4 hours. Preischemic compared with 1 hour postschemic arteriolar dilation to NMDA was 10±2% versus 1±0% at 10^{-5} mol/L and 40±4% versus 20±4% at 10^{-4} mol/L NMDA (mean±SEM; both P<0.05, n=7). In contrast, pretreatment with Act-D resulted in preservation of the arteriolar responses to NMDA 1 hour after ischemia. For 10^{-6} mol/L (n=5) of Act-D, dilations were 6±2% versus 6±2% at 10^{-5} mol/L and 51±9% versus 39±10% at 10^{-4} mol/L of NMDA. For 10^{-5} mol/L (n=5) of Act-D, arterioles dilated by 7±2% versus 7±2% at 10^{-3} mol/L and 38±4% versus 35±4% at 10^{-4} mol/L NMDA. Similarly, CHX preserved NMDA-induced vasodilation. For 0.3 mg/kg of CHX (n=5), dilations were 8±2% versus 8±1% at 10^{-5} mol/L and 39±4% versus 28±6% at 10^{-4} mol/L NMDA. For 1.0 mg/kg of CHX (n=5), arterioles dilated by 10±2% versus 6±2% at 10^{-5} mol/L and 37±7% versus 35±6% at 10^{-4} mol/L NMDA. In experiments without ischemia, NMDA-induced vasodilation before and 85 minutes after administration of Act-D or CHX was not significantly different.

Conclusions—Vascular responses of cerebral arterioles to NMDA after ischemia are preserved by pretreatment with either Act-D or CHX. Without preceding ischemia, Act-D and CHX do not potentiate neuronal-vascular responses to NMDA. Our results suggest that continued or augmented protein synthesis is involved in the transient attenuation of NMDA-induced dilation during the early reperfusion phase and that inhibitors of protein synthesis may protect neurons against ischemic stress. (Stroke. 1999;30:148-152.)

Key Words: cerebral circulation □ reperfusion □ actinomycin D □ cycloheximide □ pigs

Cerebral arteriolar dilation to glutamate and its receptor subtype–specific analogue N-methyl-D-aspartate (NMDA) involves sequential activation of neuronal NMDA receptors, neuronal production and release of nitric oxide (NO),1-5 and increased production of cGMP, which results in smooth muscle relaxation. This neuronal-vascular sequence may represent one of the mechanisms coupling local cerebral metabolism to blood flow. We have shown that the NMDA-induced vasodilation is substantially attenuated after brief episodes of global ischemia or hypoxia.6-8 Our findings suggest that the attenuation reflects the effects of processes triggered by ischemia on neurons rather than on cerebral arterioles.7,8 Accordingly, the attenuation of the NMDA-induced arteriolar dilation after ischemia may be an indicator of early posts ischemic neuronal damage. Conversely, the posts ischemic integrity of the neuronal-

Received June 3, 1998; final revision received August 31, 1998; accepted October 5, 1998.

From the Stroke Research Center (R.V.) and Department of Physiology and Pharmacology (R.V., F.D., F.B., D.W.B.), Wake Forest University School of Medicine, Winston Salem, NC; the Department of Physiology, Albert Szent-Györgyi Medical University, Szeged, Hungary (F.D., F.B.); and the Department of Anatomy and Cell Biology, East Carolina University, Greenville, NC (T.M.L.).

Correspondence to Roland Veltkamp, MD, Stroke Research Center, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157-1083. E-mail veltkamp@bgsm.edu

© 1999 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

148
vascular axis can serve as a parameter for the in vivo study of early effects of experimental interventions on neuronal function.9

The exact molecular mechanisms underlying the attenuated vascular responses to NMDA are currently unknown. The effects of ischemia on protein synthesis may be directly or indirectly involved in the attenuation of the neuronal-vascular response, because ischemia rapidly modifies gene expression.9–11 Although protein synthesis is generally suppressed during the early reperfusion period, translation of certain proteins takes place.9–11 Newly synthesized proteins may have protective or detrimental effects on neurons. The functional significance of specific proteins, however, is not clearly established at this time.11

The purpose of the present study was to examine the effect of pretreatment with inhibitors of protein synthesis on the postischemic NMDA-induced vasodilatation. Specifically, we tested the hypothesis that pretreatment with the mRNA transcription blocker actinomycin D (Act-D) or the translation inhibitor cycloheximide (CHX) preserves the vascular responsiveness to NMDA 1 hour after 10 minutes of global cerebral ischemia. We also investigated whether Act-D or CHX potentiates the vascular responses to NMDA without preceding ischemia.

Materials and Methods

Surgical Preparation

Experiments were performed on newborn pigs (1 to 7 days old) of either sex weighing 1 to 2 kg. The procedures used in the study were approved by the Institutional Animal Care and Use Committee. The piglets were initially anesthetized with sodium thiopental 30 mg/kg IP and later with α-chloralose 75 mg/kg IV. Additional amounts of α-chloralose were given as needed to maintain a stable level of anesthesia. The piglets were intubated and artificially ventilated. A femoral artery and vein were cannulated with polyethylene tubing (PE-90). Arterial blood gases and pH were measured repeatedly, and rectal temperature was continually monitored. Rectal temperature was kept within the range of 37°C to 38°C. The head of each piglet was fixed in a stereotactic apparatus. Approximately 3 mL of cerebrospinal fluid (CSF) was withdrawn from the cisterna magna. The scalp was cut, and the connective tissue over the parietal bone was removed. A circular craniectomy (19 mm in diameter) was made to the opening, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic. A hollow brass bolt was inserted into the left parietal cranium rostral to the cranial window to allow equilibration with the periarachnoid CSF. A cerebral arteriole of approximate diameter 30–50 μm was selected for observation. The window was sutured, and the dura was exposed. A toothless bit, and the dura was exposed. A hollow brass bolt was inserted into the left parietal cranium rostral to the cranial window and secured in place with cyanoacrylate ester and dental acrylic. Cerebral ischemia was produced by infusion of aCSF to maintain intracranial pressure above mean arterial pressure so that blood flow through pial vessels was stopped. Venous blood was withdrawn as necessary to maintain mean arterial pressure near normal values. At the end of the 10-minute period of ischemia, the infusion tube was clamped and the intracranial pressure was allowed to return to preischemic values. The heparinized blood was reinfused intravenously.

Experimental Design

At the beginning of each experiment, the cranial window was flushed several times with aCSF to allow equilibration with the periarachnoid CSF. A cerebral arteriole of ~100-μm diameter was chosen. When baseline arteriolar diameter was stable, arteriolar responses to NMDA 10−3 and 10−4 mol/L were determined. Each dose of NMDA was introduced into the window, the infusion was stopped, and arteriolar diameter was recorded over the next 5 to 10 minutes. Afterward, the window was flushed with aCSF. The arteriolar diameter returned to baseline within 15 to 20 minutes.

Animals were divided into 5 experimental groups. In group 1 (n = 7), arteriolar responses to NMDA were recorded before (see above) and 1, 2, and 4 hours after 10 minutes of global cerebral ischemia. In addition to this protocol, groups 2 and 3 were pretreated 15 minutes before ischemia with a topical infusion of Act-D 10−3 mol/L (group 2, n = 5) or 10−4 mol/L (group 3, n = 5) diluted in aCSF into the window. Groups 4 and 5 were pretreated with intravenous CHX 1 mg/kg (n = 5) and 0.3 mg/kg (n = 5), respectively. Act-D was applied topically because, in contrast to CHX, it does not cross the blood-brain barrier. Doses of drugs that had been shown by other investigators to be efficacious (see References 14 and 15) were chosen. The window was flushed with aCSF just before the beginning of ischemia. In additional experiments, the cerebral arteriolar responses to NMDA before and 1 hour after 15 minutes of topical application of Act-D 10−3 mol/L (n = 6) or intravenous administration of CHX 1 mg/kg (n = 6) were determined.

Drugs

We used NMDA (Sigma Chemical Co), Act-D (Calbiochem), and CHX (Sigma).

Statistics

Data are expressed as mean±SEM. A paired t test was used for comparing data between 2 groups. For repeated-measures analysis, ANOVA was used, and the Student-Newman-Keuls test was then applied. Data analyses were performed on absolute and percent-change data. A value of P<0.05 was regarded as statistically significant.

Results

Before and after ischemia, mean arterial blood pressure was stable and within normal limits for piglets. For example, in group 1 (control), arterial blood pressure was 60±3 mm Hg before ischemia and 61±3 mm Hg 1 hour after ischemia. Arterial blood pressure was not affected by the topical application of Act-D; intravenous administration of the high dose of CHX led to a brief, transient (1 to 2 minutes) increase of blood pressure (<20 mm Hg) in some but not all animals.

Arterial blood gases and pH were monitored regularly during the experiments. In the control group (n = 7), baseline pH was 7.49±0.02, PCO2 was 25±2 mm Hg, and PO2 was 96±5 mm Hg. One hour after ischemia, pH was 7.41±0.02, PCO2 was 24±2 mm Hg, and PO2 was 94±4 mm Hg. Blood gases and pH did not differ significantly among groups.

Application of NMDA before hypoxia/ischemia caused a dose-dependent cerebral arteriolar dilation (Table, Figure 1). One hour after ischemia, however, arteriolar responses to NMDA were markedly reduced (Table, Figure 1, P<0.05). Vascular responsiveness to NMDA returned toward baseline 2 to 4 hours after ischemia (Table, Figure 1).

Pretreatment with Act-D resulted in completely (10−5 mol/L) or largely (10−4 mol/L) preserved arteriolar responses to the different concentrations of NMDA 1 hour after ischemia (Table, Figure 1, all P>0.05). Similarly,
### Protection by Protein Synthesis Inhibitors

**Arteriolar Dilation to NMDA Before and After Ischemia**

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>1 h After</th>
<th>2 h After</th>
<th>4 h After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁵ mol/L</td>
<td>10⁻⁴ mol/L</td>
<td>10⁻⁵ mol/L</td>
<td>10⁻⁴ mol/L</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>100±3</td>
<td>110±5</td>
<td>140±2†</td>
<td>102±3</td>
</tr>
<tr>
<td>Δ Diameter, µm</td>
<td>10±2</td>
<td>40±3</td>
<td></td>
<td>1±0*</td>
</tr>
<tr>
<td>Act-D 10⁻⁴ mol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>103±1</td>
<td>110±3</td>
<td>142±4†</td>
<td>104±1</td>
</tr>
<tr>
<td>Δ Diameter, µm</td>
<td>7±2</td>
<td>39±4</td>
<td></td>
<td>7±2</td>
</tr>
<tr>
<td>Act-D 10⁻³ mol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>98±4</td>
<td>104±6</td>
<td>149±15†</td>
<td>99±5</td>
</tr>
<tr>
<td>Δ Diameter, µm</td>
<td>6±2</td>
<td>51±11</td>
<td></td>
<td>6±2</td>
</tr>
<tr>
<td>CHX 1.0 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>101±5</td>
<td>111±5</td>
<td>139±11†</td>
<td>99±6</td>
</tr>
<tr>
<td>Δ Diameter, µm</td>
<td>10±1</td>
<td>38±8</td>
<td></td>
<td>5±2</td>
</tr>
<tr>
<td>CHX 0.3 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>110±5</td>
<td>119±6</td>
<td>153±9†</td>
<td>111±7</td>
</tr>
<tr>
<td>Δ Diameter, µm</td>
<td>9±2</td>
<td>43±5</td>
<td></td>
<td>8±1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Δ Diameter indicates change in diameter of arteriole.
*Significantly different from respective baseline.
†Significantly different from respective baseline.

The major new finding from these experiments is that pretreatment with either Act-D or CHX preserves the neuronal vascular response to NMDA early after ischemia. In our additional experiments, neither agent potentiated arteriolar dilation to NMDA under nonischemic circumstances. Thus, inhibition of transcription or translation appears to protect the neuronal-vascular sequence against ischemic stress.

The exact mechanism by which Act-D and CHX preserve the NMDA-induced vasodilation is unclear at this time. Our earlier work suggested that ischemia affects the neuronal...
steps of the sequence proximal to nitric oxide synthase. In that study, NO synthase (NOS) activity in cerebral cortex as measured by the conversion of L-[\textsuperscript{14}C]citrulline from L-[\textsuperscript{14}C]arginine was unaffected by ischemia. Also, levels of brain NOS as determined by Western blotting techniques were not substantially altered by ischemia. Further, kainate-induced arteriolar dilation, which is partially dependent on activation of NOS, is intact after ischemia. Cerebral arteriolar responsiveness to NO donors such as sodium nitroprusside is not affected by ischemia. Indeed, the NMDA receptor complex with its multiple modulatory sites appeared to represent a readily modifiable and potentially vulnerable target for ischemia. Studies in piglets have shown that ischemic and hypoxic exposure can alter NMDA receptor characteristics.

Previous experiments also addressed the question of which pathophysiological processes affect the neuronal component of the sequence. Pretreatment with inhibitors of oxygen radical formation such as indomethacin and oxypurinol or with the oxygen radical scavenger superoxide dismutase preserved the NMDA-induced vasodilation after ischemia and hypoxia. Obviously, this suggested a role for free oxygen radicals in the posts ischemic attenuation of the sequence.

Conceivably, protection of the sequence by Act-D and CHX may be based on decreased formation of free oxygen radicals during ischemia and reperfusion. Cyclooxygenase activity is a major source of free oxygen radicals in piglet brain after ischemia. It catalyzes the synthesis of prostaglandins from arachidonic acid, with superoxide radicals as byproducts of this process. Inhibition of cyclooxygenase by indomethacin preserved the sequence in a previous study. Cyclooxygenase levels are determined by an equilibrium between continuous protein degradation and replenishment. Consequently, blockage of cyclooxygenase production can be expected to diminish cyclooxygenase levels. There is evidence from in vitro studies that this effect may be sufficiently rapid to explain our present observations. Fagan and Goldberg reported that incubation of skeletal muscle fibers with CHX for 10 to 20 minutes largely inhibited prostaglandin production in response to arachidonic acid. Using piglet astroglial culture, Nam et al demonstrated that CHX blocked the increased production of PGF\textsubscript{2\alpha} induced by interleukin-1\alpha as early as 20 minutes after treatment. Thus, rapid inhibition of cyclooxygenase synthesis may account for the protection of the sequence against ischemia-induced radical production. Alternatively, protein synthesis inhibitors may block the production of an unidentified regulatory protein that is rapidly overexpressed after ischemia, or, like hypothermia, may protect the brain by decreasing metabolic rate.

Several other investigators have also studied inhibitors of protein synthesis in experimental cerebral ischemia. Goto et al demonstrated in a global ischemia model that postischemic administration of CHX prevented delayed neuronal death in the hippocampal CA1 sector. In a study by Linnik et al, infusion of CHX into the lateral ventricle reduced infarct size in rats. Similarly, Du et al reported that pretreatment with CHX 1 mg/kg led to a decrease of infarct volume in a rat model of transient focal ischemia as measured 2 weeks after the insult. Aronowski et al found that rats exposed to 2 to 5 hours of reversible focal ischemia had significantly larger infarcts than rats undergoing longer or permanent vessel occlusion. When reperfused animals were pretreated with CHX, however, infarct size was similar to that with permanent occlusion. The authors concluded that a short-lived “noxious/killer protein” produced during the early reperfusion period may be responsible for the additional damage.

The cited studies provide evidence for a protective effect of inhibitors of protein synthesis as measured by long-term parameters (ie, extent of histological damage). In contrast, the integrity of the NMDA-induced cerebral arteriolar dilation in our paradigm may serve as an indicator of early post ischemic neuronal function. It is currently unclear whether the attenuation of the NMDA-induced vasodilation reflects an early stage of (permanent) neuronal damage and whether transient uncoupling of flow and metabolism aggravates ischemic brain damage. Specifically, we do not know how the findings in the present study relate to protective effects of protein synthesis inhibition in the longer term.

Our present findings may have clinical implications for acute global and focal cerebral ischemia. They indicate that de novo gene expression and protein synthesis have a profound and surprisingly rapid impact on neurons early after ischemia. Although we could not assess potential long-term or systemic adverse effects in our experimental model, transient inhibition of protein synthesis appeared to protect the function of neurons involved in the NMDA-induced neuronal vascular sequence. Whether this is due to inhibition of production of specific proteins such as cyclooxygenase or a less specific consequence of suppressed protein synthesis is currently unknown.

In summary, we have shown that pretreatment with the mRNA transcription inhibitor Act-D or the translation inhibitor CHX preserves the NMDA-induced neuronal-vascular coupling early after ischemia. These findings suggest that continued or augmented protein synthesis is involved in the transient attenuation of NMDA-induced vasodilation.

Acknowledgments
This research was supported by grants HL-30260, HL-46558, and HL-50587 from the National Institutes of Health.

References


Under normal conditions, relatively large quantities of NO are produced in brain by the neuronal isoform of NO-synthase (nNOS). A major stimulus for production of NO by nNOS in neurons is activation of glutamate receptors. For example, nNOS is physically coupled to one subtype of glutamate receptor, the NMDA receptor. Activation of NMDA receptors increases activity of nNOS, resulting in extracellular release of NO and local dilatation of cerebral arterioles. Interestingly, the cerebral vascular response to NMDA receptor activation may be under dynamic control. Clearly, additional studies will be needed to more fully define this effect and to identify gene products that may be involved.

Frank M. Faraci, PhD, Guest Editor
Department of Internal Medicine
Cardiovascular Division
University of Iowa College of Medicine
Iowa City, Iowa

References
Inhibitors of Protein Synthesis Preserve the N-Methyl-d-Aspartate–Induced Cerebral Arteriolar Dilation After Ischemia in Piglets
Roland Veltkamp, Ferenc Domoki, Ferenc Bari, Thomas M. Louis and David W. Busija

Stroke. 1999;30:148-152
doi: 10.1161/01.STR.30.1.148

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/30/1/148

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/