I

0031-3998/99/4506-0834
PEDIATRIC RESEARCH
Vol. 45, No. 6, 1999
Printed in U.S.A.

Cerebrovascular Reactivity Remains Intact after Cortical Depolarization in Newborn Piglets

FERENC DOMOKI, ROLAND VELTKAMP, FERENC BARI, THOMAS M. LOUIS, AND DAVID W. BUSIJA

Department of Physiology and Pharmacology[F.D., R.V., F.B., D.W.B.] and Stroke Research Center [R.V.], Wake Forest University School of Medicine, Winston-Salem, NC 27157-1083; Department of Physiology [F.D., F.B.], Albert Szent-Györgyi Medical University, Szeged, H-6720, Hungary; and Department of Anatomy and Cell Biology [T.M.L.], East Carolina University, School of Medicine, Greenville NC 27858-4353

ABSTRACT

Cerebrovascular reactivity is severely affected by ischemia, and changes in vascular responses have been reported after cortical spreading depression and head trauma as well. Cortical depolarization (CD) occurs during ischemia, cortical spreading depression, and head trauma, but its effects on cerebrovascular reactivity are unclear. We tested the hypothesis that CD induced by KCl diminishes the vascular responsiveness to various vasodilatory stimuli in piglets. Responses of pial arterioles were determined by changes in vascular diameter by use of a closed cranial window and intravital microscopy. Baseline arteriolar diameters were 105 ± 3 μm (mean ± SEM, n = 27). CD was elicited by topical administration of 1 mol/L KCl for 3 min. Vascular responses were measured before and 1 h after CD. KCl elicited CD and constricted arterioles by 54 ± 4% (n = 27). *N*-methyl-D-aspartate induced dose-dependent vasodilation that was unaffected by CD; the percent changes were 9 ± 1 *versus* 8 ± 1 (before and after CD) at 10^{-5} mol/L, 19 ± 2 *versus* 18 ± 3 at 5×10^{-5} mol/L, and 29 ± 2 *versus* 26 ± 3 at 10^{-4} mol/L (n = 9). Hypercapnic vasodilation was not diminished by CD; the percent changes were 15 ± 2 *versus* 16 ± 4 at 5%, and 27 ± 5 *versus* 27 ± 6 at 10% inspired CO_2 (n = 8). Aprikalim and forskolin caused dilation that was also resistant to prior CD; the percent change values were 21 ± 4 *versus* 18 ± 3 and 16 ± 2 *versus* 16 ± 4 at 10^{-6} mol/L, 36 ± 5 *versus* 34 ± 5 and 34 ± 7 *versus* 37 ± 7 at 10^{-5} mol/L (n = 8), respectively. Finally, calcitonin gene-related peptide-induced vasodilation was unaffected by CD; percent changes were 15 ± 3 *versus* 16 ± 2 at 10^{-7} mol/L and 26 ± 4 *versus* 22 ± 3 at 10^{-6} mol/L (n = 8). The intact vascular responses after CD suggest that this component is not responsible for decreased cerebrovascular reactivity after ischemia, head trauma, or cortical spreading depression. (*Pediatr Res* 45: 834–837, 1999)

Abbreviations

CSD, cortical spreading depression
CD, cortical depolarization
NMDA, *N*-methyl-D-aspartate
CGRP, calcitonin gene-related peptide
K_{ATP}, ATP-sensitive K^+ channels

CD is a hallmark feature of anoxic depolarization and CSD. CD is characterized by a negative direct current (DC) potential shift and cessation of EEG activity. Anoxic depolarization develops rapidly after the initiation of hypoxia or global cerebral ischemia. Neonates are commonly subjected to various lengths of asphyxia or cerebral ischemia, which severely impairs cerebrovascular reactivity. Previous studies indicate that dilator responses are vulnerable to cerebral ischemia, head trauma, and CSD, whereas constrictor responses are more resistant. The newborn pig is an appropriate model for studying neonatal cerebral circulation, because developmental stage and vascular responses are very similar to those of human babies. In piglets, global cerebral ischemia has been shown to attenuate cerebrovascular responses to various vasodilatory stimuli including NMDA, arterial hypercapnia, CGRP, and aprikalim, but not forskolin (1–4). However, an impaired vasodilation to cAMP analogues has been found after traumatic brain injury in which transient CD also occurs (5).

The above-mentioned agents dilate cerebral arterioles by different mechanisms. NMDA-induced vasodilation is a neuronally mediated multistep process involving the activation of neuronal nitric oxide synthase (6, 7). Hypercapnic vasodilation is dependent on prostanoid synthesis and requires intact endothelium in the newborn pig (8, 9). CGRP, the activation of K_{ATP} by aprikalim, and the adenyl-cyclase activator forskolin

Received October 21, 1998; accepted February 16, 1999.
Correspondence and reprint requests: Ferenc Domoki, M.D., Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1010.
Supported in part by grants HL-30260, HL-46558, and HL50587 from the National Institutes of Health, by T-026295 OTKA from the Hungarian Science Foundation, and by T-07614/97 ETT from the Hungarian Ministry of Welfare.

directly affect vascular smooth muscle cells. The transient suppression of these physiologically relevant vasodilatory responses may be of importance in determining the final neurologic outcome of the ischemic insult.

The possible involvement of CD in the mechanism of diminished vascular responsiveness is currently unknown. Diminished cerebrovascular responses after CSD have been reported. In fact, both in the lissencephalic rat and the gyrencephalic cat, an abolition of the hypercapnic vasodilation lasting up to 10 h after a single CSD has been reported (10–13), although cerebral autoregulation was preserved (10). In contrast, no change in cerebrovascular reactivity to arterial hypercapnia, CGRP, or acetylcholine was observed in the rabbit (14). In CSD, the CD is accompanied by a large increase in regional cerebral blood flow and is followed by return to baseline or by prolonged hypoperfusion (10–14). The regulatory mechanisms by which these changes of vascular tone occur are still unclear but seem to involve competing influences of nitric oxide, CGRP, and prostanoids (15–19). In contrast with the reported reduced vascular responsiveness after CSD, repeated CSD are capable of producing similar increases in cerebral blood flow without attenuation of the vascular response in all species studied (13–15). Therefore, the possible role of CD in the attenuation of cerebrovascular responses during ischemia or CSD is still controversial.

The purpose of the present study was to investigate cerebral arteriolar dilator responses after inducing CD by direct application of KCl. Unlike in adult animals of several species, CSD is difficult to elicit in the piglet (personal observations). However, topical application of KCl offers an alternative means to study the intactness of cerebrovascular responsiveness to CD. We tested the hypothesis that CD reduces pial arteriolar dilation in response to NMDA, hypercapnia, CGRP, aprikalim, and forskolin.

METHODS

In these experiments, newborn piglets of either sex (1–7 d old, body weight 1–2 kg) were used. All procedures were approved by the Institution Animal Care and Use Committee. The animals were anesthetized with sodium thiopental (30–40 mg/kg i.p.) followed by intravenous injection of α-chloralose (75 mg/kg). Supplemental doses of α-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. The piglets were intubated via tracheotomy and were artificially ventilated with room air. The ventilation rate (~20/min) and tidal volume (~20 mL) were adjusted to maintain arterial blood gas values and pH in the physiologic range. In the present study, the piglets had normal values for arterial pH (7.40 ± 0.03), Pco_2 (29.5 ± 1 mm Hg), and Po_2 (99 ± 3 mm Hg) (n = 27).

Body temperature was maintained at 37–38°C by a water-circulating heating pad. The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19 mm in diameter) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic.

Extracellular DC potential was recorded in some animals to demonstrate CD. The DC potential was derived from the potential between a silver/silver-chloride electrode placed on the frontoparietal cortex 5 mm anterior to the cranial window and a grounded reference electrode placed in the subcutaneous connective tissue of the scalp, amplified (EXT-MC, Experimetria Ltd., Hungary), and stored with an on-line data acquisition software.

The closed window was filled with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O_2 and 6.5% CO_2 in balance N_2 to give pH = 7.33, Pco_2 = 46 mm Hg, and Po_2 = 43 mm Hg. The aCSF consisted of (in mmol/L) NaCl 132, KCl 2.9, $CaCl_2$ 1.2, $MgCl_2$ 1.4, $NaHCO_3$ 24.6, urea 6.7, and glucose 3.7. Diameters of pial arterioles were measured using a microscope (Wild M36, Switzerland) equipped with a video camera (Panasonic, Japan) and a video micro scaler (IV-550, For-A-Co., Newton, MA). After surgery, the cranial window was gently perfused with aCSF until a stable baseline was obtained. At the end of the experiments, the animals were killed while anesthetized with an intravenous bolus of KCl.

Experimental protocol. We examined the responses of cerebral arterioles to NMDA (10^{-5}, 5×10^{-5}, 10^{-4} mol/L, n = 9), hypercapnia (5 or 10% inhaled CO_2, n = 8), aprikalim (10^{-6}, 10^{-5} mol/L, n = 8), CGRP (10^{-7}, 10^{-6} mol/L, n = 8), and forskolin (10^{-6}, 10^{-5} mol/L, n = 8) before and 1 h after 3 min of CD. These doses of drugs and CO_2 were selected to yield intermediate and large changes in diameter. Whenever possible, we obtained data for two different drugs in each animal. With this protocol, we obtained arteriolar responses to aprikalim, forskolin, CGRP, and CO_2 similar to those we have observed before when only one of these drugs was given. Further, NMDA was not given with any other drug. The drugs were dissolved in aCSF and administered topically through the injectable ports of the cranial window onto the brain surface with a single application. Arteriolar diameters were measured continuously for 5 min for each dose. Then the window was flushed with aCSF. The second substance was administered when the arteriolar diameters returned to baseline values. Hypercapnia was elicited by artificially ventilating the animal with a gas mixture (5 or 10% CO_2, 21% O_2, balance N_2) for 5 min. Our previous results indicate that inhalation of 5 or 10% CO_2 in air results in elevation of Pco_2 to 45–50 and 70–75 mm Hg, respectively, with a simultaneous drop in pH (2). CD was achieved by single topical application of 1 mol/L KCl dissolved in aCSF for 3 min. After CD, the cranial window was infused several times with aCSF until arteriolar diameters returned to baseline.

Drugs. The drugs used in this study were NMDA, forskolin (both from Sigma Chemical Co.), aprikalim (Rhone-Poulenc-Rohrer), and CGRP (Research Biochemical International).

Statistics. Data are expressed as mean ± SEM. Data were analyzed using repeated measures ANOVA, followed by pairwise comparisons using the Student-Newman-Keuls test where appropriate.

RESULTS

Mean arterial blood pressure was in normal range (57 ± 2 mm Hg, $n = 27$) and did not change by topical drug application or hypercapnia throughout the measurements. In all animals, topical application of 1 mol/L KCl resulted in a large reduction of diameters of pial arterioles from 105 ± 3 to 48 ± 4 μm ($n = 27$). Also, there was a negative deflection in the extracellular DC potential (maximum 5–7 mV) indicating CD (Fig. 1). After 3 min, the KCl was washed out by repeated infusion of aCSF, and arteriolar diameters returned to baseline within 30–40 min.

Topical application of NMDA ($n = 9$) resulted in a dose-dependent arteriolar vasodilation (Fig. 2.). The baseline diameters were very similar (102 ± 2 *versus* 99 ± 3 μm before and after CD). Only at the highest dose was the absolute change in diameter slightly (~10%) decreased. However, the percent changes were intact (9 ± 1 *versus* 8 ± 1 at 10^{-5} mol/L, 19 ± 2 *versus* 18 ± 3 at 5×10^{-5} mol/L, and 29 ± 2 *versus* 26 ± 3 at 10^{-4} mol/L). Thus, the NMDA-induced vasodilation was not significantly affected by prior CD.

Inspiration of a gas mixture containing 5 or 10% CO_2 ($n = 8$) resulted in a concentration-dependent cerebral vasodilation (Fig. 2); the baseline diameters were not significantly different (97 ± 3 *versus* 91 ± 4 before and after CD). The percent changes were 15 ± 2 *versus* 16 ± 4 at 5% and 27 ± 5 *versus* 27 ± 6 at 10% inspired CO_2. In addition, the arteriolar dilation to hypercapnia was sustained and did not wane after CD similarly to the first challenge. We conclude that hypercapnic vasodilation remains intact at 1 h after topical KCl application.

CGRP ($n = 8$) caused a dose-dependent increase in arteriolar diameter; the response remained largely intact after 1 h of topical KCl application. Baseline diameters were 108 ± 5 *versus* 103 ± 4 μm before and after CD, and the percent changes were 15 ± 3 *versus* 16 ± 2 at 10^{-7} mol/L and 26 ± 4 *versus* 22 ± 3 at 10^{-6} mol/L. The aprikalim-induced vascular dilation ($n = 8$) was dose dependent and completely resistant to CD (Fig. 2). The baseline diameters were 102 ± 5 *versus* 103 ± 6 μm before and after CD. The percent changes for aprikalim were 21 ± 4 *versus* 18 ± 3 at 10^{-6} mol/L and 36 ± 5 *versus* 34 ± 5 at 10^{-5} mol/L. Similarly, there was no change in the responsiveness of pial arterioles to forskolin ($n = 8$). Baseline diameters were 101 ± 5 *versus* 96 ± 6 μm before and after CD, and percent changes were 16 ± 2 *versus* 16 ± 4 at 10^{-6} mol/L and 34 ± 5 *versus* 37 ± 7 at 10^{-5} mol/L.

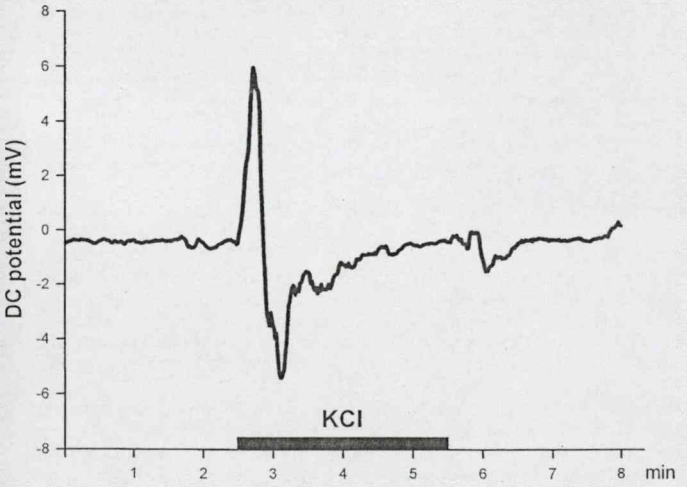

Figure 1. CD induced by 1 mol/L KCl solution. On the tracing, negative DC potential shift can be observed during KCl application. The CD lasts approximately 3 min.

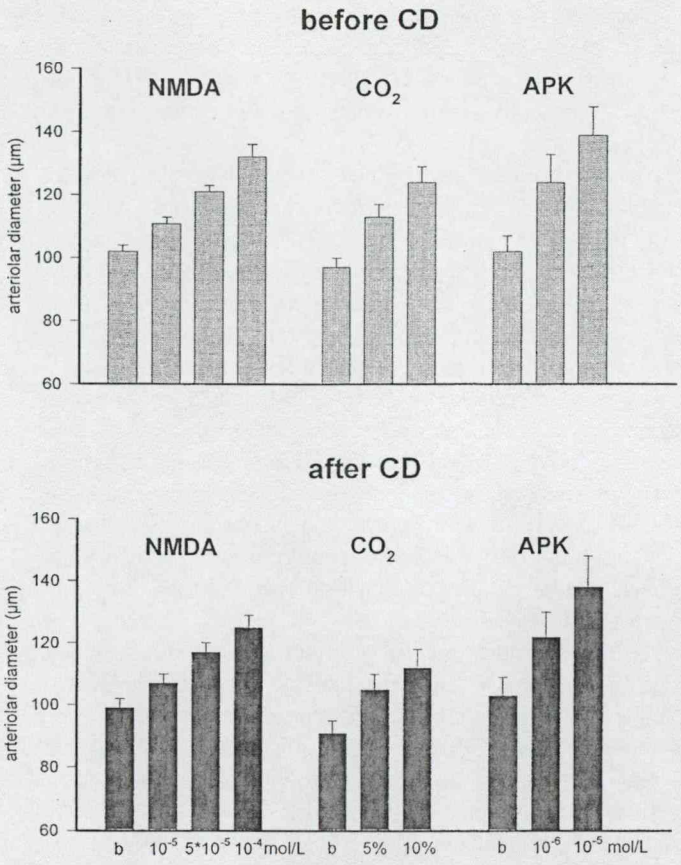

Figure 2. Effect of CD on pial arteriolar dilation to NMDA ($n = 9$), hypercapnia (CO_2, $n = 8$), and aprikalim (*APK*, $n = 8$). Baseline vascular diameters (*b*) were similar before and 1 h after 3 min of CD. All stimuli induced dose-dependent pial arteriolar vasodilation that was essentially unchanged after CD by 1 M KCl. Values are expressed as mean ± SEM.

DISCUSSION

The major finding from the present study is that cerebrovascular reactivity remains intact after CD in piglets. Although there may be a tendency for slight decreases in the vascular responses, especially at the highest doses (NMDA, hypercapnia, CGRP, but not aprikalim or forskolin), these changes were not significant. The overall picture indicates intact responsiveness to all dilator stimuli studied after CD.

Topical NMDA administration induces pial arteriolar vasodilation. This effect is indirect because cerebral vessels lack NMDA receptors (20–22). The activation of neuronal NMDA receptors leads to the synthesis of nitric oxide by neuronal nitric oxide synthase, resulting in a dilation of cerebral arterioles (6, 7). This mechanism was shown to be vulnerable to even short periods of hypoxia, ischemia, or asphyxia (23–25). In contrast, NMDA-induced vasodilation proved to be resistant to CD, indicating that other mechanisms are responsible for the

attenuation of the vascular response to NMDA after a short hypoxic/ischemic insult. Indeed, our results suggest that cortical neurons can be activated and show normal responses to external stimuli such as NMDA soon after recovery from depolarization.

Hypercapnic acidosis produces cerebral vasodilation by mechanisms that have not been identified. Extracellular acidosis probably plays a crucial role. In the newborn piglet, hypercapnic vasodilation is dependent on prostanoids and requires intact endothelium (8, 9). Hypercapnic vasodilation is abolished at 1 h after global cerebral ischemia in piglets (2). Also, after a single CSD, a complete abolition of the response to hypercapnia was reported in the rat and the cat (10–12) but not in the rabbit (14). In the present study, CD in piglets did not have any effect on the pial arteriolar vasodilation to hypercapnia, suggesting that endothelial function and prostanoid synthesis are unaltered after transient depolarization. However, changes in pial arteriolar diameter and those of cortical blood flow may not be parallel to all vasoactive stimuli.

Aprikalim causes vasodilation by opening K_{ATP} channels on vascular smooth muscle cells. Activation of potassium channels is an important cellular mechanism in a number of vasodilatory substances regulating vascular tone. K_{ATP} channels are sensitive to ischemic stress (4). Our present results show that the response to aprikalim is unchanged after CD. Forskolin is an activator of the adenyl-cyclase-cAMP system and the Ca^{2+}-sensitive K^+ channels (K_{Ca}) as well. The sensitivity of K_{Ca} channels is reduced by experimental head trauma in piglets (5). But our results show intact vascular responses to forskolin, indicating that these regulatory pathways are also unaltered after CD. CGRP induces vasodilation by activating receptors on the vascular smooth muscle cells. The cellular mechanisms of vasodilation include the activation of adenyl-cyclase and the activation of K_{ATP} channels as well (4). CGRP-induced vasodilation is severely reduced after global cerebral ischemia (3). In the present study, we found no significant attenuation in the vascular responses to CGRP after CD. These results suggest that vascular smooth muscle cells are functioning and capable of responding to different stimuli after CD.

The discrepancy between the results of the present study and those of the literature (10–13) remains to be explained. The CD achieved in this study was similar to the time course and magnitude of normal CSD recorded in other species. However, CD by KCl is different from CSD because, during CD, not only the cerebral cortex but also the pial vasculature has been depolarized. By use of intravital microscopy, a transient hypoperfusion has been observed by the sluggish blood flow in the constricted arterioles or recorded by laser-Doppler flowmetry (unpublished observations). Thus, direct depolarization of the brain surface by KCl is a significant stress, whereas CSD is usually regarded as a major but transient and ultimately benign perturbation of brain blood flow and metabolism. The fact that we do not see any substantial change in the arteriolar response to either vasodilatory stimuli at 1 h after 3 min of CD makes a pronounced effect of a single CSD unlikely. Further, if blood flow and/or cerebral metabolism are already compromised, CD/CSD may be more deleterious to the integrity of cerebrovascular regulatory mechanisms.

In summary, our data indicate that CD by itself does not change the responsiveness of the cerebral vasculature to several stimuli. This finding may have implications in the pathophysiology of the reduced cerebrovascular reactivity after ischemic conditions.

Acknowledgment. The authors thank Rhone-Poulenc-Rohrer for their generous gift of aprikalim.

REFERENCES

1. Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM 1996 Effects of ischemia on cerebrovascular responses to *N*-methyl-D-aspartate in piglets. Am J Physiol 270:H1225–H1230
2. Bari F, Louis T, Busija DW 1998 Effects of ischemia on cerebral arteriolar dilation to arterial hypoxia in piglets. Stroke 29:222–228
3. Louis TM, Meng W, Bari F, Errico RA, Busija DW 1996 Ischemia reduces CGRP-induced cerebral vascular dilation in piglets. Stroke 27:134–139
4. Bari F, Louis TM, Meng W, Busija DW 1996 Global ischemia impairs ATP-sensitive K^+ channel function in cerebral arterioles in piglets. Stroke 27:1874–1881
5. Armstead WM 1997 Role of impaired cAMP and calcium-sensitive K^+ channel function in altered cerebral hemodynamics following brain injury. Brain Res 768:177–184
6. Meng W, Tobin JR, Busija DW 1995 Glutamate-induced cerebral vasodilation is mediated by nitric oxide through *N*-methyl-D-aspartate receptors. Stroke 26:857–862; discussion 863
7. Faraci FM, Breese KR 1993 Nitric oxide mediates vasodilation in response to activation of *N*-methyl-D-aspartate receptors in brain. Circ Res 72:476–480
8. Leffler CW, Busija DW, Armstead WM, Shanklin DR, Mirro R, Thelin O 1990 Activated oxygen and arachidonate effects on newborn cerebral arterioles. Am J Physiol 259:H1230–H1238
9. Leffler CW, Mirro R, Shanklin DR, Armstead WM, Shibata M 1994 Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in newborn pigs. Am J Physiol 266:H623–H630
10. Lauritzen M 1984 Long-lasting reduction of cortical blood flow of the rat brain after spreading depression with preserved autoregulation and impaired CO_2 response. J Cereb Blood Flow Metabol 4:546–554
11. Wahl M, Lauritzen M, Schilling L 1987 Change of cerebrovascular reactivity after cortical spreading depression in cats and rats. Brain Res 411:72–80
12. Piper RD, Lambert GA, Duckworth JW 1991 Cortical blood flow changes during spreading depression in cats. Am J Physiol 261:H96–H102
13. Lacombe P, Sercombe R, Coreze JL, Springhetti V, Seylaz J 1992 Spreading depression induces prolonged reduction of cortical blood flow reactivity in the rat. Exp Neurol 117:278–286
14. Busija DW, Meng W 1993 Retention of cerebrovascular dilation after cortical spreading depression in anesthetized rabbits. Stroke 24:1740–1744
15. Read SJ, Smith MI, Hunter AJ, Parsons AA 1997 The dynamics of nitric oxide measured directly and in real time following repeated waves of cortical spreading depression in the anaesthetised cat. Neurosci Lett 232:127–130
16. Goadsby PJ, Kaube H, Hoskin KL 1992 Nitric oxide synthesis couples cerebral blood flow and metabolism. Brain Res 595:167–170
17. Wahl M, Schilling L, Parsons AA, Kaumann A 1994 Involvement of calcitonin gene-related peptide (CGRP) and nitric oxide in the pial artery dilatation elicited by cortical spreading depression. Brain Res 637:204–210
18. Colonna DM, Meng W, Deal DD, Gowda M, Busija DW 1997 Neuronal NO promotes cerebral cortical hyperemia during cortical spreading depression. Am J Physiol 272:H1315–H1322
19. Meng W, Colonna DM, Tobin JR, Busija DW 1995 Nitric oxide and prostaglandins interact to mediate arteriolar dilatation during cortical spreading depression. Am J Physiol 269:H176–H181
20. Beart PM, Sheehan KAM, Mannalack DT 1988 Absence of *N*-methyl-D-aspartate receptors on ovine cerebral microvessels. J Cereb Blood Flow Metab 8:879–882
21. Wendling WW, Chen D, Daniels FB, Monteforte MR, Fischer MB, Harakal C, Carlsson C 1996 The effects of *N*-methyl-D-aspartate agonists and antagonists on isolated bovine cerebral arteries. Anesth Analg 82:264–268
22. Morley P, Small DL, Murray CL, Mealing GA, Poulter MO, Durkin JP, Stanimirovic DB 1998 Evidence that functional glutamate receptors are not expressed on rat or human cerebromicrovascular endothelial cells. J Cereb Blood Flow Metab 18:396–406
23. Busija DW, Meng W 1993 Altered cerebral vascular responsiveness to *N*-methyl-D-aspartate after asphyxia in piglets. Am J Physiol 265:H389–H394
24. Bari F, Errico RA, Louis TM, Busija DW 1996 Differential effects of short term hypoxia and hypercapnia on *N*-methyl-D-aspartate-induced cerebral vasodilation in piglets. Stroke 27:1634–1640
25. Hoffman DJ, McGowan JE, Marro PJ, Mishra OP, Delivoria-Papadopoulos M 1994 Hypoxia-induced modification of the *N*-methyl-D-aspartate receptor in the brain of the newborn piglet. Neurosci Lett 167:156–160

II

Potassium Channel Activators Protect the *N*-Methyl-D-Aspartate–Induced Cerebral Vascular Dilation After Combined Hypoxia and Ischemia in Piglets

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

Background and Purpose—Cerebral arteriolar dilation to *N*-methyl-D-aspartate (NMDA) is a neuronally mediated multistep process that is sensitive to cerebral hypoxia and ischemia (H/I). We tested the hypothesis that topical pretreatment with the selective potassium channel agonists NS1619 and aprikalim preserves the vascular response to NMDA after consecutive H/I.

Methods—Pial arteriolar diameters were measured in anesthetized piglets with the use of a closed cranial window and intravital microscopy. Arteriolar responses to NMDA (10^{-5}, 5×10^{-5}, and 10^{-4} mol/L) were recorded before and 1 hour after 10 minutes of hypoxia (8.5% O_2 in N_2) plus 10 minutes of ischemia (H/I). Ischemia was induced by increasing intracranial pressure. Subgroups were topically pretreated with 10^{-5} mol/L NS1619, 10^{-6} mol/L aprikalim, 10^{-6} mol/L calcitonin gene–related peptide (CGRP), or 10^{-5} mol/L papaverine. We also examined the effects of H/I on vascular responses to kainate (10^{-4} mol/L) to assess specificity of neuronal injury.

Results—Arteriolar responses to NMDA were significantly attenuated after H/I. Baseline compared with post-H/I arteriolar diameters were 9±4% versus 3±2% at 10^{-5} mol/L, 22±4% versus 4±2% at 5×10^{-5} mol/L, and 33±4% versus 7±2% at 10^{-4} mol/L (mean±SE; all $P<.05$, n=7). Pretreatment with NS1619 and aprikalim preserved the arteriolar responses to NMDA after H/I. For NS1619 (n=6), values were as follows: 9±2% versus 6±4% at 10^{-5} mol/L, 19±6% versus 21±5% at 5×10^{-5} mol/L, and 35±3% versus 31±5% at 10^{-4} mol/L. For aprikalim (n=7), values were as follows: 6±2% versus 8±2% at 10^{-5} mol/L, 22±6% versus 15±3% at 5×10^{-5} mol/L, and 41±5% versus 32±6% at 10^{-4} mol/L. In contrast, piglets pretreated with CGRP (n=6) or papaverine (n=5) showed no preservation of the vascular response to NMDA after H/I, although these compounds dilated the arterioles to an extent similar to that with NS1619/aprikalim. Kainate-induced arteriolar dilation (n=6) was largely preserved after H/I compared with preischemic responses.

Conclusions—(1) Vascular responses of cerebral arterioles to NMDA after H/I are preserved by pretreatment with NS1619 or aprikalim, indicating a neuroprotective effect. (2) CGRP and papaverine do not preserve the vascular response to NMDA despite causing vasodilation similar to that with NS1619 or aprikalim. This suggests that activation of potassium channels on neurons accounts for the protective effect of potassium channel agonists. (3) Preserved arteriolar dilation to kainate suggests largely intact functioning of neuronal nitric oxide synthase after H/I. (***Stroke.*** **1998;29:837-843.)**

Key Words: cerebral arterioles ■ neuronal protection ■ *N*-methyl-D-aspartate ■ potassium channels

Glutamate is an important excitatory amino acid neurotransmitter in the central nervous system. It can bind to three different ionotropic glutamate receptor subtypes on neurons named after specific synthetic analogues: NMDA, kainate, and AMPA. Activation of neuronal NMDA and kainate receptors causes cerebral arteriolar dilation in different animal species, which is mediated in part or even totally by NO-dependent mechanisms.[1-6] This sequence may represent one of the mechanisms coupling local cerebral metabolism to blood flow.

See Editorial Comment, page 842

We and others have shown that global H/I severely attenuates or abolishes the arteriolar response to NMDA[7-9] as well as to other vasodilating agents such as CGRP and aprikalim.[10,11] Impaired neuronal-vascular coupling in combination with pathological metabolic and electrophysiological processes may contribute to delayed cerebral damage in brain H/I.[12]

From an experimental point of view, the integrity of the neuronal-vascular axis can be used as a model for the study of

Received October 1, 1997; final revision received December 18, 1997; accepted January 13, 1998.

From the Stroke Research Center (R.V.) and Department of Physiology and Pharmacology (R.V., F.D., F.B., D.W.B.), Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC; and Department of Physiology, Albert Szent-Györgyi Medical University, Szeged, Hungary (F.D., F.B.).

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

Selected Abbreviations and Acronyms

aCSF = artificial cerebrospinal fluid
AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
CGRP = calcitonin gene–related peptide
H/I = hypoxia/ischemia
K_{ATP} = ATP-dependent potassium channels
K_{Ca} = calcium-dependent potassium channels
NMDA = *N*-methyl-D-aspartate
NO = nitric oxide

potential neuroprotective agents. The rationale of this model is based on the observation that the arteriolar response to a neuron-mediated vasodilator (eg, NMDA) is attenuated after ischemia compared with preischemic baseline measurements. An experimental intervention is considered neuroprotective if it preserves the original vascular responsiveness to NMDA after ischemia.

Recent advances in the molecular biology and pharmacology of potassium channels[13] have enabled the investigation of potential therapeutic effects of K^+ channel agonists. Different types of ligand-operated channels have been characterized according to their primary regulatory or gating mechanism. Opening of K^+ channels leads to efflux of K^+ ions, which is a mechanism for recovering (repolarization) and/or enhancing (hyperpolarization) the membrane potential of a cell.[14] Potassium channel activation may therefore counteract ischemia-induced depolarizations. In previous studies K^+ channel agonists blocked ischemia-induced glutamate release in rat hippocampal slices[15] and prevented the ischemia-induced expression of immediate early genes in a rat model of global ischemia.[16]

The purpose of the present study was to test the hypothesis that potassium channel agonists preserve the NMDA-induced cerebral arteriolar dilation after consecutive H/I. Specifically, we examined the effects of NS1619 and aprikalim, putatively specific openers of calcium-dependent potassium channels (K_{Ca}) and ATP-dependent potassium channels (K_{ATP}), respectively. We also examined the effects of CGRP and papaverine to determine whether protective effects were independent of arteriolar dilation. Furthermore, we investigated whether the combined H/I protocol attenuated the kainate-induced arteriolar dilation, which had been resistant to ischemia alone in an earlier study.[6] Kainate-induced arteriolar dilation shares features of NMDA-induced arteriolar responses in that at least one half of the vascular response is NO dependent.

Materials and Methods

Surgical Preparation

Experiments were performed on newborn pigs (1 to 7 days) 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 by a tracheotomy and artificially ventilated. A femoral artery and vein were cannulated with polyethylene tubing (PE-90). Arterial blood gases and pH were repetitively measured, and rectal temperature was continually monitored. These parameters were kept within the normal physiological range. The head of each piglet was fixed in a stereotaxic apparatus. Approximately 3 mL of 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 in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel and glass cranial window with three ports was put into the opening, sealed with bone wax, and cemented with cyanoacrylate ester (SuperGlue) followed by one or two layers of dental acrylic. The closed window was filled with aCSF that was warmed to 37°C and equilibrated with 6% O_2, 6.5% CO_2, balance N_2. Arterioles were observed with a microscope (Wild M36) equipped with a television camera (Panasonic), and arteriolar diameter was measured with a video microscaler (IV-550, For-A Co).

Cerebral H/I

Cerebral hypoxia was induced by artificial ventilation with 8.5% O_2, balance N_2 over 10 minutes. Arterial blood gases were measured 8 minutes after hypoxia was started. Ten minutes of hypoxia were immediately followed by 10 minutes of global cerebral ischemia.

Cerebral ischemia was produced by implantation of a hollow brass bolt in the left parietal cranium 20 mm rostral to the cranial window. A 3-mm hole was drilled in the skull with an electric drill with a toothless bit, and the dura was exposed. The hollow bolt was inserted and secured in place with cyanoacrylate ester and dental acrylic. After implantation of the window and the bolt, aCSF was allowed to equilibrate with the periarachnoid CSF for 20 minutes. To induce ischemia, aCSF was infused 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 preischemia values. The heparinized blood was reinfused intravenously.

Experimental Design

At the beginning of each experiment the cranial window was flushed several times with aCSF until a stable baseline was observed. Then arteriolar responses to NMDA (10^{-5}, 5×10^{-5}, 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 five experimental groups. In group 1 (n=7) arteriolar responses to NMDA were recorded before (see above) and 1 hour after 10 minutes of hypoxia plus 10 minutes of global ischemia. In addition to this protocol, the other four groups were pretreated with a topical infusion 10 minutes before H/I of either NS1619 10^{-5} mol/L (group 2, n=6); aprikalim 10^{-6} mol/L (group 3, n=7); CGRP 10^{-6} mol/L (group 4, n=6); or papaverine, 10^{-5} mol/L (group 5, n=5). Arteriolar responses to these drugs were recorded during the infusion period. The drugs were washed away just before the beginning of hypoxia. In groups 1 and 4, 10^{-4} mol/L sodium nitroprusside was applied topically after the NMDA responses 1 hour after H/I were measured to examine the vascular responsiveness to exogenous NO.

In another experiment, the cerebral arteriolar responses to 10^{-4} mol/L kainate (group 5, n=6) before and 1 hour after H/I were determined.

Drugs

We used NMDA (Sigma), kainate (Sigma), sodium nitroprusside (Sigma), NS1619 (Research Biochemicals International), aprikalim (Rhone-Roulenc Rorer), CGRP (Research Biochemicals International), and papaverine (Sigma).

Statistical Analysis

Data are expressed as mean±SEM. A paired *t* test was used for comparing data between two groups. For repeated-measurement analysis, ANOVA was used, and the Student-Newman-Keuls test was then performed. Data analyses were performed on absolute and

Arteriolar Dilation to NMDA Before and After H/I

	NMDA Application Before H/I				NMDA Application 1 h After H/I			
	Baseline	10^{-5} mol/L	5×10^{-5} mol/L	10^{-4} mol/L	Baseline	10^{-5} mol/L	5×10^{-5} mol/L	10^{-4} mol/L
Group 1 (n=7): none								
Diameter	101±2	109±3	123±6†	134±5†	104±4	105±6	109±6*	111±6*
Δ Diameter		9±1	22±4†	33±4†		3±2	5±2*	7±2*
Group 2 (n=6): NS1619								
Diameter	103±3	112±3	123±7†	139±3†	104±3	109±3	125±5†	135±5†
Δ Diameter		9±1	20±6†	36±3†		6±2	25±5†	32±5†
Group 3 (n=7): aprikalim								
Diameter	101±4	107±5	123±8†	142±9†	104±4	113±6	120±6†	138±9†
Δ Diameter		6±2	22±6†	41±6†		9±2	15±3†	34±7†
Group 4 (n=6): CGRP								
Diameter	104±4	110±5	127±7†	133±8†	112±7	113±7	114±8*	118±9*
Δ Diameter		6±2	23±5†	29±7†		1±1	2±1*	3±1*

Values are mean±SEM, expressed in micrometers.
*Significantly different from respective preischemic value ($P<.05$).
†Significantly different from respective baseline.

percent change data. A P value $<.05$ was regarded as statistically significant.

Results

Before and after H/I, mean arterial blood pressures were stable and within normal limits for piglets. For example, in the NS1619 group, during baseline conditions arterial blood pressures were 64±4 mm Hg before H/I and 63±2 mm Hg 1 hour after H/I. Arterial blood pressures were not affected by the topical application of the drugs used in these experiments.

Arterial blood gases and pH were monitored regularly during the experiments and were generally kept within the physiological range. At baseline, pH was 7.45±0, P_{CO_2} was 32±1 mm Hg, and P_{O_2} was 106±4 mm Hg (n=32). After 8 minutes of hypoxia, pH was 7.40±0, P_{CO_2} was 32±2 mm Hg, and P_{O_2} was 27±1 mm Hg (n=24). Arterial blood gases were similar to baseline after recovery from H/I. Blood gases and pH did not differ significantly among groups.

Application of NMDA before H/I caused a reproducible, dose-dependent cerebral arteriolar dilation (Table, Fig 1). After H/I, however, arteriolar responses to NMDA were markedly reduced (Table, Fig 1). Subsequent administration of sodium nitroprusside (10^{-4} mol/L) caused consistent dilation of the same vessels by 25±2% compared with baseline.

Topical administration of the drugs 10 minutes before H/I caused arteriolar dilation by 15±4% for NS1619, 26±7% for aprikalim, 15±4% for CGRP, and 20±2% for papaverine.

Pretreatment with NS1619 (10^{-5} mol/L) resulted in an almost complete preservation of the arteriolar dilation to the different concentrations of NMDA 1 hour after H/I (Table, Fig 2), indicating no statistically significant difference between the preischemic and postischemic arteriolar responses. Similarly, administration of aprikalim (10^{-6} mol/L) preserved the post-H/I vascular response to NMDA (Table). Percent changes in vascular diameter to NMDA before and after H/I were as follows: 6±2% versus 8±2% at 10^{-5} mol/L, 22±6% versus 15±3% at 5×10^{-5} mol/L, and 41±5% versus 32±6% at 10^{-4} mol/L (n=7, $P>.05$). In contrast, piglets pretreated with CGRP (10^{-6} mol/L) showed a severe attenuation of the vascular response to NMDA comparable to the group that did not receive any pretreatment (Table). Vascular diameter increased by 6±2% versus 0±0% at 10^{-5} mol/L, 22±5% versus 2±1% at 5×10^{-5} mol/L, and 28±7% versus 2±1% at 10^{-4} mol/L of NMDA. In addition, vascular responses to NMDA were markedly attenuated after pretreatment with papaverine (10^{-5} mol/L). Arteriolar dilation before compared with after H/I was 7±1% versus 3±1% at 10^{-5} mol/L NMDA and 38±4% versus 16±6% at 5×10^{-5} mol/L NMDA (n=5, $P<.05$).

Administration of kainate (10^{-4} mol/L) before H/I dilated cerebral arterioles from a baseline of 106±3 μm by 27±4%. One hour after H/I kainate induced a 21±3% dilation from a baseline of 110±6 μm ($P>.05$).

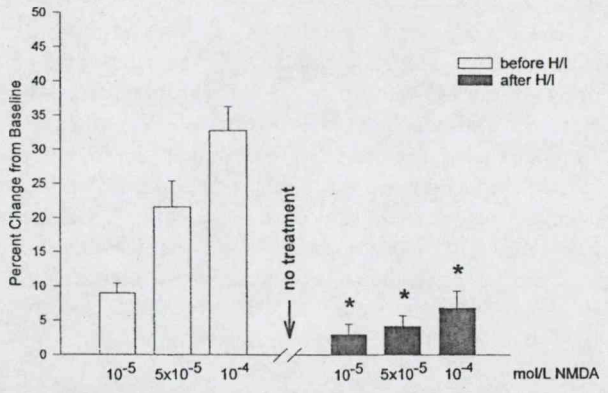

Figure 1. Effects of H/I on cerebral arteriolar dilation to NMDA. Cerebral arteriolar dilator responses to NMDA were significantly decreased 1 hour after 10 minutes of hypoxia plus 10 minutes of ischemia. Values are mean±SEM for 7 piglets. *Significantly different from preischemic dilation ($P<.05$).

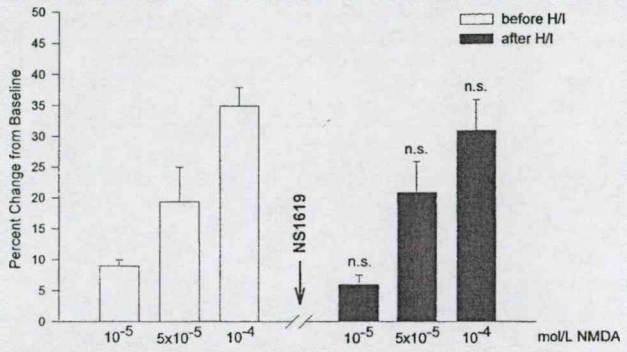

Figure 2. Effects of NS1619 pretreatment on cerebral arteriolar dilation to NMDA after H/I. Cerebral arteriolar responses to NMDA were preserved 1 hour after 10 minutes of hypoxia plus 10 minutes of ischemia by NS1619 (10^{-6} mol/L). Values are mean±SEM for 6 piglets. n.s. indicates not significantly different from preischemic dilation ($P>.05$).

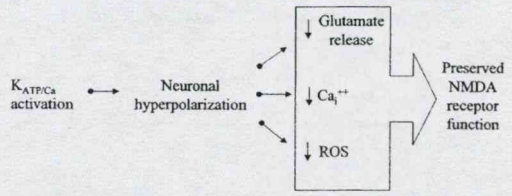

Figure 3. Potential mechanisms by which potassium channel activators may protect the NMDA-induced cerebral arteriolar dilation during and after H/I. Efflux of K^+ through activated K^+ channels leads to hyperpolarization of neurons. $K_{ATP/Ca}$ indicates ATP-dependent/calcium-dependent potassium channels; Ca_i^{++}, intracellular calcium concentration; and ROS, reactive oxygen species.

Discussion

There are three major new findings from these experiments: First, the vascular responsiveness of cerebral arterioles to NMDA, which is attenuated after consecutive H/I, was preserved by topical pretreatment with either NS1619 or aprikalim. Second, CGRP and papaverine did not preserve the vascular response to NMDA, although they caused dilation of the cerebral arterioles to an extent similar to that of the potassium channel agonists before H/I. Third, the kainate-induced arteriolar dilation was largely preserved after H/I.

Cerebral arterioles do not possess NMDA receptors.[17,18] Consequently, the NMDA-induced cerebral arteriolar dilation can only take place through an indirect pathway involving production, release, and action of NO. While the complete sequence of mechanisms involved is currently unknown, NMDA mainly causes cerebral arteriolar dilation through the sequential production of neuronal NO and vascular smooth muscle cGMP.[1-5,19]

In the present study the combined stress of H/I markedly attenuated the response of cerebral arterioles to NMDA as measured 1 hour after the insult. Previously, we have shown that a period of 5 to 15 minutes of either ischemia, asphyxia, or hypoxia is able to attenuate the NMDA-induced arteriolar dilation.[7,8,20] Administration of either NS1619 or aprikalim, putatively selective agonists of K_{Ca} channels[13,21,22] and K_{ATP} channels,[23-25] respectively, essentially preserved the response of cerebral arterioles to NMDA. The mechanism leading to this unprecedented finding is as yet unclear. NS1619 and aprikalim can dilate cerebral arterioles by the direct stimulation of potassium channels on vascular smooth muscle cells.[25-28] Therefore, hypothetically, the preservation of the NMDA-induced vascular response may take place as a consequence of vasodilation before ischemia. This hypothesis, however, is hard to reconcile with the observation that the similar dilation of cerebral arterioles to CGRP and papaverine before ischemia had little or no effect on the postischemic vascular response to NMDA.

Several different subtypes of potassium channels (eg, voltage-gated, ATP-dependent, calcium-dependent, inward rectifier potassium channels) have been identified on cells,[14] and all subtypes are present on neurons.[29] The interaction of NS1619 and aprikalim with their respective potassium channels on neurons therefore represents an alternative mechanism through which their preservative effect on the NMDA-mediated neuronal-vascular coupling may have taken place. Although the gating mechanism and conduction properties differ between subtypes, opening of potassium channels generally leads to hyperpolarization of excitable cells by increasing the efflux of potassium ions from the relatively negatively charged intracellular compartment into the extracellular space. Current concepts of H/I brain damage include the deleterious effects of excessive secretion of neurotoxic excitatory neurotransmitters[30] and the intracellular accumulation of calcium.[31] Theoretically, potassium channel openers may counteract these effects by hyperpolarizing presynaptic and postsynaptic neurons.[14,29] Potassium channel openers also may inhibit the release of calcium from intracellular stores.[14] This may lead to decreased glutamate secretion, slowing of the depolarization rate, diminished intracellular calcium accumulation, lower energy consumption, and reduced production of free oxygen radicals (Fig 3).

Experimental data supporting this attractive neuroprotective concept are sparse. Wind et al[32] found protection of cultured neurons against chemically induced hypoxia by bimakalim, a K_{ATP} activator. Suzuki and coworkers[33] showed that pretreatment with pinacidil, a partial K_{ATP} agonist, shortened the recovery time of spinal reflexes after spinal ischemia in cats. When glibenclamide, an inhibitor of K_{ATP}, was coadministered, the protective effect was absent. Similarly, in a study by Riepe et al,[34] glibenclamide partly reversed increased hypoxic tolerance after chemical inhibition of oxidative phosphorylation in hippocampal slices. Zini et al[15] reported that K^+ channel agonists blocked ischemia-induced glutamate release in rat hippocampal slices. Using a rat model of global ischemia, Heurteaux et al[16] demonstrated that several K_{ATP} openers administered intracerebroventricularly blocked the expression of immediate early genes in the hippocampus. The same and a similar study by these authors[16,35] also showed a decrease in delayed neuronal death in hippocampal CA1 neurons. We are not aware of other studies examining the

neuroprotective effect of K_{Ca} openers in cerebral hypoxia and ischemia.

The methods in the present study do not allow us to identify the precise molecular site at which NS1619 and aprikalim preserved the NMDA-mediated neuronal-vascular coupling after H/I. We speculate that both types of potassium channel openers preserved the NMDA-mediated response by indirectly protecting the NMDA receptor complex. Based on the results of previous work, we suggested that the affected step in the NMDA-vasodilating sequence may be the neuronal NMDA receptor rather than NO synthesis or the action of subsequent metabolites.[8,20] The present study partly supports this concept since the arterioles, which did not respond to NMDA after H/I, still dilated to sodium nitroprusside, an NO donor. Indeed, the postischemic dilation to sodium nitroprusside (25±2%) was of an extent similar to that of previous preischemic control measurements in the same experimental setup. Moreover, the partially (approximately 80%) preserved arteriolar response to kainate, which has been shown to be mediated equally by NO and prostaglandins[6] in this model, suggests that neurons carrying glutamate receptors maintain some signaling mechanisms including stimulus-dependent NO synthesis. However, we cannot rule out a compensatory role of prostaglandins in kainate-induced arteriolar dilation after H/I.

Holland et al[36] demonstrated a neuroprotective effect of intravenous CGRP in a rat model of focal ischemia. Since CGRP does not easily cross the blood-brain barrier, the authors speculated that the recorded increase in cerebral blood flow may account for their findings. In contrast, CGRP failed to preserve the NMDA-mediated neuronal-vascular coupling in our experiments, although it dilated cerebral arterioles before ischemia. CGRP exerts its vasodilating effect largely through activation of K_{ATP} on smooth muscle cells.[37] In contrast to direct activators of potassium channels (eg, aprikalim, NS1619), which are known to hyperpolarize neurons, the effect of CGRP on neurons is probably more complex and may depend on the neuronal circuit involved. CGRP receptors have been shown to be present in the porcine cortex,[38] but we are not aware of any study investigating the receptor density in certain cortical layers or even colocalization with NMDA receptor–positive neurons. To our knowledge the relationship of CGRP to neuronal potassium channels has never been studied. From our experiments we can only conclude that CGRP does not have a sufficient (if any) effect on neuronal K^+ channels to preserve the NMDA-mediated vasodilating sequence. The modest preservation of NMDA-induced dilation by papaverine may be due to effects on neuronal K^+ channels or to other yet undisclosed neuroprotective effects.

Even after the severe consecutive H/I stress, the vascular responses to kainate were only attenuated by approximately 20%. This confirms the remarkable resistance of the kainate-mediated neuronal-vascular sequence in general and of the kainate receptor in particular, which we reported recently.[6] It also illustrates the selectivity of damage caused by cerebral H/I affecting the response linked to one glutamate receptor subtype while in part sparing another.

We cautiously interpret our findings as indicative of a neuroprotective effect of potassium channel agonists on the postischemic function of neurons carrying the NMDA receptor. The immediate implications of this specific preservative effect for the overall protection of the brain against ischemia are currently unknown. We suggest, however, that the protective mechanisms of potassium channel openers are unlikely to be confined to the NMDA receptor but rather express their beneficial effect on another, more general pathophysiological mechanism in H/I.

The presented results may have clinical implications for focal ischemic stroke or cerebral global ischemia after heart arrest. Our experimental paradigm circumvents some of the problems associated with the systemic administration of central nervous system drugs.[39] In addition, postischemic treatment may encounter altered receptor interfaces favoring or disfavoring particular therapeutic strategies. In the present study the drugs were applied before H/I when receptors and signaling mechanisms were evidently intact. Our recent work has shown that postischemic vasodilation to K_{Ca} openers[40] but not to K_{ATP} agonists is resistant to ischemia.[11] Although the vasodilation induced by potassium channel openers is probably caused mainly by immediate interaction with potassium channels on vascular smooth muscle cells, the attenuated response to K_{ATP} agonists may indicate the vulnerability and transient nonresponsiveness of the targeted receptor and pathway. If this also involved neuronal K_{ATP} channels, they would become a less attractive target for neuroprotection than K_{Ca} channels for treatment started in the early postischemic period.

In summary, our findings show that topical pretreatment with the selective potassium channel openers NS1619 and aprikalim preserves the NMDA-mediated neuronal-vascular coupling after H/I in newborn piglets. This finding suggests considerable neuroprotective potential of these agents.

Acknowledgments

This study was supported by grants HL-30260, HL-46558, and HL-50587 from the National Institutes of Health and a grant from the Hungarian Ministry of Education (FKFP 0713/1997). We gratefully acknowledge that aprikalim was a gift from Rhone-Poulenc-Rohrer.

References

1. Busija DW, Leffler CW. Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol.* 1989;257:H1200–1203.
2. Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature.* 1988;336:385–388.
3. Armstead WM, Mirro R, Zuckerman S, Busija DW, Leffler CW. The influence of opioids on local cerebral glucose utilization in newborn pig. *Brain Res.* 1992;571:97–102.
4. Faraci FM, Breese KR. Nitric oxide mediates vasodilation in response to activation of *N*-methyl-D-aspartate receptors in brain. *Circ Res.* 1993;72:476–480.
5. Faraci FM, Breese KR, Heistead DD. Responses of cerebral arterioles to kainate. *Stroke.* 1994;25:2080–2084.
6. Bari F, Louis TM, Busija DW. Kainate-induced cerebrovascular dilation is resistant to ischemia in piglets. *Stroke.* 1997;28:1272–1277.

7. Busija DW, Meng W. Altered cerebral vascular responsiveness to *N*-methyl-D-aspartate after asphyxia in piglets. *Am J Physiol.* 1993;265: H389–H394.
8. Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM. Effects of ischemia on cerebrovascular responses to *N*-methyl-D-aspartate in piglets. *Am J Physiol.* 1996;270:H1225–1230.
9. Hoffman DJ, McGowan JE, Marro PJ, Mishra OP, Delivoria-Papadopoulos M. Hypoxia-induced modification of the *N*-methyl-D-aspartate receptor in the brain of the newborn piglet. *Neurosci Lett.* 1994;167:156–160.
10. Louis TM, Meng W, Bari F, Errico RA, Busija DW. Ischemia reduces CGRP-induced cerebral vascular dilation in piglets. *Stroke.* 1996;27: 134–139.
11. Bari F, Louis TM, Meng W, Busija DW. Global ischemia impairs ATP-sensitive K^+ channel function in cerebral arterioles in piglets. *Stroke.* 1996;27:1874–1881.
12. Hossmann KA. Periinfarct depolarizations. *Cerebrovasc Brain Metab Rev.* 1996;8:195–208.
13. Garcia ML, Hanner M, Knaus HG, Koch R, Schmalhofer W, Slaughter RS, Kaczorowski GJ. Pharmacology of potassium channels. *Adv Pharmacol.* 1997;39:425–471.
14. Lawson K. Potassium channel activation: a potential therapeutic approach? *Pharmacol Ther.* 1996;70:39–63.
15. Zini S, Roisin AAP, Armengaud C, Ben-Ari Y. Effect of potassium channel modulators on the release of glutamate induced by ischaemic-like conditions in rat hippocampal slices. *Neurosci Lett.* 1993;153:202–205.
16. Heurteaux C, Bertaina V, Widmann C, Ladzunski M. K^+ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein, and amyloid β-protein precursor genes and neuronal death in rat hippocampus. *Proc Natl Acad Sci U S A.* 1993;90:9431–9435.
17. Small D, Morley P, Lafferiere N, Mealing G, Poulter M, Durkin J, Stanimirovic D. Evidence that glutamate receptors are not expressed on rat or human cerebromicrovascular endothelial cells. *Stroke.* 1997;28:261. Abstract 152.
18. Beart PM, Sheehan KAM, Manallack DT. Absence of *N*-methyl-D-aspartate receptors on ovine cerebral microvessels. *J Cereb Blood Flow Metab.* 1988;8:879–882.
19. East SJ, Garthwaite J. NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. *Neurosci Lett.* 1991;123:17–19.
20. Bari F, Errico RA, Louis TM, Busija DW. Differential effects of short-term hypoxia and hypercapnia on *N*-methyl-D-aspartate–induced cerebral vasodilatation in piglets. *Stroke.* 1996;27:1634–1640.
21. Olesen SP, Munch E, Moldt P, Drejer J. Selective activation of Ca^{2+}-dependent K^+ channels by novel benzimidazolone. *Eur J Pharmacol.* 1994;251:53–59.
22. Edwards G, Niederste-Hollenberg A, Schneider J, Noack T, Weston AH. Ion channel modulation by NS1619, the putative BKCa channel opener in vascular smooth muscle. *Br J Pharmacol.* 1994;113:1538–1547.
23. Aloup JC, Farge D, James C, Mondot S, Cavero I. 2-(3-Pyridyl)-tetrahydrothiopyran-2-carbonthianmide derivatives and analogues: a novel family of potent potassium channel openers. *Drugs Future.* 1990; 15:1097–1108.
24. Richer J, Pratz J, Mulder P, Mondot S, Giudicelli JF, Cavero I. Cardiovascular and biological effects of K^+ channel openers, a class of drugs with vasorelexant and cardioprotective properties. *Life Sci.* 1990;47: 1693–1705.
25. Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y, Nelson MT. Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science.* 1989;245:177–180.
26. Quast U, Guillon JM, Cavero I. Cellular pharmacology of potassium channel openers in vascular smooth muscle. *Cardiovasc Res.* 1994;28: 805–810.
27. Faraci FM, Sobey CG. Potassium channels and the cerebral circulation. *Clin Exp Pharmacol Physiol.* 1996;23:1091–1095.
28. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol.* 1995;268:C799–C822.
29. Obrenovitch TP. Sodium and potassium channel modulators: their role in neuroprotection. *Int Rev Neurosci.* 1997;40:109–133.
30. Choi DW. Glutamate receptors and the induction of excitotoxic neuronal death. *Prog Brain Res.* 1994;100:47–51.
31. Kristian T, Siesjo BK. Calcium related damage in ischemia. *Life Sci.* 1996;59:357–367.
32. Wind T, Prehn JH, Peruche B, Krieglstein J. Activation of ATP-sensitive potassium channels decreases neuronal injury caused by chemical hypoxia. *Brain Res.* 1997;751:295–299.
33. Suzuki T, Sekikawa T, Nemoto T, Mriya H, Nakaya H. Effects of nicorandil on the recovery of reflex potentials after spinal cord ischaemia in cats. *Br J Pharmacol.* 1995;116:1815–1820.
34. Riepe MW, Esclaire F, Kaischke K, Schreiber S, Nakase H, Kempski O, Ludolph AC, Dirnagl U, Hugon J. Increased hypoxic tolerance by chemical inhibition of oxidative phosphorylation: 'chemical preconditioning.' *J Cereb Blood Flow Metab.* 1997;17:257–264.
35. Heurteaux C, Lauritzen I, Widmann C, Lazdunski M. Essential role of adenosine, adenosine A1 receptors and ATP-sensitive K^+ channels in cerebral ischemic preconditioning. *Proc Natl Acad Sci U S A.* 1995;92: 4666–4670.
36. Holland JP, Sydserff SGC, Taylor WAS, Bell BA. Calcitonin gene-related peptide reduces brain injury in a rat model of focal ischemia. *Stroke.* 1994;25:2055–2059.
37. Kitazono T, Heistad DD, Faraci FM. Role of ATP-sensitive K^+ channels in CGRP induced dilatation of basilar artery in vivo. *Am J Physiol.* 1993;265:H581–H585.
38. Wimalawansa SJ, El-Kholy AA. Comparative study of distribution and biochemical characterization of brain calcitonin gene-related peptide receptors in five different species. *Neuroscience.* 1993;54:513–519.
39. Pardridge WM. Drug delivery to the brain. *J Cereb Blood Flow Metab.* 1997;17:713–731.
40. Bari F, Louis TM, Busija DW. Calcium-activated K^+ channels in cerebral arterioles in piglets are resistant to ischemia. *J Cereb Blood Flow Metab.* 1997;17:1152–1156.

Editorial Comment

Acute episodes of cerebral ischemia followed by reperfusion produce reactive hyperemia[1] with subsequent reductions in cerebral blood flow,[2] disruption of the blood-brain barrier,[3] damage to cerebrovascular endothelium,[4] and impairment of NO synthase–dependent reactivity of cerebral arteries.[5] Mechanisms that contribute to impaired NO synthase–dependent reactivity of cerebral arteries after cerebral ischemia are unclear.

Recent evidence suggests that activators of K^+ channels may have potential therapeutic implications. It is possible that K^+ channel activators, by stimulating the efflux of potassium ions from cellular compartments, may counteract ischemia-induced depolarizations and thus protect the cerebral circulation. Glutamate is an important neurotransmitter in the brain and produces dilation of cerebral blood vessels, in part, through activation of NMDA receptors and synthesis/release of NO. Previous studies by this group[6] and others[7] have shown that cerebral H/I impairs or abolishes dilation of cerebral arterioles in response to NMDA. The purpose of the present study was to determine whether treatment with K^+ channel activators before cerebral H/I preserves NMDA-induced arteriolar dilation.

These investigators measured in vivo responses of piglet cerebral arterioles to topical application of NMDA before and after periods of cerebral H/I. The authors report impaired responses of cerebral arterioles to NMDA after H/I. Pretreatment with K^+ channel activators (aprikalim and NS1619), however, preserved arteriolar dilation to NMDA after cerebral H/I. Restoration in responses to NMDA after treatment with K^+ channel activators was specific since CGRP did not alter responses to NMDA.

Thus, it appears that activation of K^+ channels, presumably on neurons, accounts for preservation in cerebrovascular responses to NMDA after cerebral H/I. These findings suggest a potentially important therapeutic role for activation of K^+ channels after cerebral ischemia.

William G. Mayhan, PhD, *Guest Editor*
Department of Physiology and Biophysics
University of Nebraska Medical Center
Omaha, Nebraska

References

1. Gourley J, Heistad DD. Characteristics of reactive hyperemia in the cerebral circulation. *Am J Physiol.* 1984;246:H52–H58.
2. Levy DE, van Uitert RL, Pike CL. Delayed postischemic hypoperfusion: a potentially damaging consequence of stroke. *Neurology.* 1979;29: 1245–1252.
3. Sage JI, van Uitert RL, Duffy TE. Early changes in blood-brain barrier permeability to small molecules after transient cerebral ischemia. *Stroke.* 1984;15:46–50.
4. Paljarvi L, Rehncrona S, Soderfeldt B, Olsson Y, Kalimo H. Brain lactic acidosis and ischemic cell damage: quantitative ultrastructural changes in capillaries of rat cerebral cortex. *Acta Neuropathol (Berl).* 1983;60: 232–240.
5. Mayhan WG, Amundsen SM, Faraci FM, Heistad DD. Responses of cerebral arteries after ischemia and reperfusion in cats. *Am J Physiol.* 1988;255:H879–H884.
6. Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM. Effects of ischemia on cerebrovascular responses to *N*-methyl-D-aspartate in piglets. *Am J Physiol.* 1996;270:H1225–H1230.
7. Hoffman DJ, McGowan JE, Marro PJ, Mishra OP, Delivoria-Papadopoulos M. Hypoxia-induced modification of the *N*-methyl-D-aspartate receptor in the brain of the newborn piglet. *Neurosci Lett.* 1994; 167:156–160.

III

Mitochondrial Potassium Channel Opener Diazoxide Preserves Neuronal-Vascular Function After Cerebral Ischemia in Newborn Pigs

Ferenc Domoki, MD; James V. Perciaccante, MD; Roland Veltkamp, MD; Ferenc Bari, PhD; David W. Busija, PhD

Background and Purpose—*N*-Methyl-D-aspartate (NMDA) elicits neuronally mediated cerebral arteriolar vasodilation that is reduced by ischemia/reperfusion (I/R). This sequence has been preserved by pretreatment with the ATP-sensitive potassium (K_{ATP}) channel opener aprikalim, although the mechanism was unclear. In the heart, mitochondrial K_{ATP} channels (mitoK_{ATP}) are involved in the ischemic preconditioning-like effect of K^+ channel openers. We determined whether the selective mitoK_{ATP} channel opener diazoxide preserves the vascular dilation to NMDA after I/R.

Methods—Pial arteriolar diameters were determined with the use of closed cranial window/intravital microscopy in anesthetized piglets. Vascular responses to NMDA were assessed before and 1 hour after 10 minutes of global cerebral ischemia induced by raising intracranial pressure. Subgroups received 1 of the following pretreatments before I/R: vehicle; 1 to 10 μmol/L diazoxide; and coapplication of 100 μmol/L 5-hydroxydecanoic acid (5-HD), a K_{ATP} antagonist with diazoxide.

Results—NMDA-induced dose-dependent pial arteriolar dilation was not affected by diazoxide treatment only but was severely attenuated by I/R. In contrast, diazoxide dose-dependently preserved the NMDA vascular response after I/R; at 10 μmol/L, diazoxide arteriolar responses were unaltered by I/R. The effect of diazoxide was antagonized by coapplication of 5-HD with diazoxide. Percent preservation of 100 μmol/L NMDA–induced vasodilation after I/R was 53±19% (mean±SEM, n=8) in vehicle-treated controls versus 55±10%, 85±5%, and 99±15% in animals pretreated with 1, 5, and 10 μmol/L diazoxide (n=8, n=8, and n=12, respectively) and 60±15% in the group treated with 5-HD+diazoxide (n=5).

Conclusions—The mitoK_{ATP} channel opener diazoxide in vivo preserves neuronal function after I/R, shown by pial arteriolar responses to NMDA, in a dose-dependent manner. Thus, activation of mitoK_{ATP} channels may play a role in mediating the protective effect of other K^+ channel openers. (***Stroke.*** **1999;30:2713-2719.)**

Key Words: cerebral ischemia, global ■ *N*-methyl-D-aspartate ■ potassium channels ■ reperfusion injury ■ pigs

Glutamate elicits cerebral arteriolar vasodilation in piglets via a multistep process, involving activation of neuronal *N*-methyl-D-aspartate (NMDA) receptors, stimulation of nitric oxide (NO) production by neuronal NO synthase, and actions of NO on vascular smooth muscle cells.[1-3] This sequence of events may represent an important mechanism coupling local blood flow to metabolism and neuronal activity.

NMDA-induced vasodilation is attenuated by hypoxia and ischemia/reperfusion (I/R) in a dose- and time-dependent manner.[4-6] For example, 10 minutes of global ischemia followed by reperfusion reduces NMDA-induced vasodilation by ≈50%. However, arteriolar dilator responses to exogenously applied NO are intact,[5,6] thereby implying that the attenuation of the vascular response to NMDA is due to

See Editorial Comment, page 2718

effects of ischemia at the level of the neurons. Furthermore, results from other laboratories as well as our own indicate that dysfunction of the NMDA receptor rather than of general neuronal injury is the primary reason for attenuated arteriolar responsiveness to NMDA.[5,7] The mechanisms involved in attenuated arteriolar dilation to NMDA are not known with certainty but appear to involve actions of reactive oxygen species (ROS), such as superoxide anion. Thus, pharmacological agents that prevent production of superoxide anion or that scavenge this radical prevent attenuation of NMDA-induced dilator responses.[4,5,8]

In our laboratory, NMDA-induced vasodilation has been used as a sensitive bioassay to assess the functional integrity

Received June 16, 1999; final revision received August 2, 1999; accepted August 31, 1999.

From the Department of Physiology and Pharmacology (F.D., R.V., F.B., D.W.B.), Department of Pediatrics (J.V.P.), and Stroke Research Center (R.V.), Wake Forest University School of Medicine, Winston-Salem, NC; and Department of Physiology, Albert Szent-Györgyi Medical University, Szeged, Hungary (F.D., F.B.).

Correspondence to David W. Busija, PhD, Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157-1010. E-mail dbusija@wfubmc.edu

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

of the neuronal-vascular axis. For instance, we have shown that activation of ATP-sensitive potassium (K_{ATP}) channels with aprikalim for a short period immediately before combined hypoxic/ischemic stress preserves pial arteriolar dilation to NMDA.[6] Possible mechanisms of action of K_{ATP} activation may be via hyperpolarization of neurons through plasmolemmal K_{ATP} channels, which may result in (1) reduced glutamate release, (2) smaller increases in intracellular Ca^{2+} levels during ischemia, or possibly (3) less ROS production during reperfusion. However, intracellular sites of action of K^+ channel activators have not been considered previously.

Mitochondrial K_{ATP} (mitoK_{ATP}) channels have been found in the inner membrane of mitochondria[9] and represent a pharmacologically distinct population of K_{ATP} channels.[10] There is increasing evidence about the diverse functions of mitoK_{ATP} channels in the regulation of mitochondrial matrix volume, ATP production, and Ca^{2+} homeostasis in mitochondria, essential factors determining the outcome of ischemic stress on cellular function and survival.[11–14] In fact, several K^+ channel openers can mimic ischemic preconditioning (IPC) in the heart,[15] and mitoK_{ATP} channels are certainly involved in mediating these effects.[16–18] However, no study has investigated the possible beneficial role of mitoK_{ATP} channel activation in vivo in the brain and the cerebral circulation.

In this study our purpose was to determine whether diazoxide, a selective mitoK_{ATP} channel opener, would preserve the NMDA-induced arteriolar dilation 1 hour after 10 minutes of global cerebral ischemia. Additionally, we investigated whether 5-hydroxydecanoic acid (5-HD), a relatively selective inhibitor of mitoK_{ATP} channels, would reduce the effect of diazoxide.

Materials and Methods

Animals

Newborn piglets of either sex (age, 1 to 7 days; body weight, 1 to 2 kg) were used. All procedures were approved by the Institutional Animal Care and Use Committee. The animals were anesthetized with sodium thiopental (30 to 40 mg/kg IP) followed by injection of α-chloralose (75 mg/kg IV). Supplemental doses of α-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. The piglets were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (≈20/min) and tidal volume (≈20 mL) were adjusted to maintain arterial blood gas values and pH in the physiological range. Body temperature was maintained at 37°C to 38°C by a water-circulating heating pad. Body temperature, arterial pH, and blood gases were also in the normal ranges and did not vary significantly among different groups. For instance, in group 5, the values were as follows: body temperature, 37.9±0.2°C; pH, 7.51±0.03; P_{CO_2}, 33.3±1.9 mm Hg; and P_{O_2}, 97±4 mm Hg.

The head of the piglet was fixed in a stereotaxic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19 mm in diameter) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with 3 needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester (Super Glue) and dental acrylic.

The closed window was filled with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O_2 and 6.5% CO_2 in balance N_2 to give pH=7.33, P_{CO_2}=46 mm Hg, and P_{O_2}=43 mm Hg. The aCSF consisted of the following (mmol/L): NaCl 132, KCl 2.9, $CaCl_2$ 1.2, $MgCl_2$ 1.4, $NaHCO_3$ 24.6, urea 6.7, and glucose 3.7. Diameters of pial arterioles were measured with a microscope (Wild M36) equipped with a video camera (Panasonic) and a video micro scaler (IV-550, For-A-Co). After surgery, the cranial window was gently perfused with aCSF until a stable baseline was obtained. At the end of the experiments, the animals were killed while anesthetized with an intravenous bolus of KCl.

Cerebral Ischemia

To induce global cerebral ischemia, a 3-mm hole was made by an electric drill with a toothless bit, and the dura was exposed. A hollow brass bolt was inserted in the left frontal 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 raise intracranial pressure above arterial pressure. Ischemia was verified by the cessation of blood flow in the observed vessels. Previously, we have shown using microspheres that cerebral blood flow is virtually zero in all brain areas examined during the ischemic period.[19] Venous blood was withdrawn as necessary to maintain mean arterial blood pressure near normal values. At the end of the ischemic period, the infusion tube was clamped, and the intracranial pressure returned to preischemic values. The heparinized blood was reinfused intravenously.

Experimental Design

After obtaining stable baseline arteriolar diameters, we examined the responses of cerebral arterioles to NMDA (10, 50, 100 μmol/L, except in group 7). NMDA and all other drugs were dissolved in aCSF and administered topically through the injectable ports of the cranial window onto the brain surface with single application. Arteriolar diameters were measured continuously for 5 to 7 minutes for each dose of NMDA. Then the window was flushed with aCSF, and the arteriolar diameters returned to baseline values. Instrumented piglets (n=49) were then divided into 7 groups, as follows.

Group 1 (n=4)

To assess whether diazoxide may have direct effect on NMDA-induced vasodilation, in the first group the animals were treated with 10 μmol/L diazoxide for 10 minutes but did not undergo ischemia. NMDA challenge was repeated 1 hour after treatment with diazoxide.

Group 2 (n=8)

To repeat our previous findings on attenuation of NMDA-induced vasodilation by I/R, in this group the piglets received vehicle (aCSF) and were exposed to 10 minutes of global cerebral ischemia followed by reperfusion. In all ischemia groups, NMDA-induced changes in pial arteriolar diameters were reexamined after the first hour of reperfusion. We have shown that attenuation of cerebral vasodilation to NMDA is greatest 1 hour after I/R (1 hour is also the shortest time after I/R at which the measurements are technically feasible).

Groups 3 to 5 (n=8, n=8, and n=12, Respectively)

To investigate the effect of diazoxide on preservation of NMDA-induced vasodilation, in these groups the piglets were pretreated with 1, 5, and 10 μmol/L diazoxide, respectively, for 10 minutes before the initiation of 10 minutes of global cerebral ischemia. The diazoxide was removed by flushing the window with aCSF just before the initiation of ischemia.

Group 6 (n=5)

To investigate the inhibitory effect of 5-HD on K_{ATP} channels activated by diazoxide, the piglets were pretreated with 100 μmol/L 5-HD for 5 minutes, followed by coapplication of 100 μmol/L 5-HD and 10 μmol/L diazoxide for 10 minutes before 10 minutes of ischemia. The diazoxide and 5-HD were removed by flushing the window with aCSF just before the initiation of ischemia.

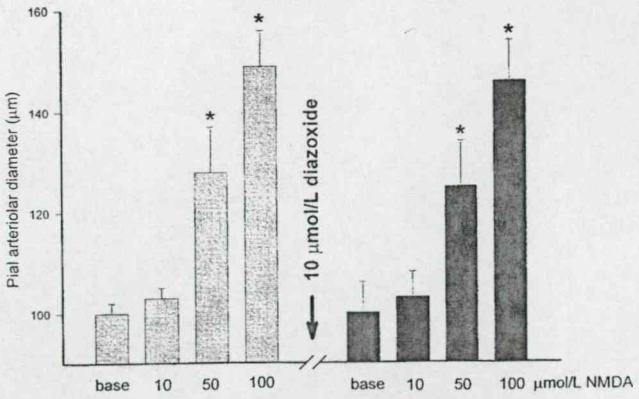

Figure 1. Effect of 10 μmol/L diazoxide on pial arteriolar responses to NMDA. NMDA induced dose-dependent vasodilation that was unaffected 1 hour after topical application of diazoxide for 10 minutes (n=4). *$P<0.05$, significantly different from corresponding baseline diameter (base).

Group 7 (n=4)

To study the effect of 5-HD on the sarcolemmal K_{ATP} channels and the vascular response to NMDA, we examined the cerebral arteriolar responses to the nonselective K_{ATP} channel opener aprikalim (10 μmol/L) followed by 100 μmol/L NMDA. Then we coapplied 10 μmol/L aprikalim and 100 μmol/L 5-HD for 10 minutes. We repeated the NMDA challenge 1 hour after pretreatment with 5-HD+aprikalim. Previously we have shown that aprikalim treatment does not affect the vascular response to NMDA.[6] Between each drug application we flushed the window several times with aCSF, until arteriolar diameters returned to baseline values.

Drugs

The drugs used in this study were NMDA (Sigma), diazoxide (Sigma), 5-HD (H135, Research Biochemicals International), and aprikalim (Rhone-Roulenc-Rohrer).

Statistical Analysis

Data are expressed as mean±SEM. Pial arteriolar diameter data were analyzed with repeated-measures ANOVA, followed by pairwise comparisons using the Student-Newman-Keuls test when appropriate. Percent preservations of preischemic vasodilation data were analyzed with 1-tailed t test. P values of <0.05 were considered statistically significant.

Results

Arterial blood pressure was in the normal range and was not significantly different before and 1 hour after ischemia; for instance, in group 5 arterial pressure was 70±4 mm Hg before and 68±4 mm Hg after I/R (n=12).

Topical application of diazoxide did not affect pial vascular diameters significantly. Typically, there was only a transient dilation immediately on application of diazoxide. Percent changes from baseline diameters were as follows: group 3, no vasoactivity was observed; group 4, 2±1%; and group 5, 9±3%. Vascular diameters quickly returned to baseline values in 2 to 3 minutes, and none of these changes were significantly different from baseline values.

NMDA elicited dose-dependent pial arteriolar vasodilation (Figures 1 and 2). In group 1, 10 μmol/L diazoxide did not potentiate or attenuate vascular dilations to NMDA 1 hour after diazoxide treatment (Figure 1). Baseline arteriolar diameters were 100±2 μm before and 100±6 μm 1 hour after diazoxide treatment. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after diazoxide treatment) were 3±1% versus 4±1%, 28±7% versus 26±9%, and 50±8% versus 47±8%, respectively.

Global cerebral ischemia (10 minutes) followed by reperfusion significantly reduced pial arteriolar responses to NMDA (Figure 2). In group 2, baseline arteriolar diameters were 100±3 μm before and 103±4 μm 1 hour after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after ischemia) were 6±2% versus 2±1%, 28±5% versus 9±3%, and 38±5% versus 16±4%, respectively. Thus, vascular dilations to 100 μmol/L NMDA were diminished by ≈50% (Figure 3).

Diazoxide exhibited a dose-dependent effect on preservation of NMDA-induced vasodilation after I/R. In group 3, decreases in pial arterial responsiveness to NMDA were similar to those observed in group 2 (Figures 2 and 3). In group 3, baseline arteriolar diameters were 102±3 μm before and 104±3 μm 1 hour after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after ischemia) were 5±2% versus 3±1%, 20±7% versus 8±2%, and 38±5% versus 19±3%, respectively. In contrast, in groups 4 and 5 we found a dose-dependent preservation of pial vascular responses to NMDA (Figures 2 and 3). More specifically, in group 4, baseline arteriolar diameters were 95±3 μm before and 95±4 μm 1 hour after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after ischemia) were 4±0% versus 7±2%, 30±10% versus 23±6%, and 45±6% versus 37±3%, respectively. In group 5, baseline arteriolar diameters were 102±6 μm before and 106±5 μm 1 hour after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after ischemia) were 7±1% versus 6±2%, 28±5% versus 24±4%, and 36±5% versus 32±4%, respectively. Therefore, pretreatment with 10 μmol/L diazoxide resulted in virtually full preservation of pial arteriolar responses to NMDA 1 hour after I/R compared with preischemic values.

Topical application of the K_{ATP} channel antagonist 5-HD and coapplication of 5-HD with diazoxide did not alter pial arteriolar diameters. In addition, 5-HD treatment did not affect pial arteriolar responses to NMDA. In group 7, baseline arteriolar diameters were 105±9 μm before and 103±7 μm 1 hour after pretreatment with 5-HD. Percent changes in pial arteriolar diameter from baseline to 100 μmol/L NMDA (before versus 1 hour after 5-HD treatment) were 52±3% versus 56±7%. However, pretreatment with 5-HD and diazoxide abolished the protection on NMDA-induced vasodilation achieved by diazoxide alone (Figures 2 and 3). In group 6, baseline arteriolar diameters were 90±6 μm before and 92±6 μm 1 hour after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before versus 1 hour after ischemia) were 3±1% versus 0±0%, 40±12% versus 19±6%, and 61±7% versus 33±5%, respectively. Interestingly, coapplication of 5-HD with aprikalim did not block the vasodilation elicited by aprikalim. In group 7, pial baseline arteriolar diameters were

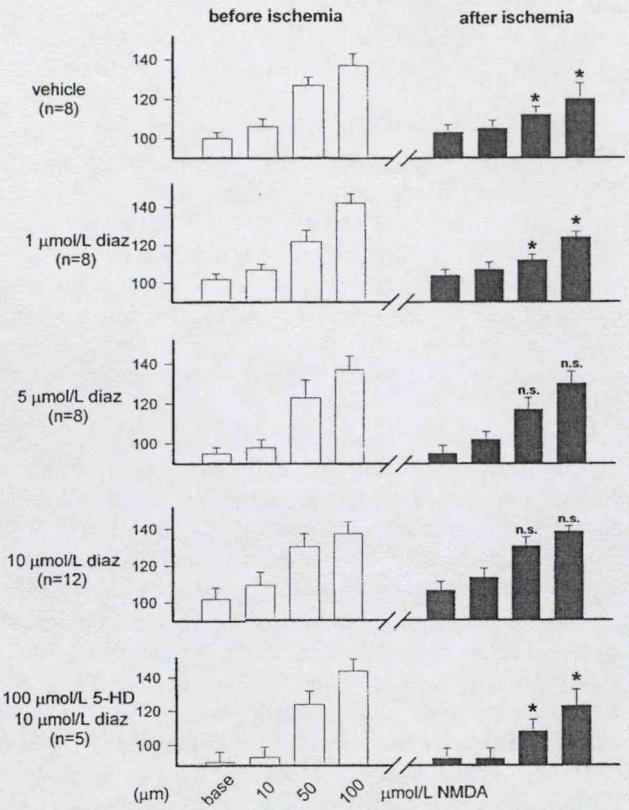

Figure 2. Changes in pial arteriolar diameters in response to NMDA 1 hour after 10 minutes of cerebral ischemia. Baseline diameters (base) did not change significantly in any groups after I/R. However, in the nontreated animals arteriolar responses to 50 and 100 μmol/L NMDA were severely reduced by ≈50%. Pretreatment with 1 μmol/L diazoxide (diaz) did not affect the reduction in NMDA-induced vascular dilation by I/R. In contrast, pretreatment with 5 or 10 μmol/L diazoxide resulted in preserved vascular responses; the changes in pial arteriolar diameters were not significantly different compared with preischemic values. Coapplication of 100 μmol/L 5-HD, a relatively specific inhibitor of mitoK$_{ATP}$ channels with 10 μmol/L diazoxide attenuated the protective effect of diazoxide. *$P<0.05$, significantly different from corresponding preischemic value.

102±8 μm before application of aprikalim alone and 101±7 μm before coapplication of aprikalim and 5-HD. Percent changes in pial arteriolar diameter from baseline to 10 μmol/L aprikalim were 65±6% versus 65±6% (aprikalim alone versus aprikalim+5-HD, respectively).

Discussion

The major finding of the present study is that the selective mitoK$_{ATP}$ channel opener diazoxide dose-dependently preserves NMDA-induced cerebral arteriolar vasodilation after I/R in piglets. Since NMDA-induced vasodilation is dependent on intact neuronal function, we present evidence for the first time showing an in vivo protective effect of diazoxide after I/R in the central nervous system.

Previously, we found that the nonselective K$_{ATP}$ channel opener aprikalim protected NMDA-induced vasodilation after combined hypoxia-ischemia.[6] The protective effect of aprikalim was shown to be mediated by neuronal rather than vascular K$_{ATP}$ channels and was independent of the vasodilation elicited by aprikalim. Our present data confirm that the protective effect of pretreatment with K^+ channel openers is independent of vasodilation accompanied by the administration of such drugs: diazoxide showed no significant vasoactivity but preserved NMDA-induced dilation. The beneficial effects of K$_{ATP}$ channel openers reducing injury by I/R have been most extensively studied in the heart. K$_{ATP}$ channels serve as the final common pathway in the event of IPC, a phenomenon in which short periods of ischemia protect the heart from subsequent exposure of a more prolonged period of ischemia. K^+ channel openers mimic IPC,[15–18] and the protection by IPC is blocked by K$_{ATP}$ channel inhibitors.[20–22] The exact mechanism of this remarkable effect has not been elucidated.

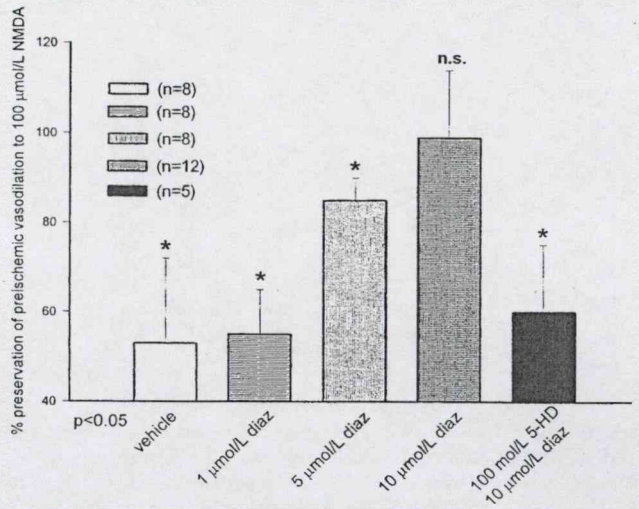

Figure 3. Protective effect of diazoxide (diaz) on 100 μmol/L NMDA–induced pial arteriolar dilation. Data are expressed as percent preservation of dilation compared with preischemic values. Note the dose-dependent preservation of vascular responses in the diazoxide-treated groups; at 10 μmol/L diazoxide the vascular response was virtually identical to preischemic value. 5-HD antagonized the effect of diazoxide. *Significantly different from preischemic value.

The discovery of mitoK$_{ATP}$ channels added further complexity to the interpretation of experimental data from pharmacological interventions on these channels. Unfortunately, there are no absolutely selective pharmacological tools to assess the mitoK$_{ATP}$ channels in vivo. However, a consistent and unique feature of these channels is their remarkably selective sensitivity to opening by diazoxide. The mitoK$_{ATP}$ channel was found to be >2000-fold more sensitive to diazoxide than the sarcolemmal K$_{ATP}$ channel in bovine cardiac myocytes ($K_{1/2}$ was 0.4 μmol/L for mitoK$_{ATP}$ channel versus 855 μmol/L for sarcolemmal K$_{ATP}$ channel). In contrast, cromakalim was an equally potent opener of both mitochondrial and plasma membranes.[10] Subsequently, mitoK$_{ATP}$ channel selective concentrations (5 to 20 μmol/L) of diazoxide have been demonstrated to improve functional recovery in isolated rat hearts after I/R in a manner similar to that of a nonselective K$_{ATP}$ channel opener, cromakalim. The cardioprotection by diazoxide was inhibited by K$_{ATP}$ channel inhibitors glibenclamide and 5-HD, confirming the effect of diazoxide via K$_{ATP}$ channels.[17] In a different study, in intact

rabbit ventricular myocytes, diazoxide induced mitochondrial depolarization, demonstrated by flavoprotein fluorescence with a $K_{1/2}$ of 27 μmol/L, but did not affect the simultaneously measured sarcolemmal K_{ATP} channel current.[16] These findings and others in the literature (for recent review, see Reference 15) strongly indicate the involvement of mitoK_{ATP} channels in the development of acute and perhaps delayed IPC in the heart.

In our present experiments we used topical diazoxide (1 to 10 μmol/L) in the mitoK_{ATP} channel–selective dose range. We did not test directly whether only mitoK_{ATP} channels were activated by diazoxide, but fortunately a good indication of selective activation was the absence of significant vasodilation accompanied by application of diazoxide. The vasodilatory effect of K^+ channel openers on cerebral arterioles was directly mediated by the sarcolemmal K^+ channels. Administration of 5 to 10 μmol/L diazoxide elicited only 2% to 9% arteriolar dilation, and the response was transient, ie, it did not last for >1 to 2 minutes. In contrast, we found that the nonselective K_{ATP} channel opener aprikalim (10 μmol/L) elicits ≈60% to 70% increases in vascular diameters, and the vasodilation does not wane. Moreover, the dose-dependent effect of diazoxide on preservation of the NMDA-induced vasodilation after I/R was inhibited by the selective K_{ATP} channel antagonist 5-HD, and 5-HD was found to be selective for mitoK_{ATP} channels, at least in some experimental designs.[16,17,23] Additionally, in our experimental model 5-HD did not inhibit the vasodilation induced by aprikalim, suggesting minor effects on plasmolemmal K_{ATP} channel channels. These observations, together with those of the literature, lead us to conclude that the protective effect of diazoxide on neuronal-vascular function after I/R is probably mediated by activation of mitoK_{ATP} channels.

The mechanism by which activation of mitoK_{ATP} channels may lead to increased resistance to I/R remains to be clarified. In our experimental model, NMDA-induced vascular response is severely attenuated at 1 hour after I/R, and responsiveness gradually returns over the time course of 2 to 4 hours.[5,24] The duration of global cerebral ischemia (10 minutes) used in the present study has been thought to cause only reversible mitochondrial alterations, ie, mitochondria have been shown to recover full function 1 to 2 hours after reperfusion.[25,26] Thus, the attenuation of the NMDA-mediated cerebral arteriolar response is not likely due to energy failure by inhibited mitochondrial function. This statement is further supported by our previous findings that kainate-induced vasodilation is resistant to ischemia in the same experimental model.[27] Additionally, neuronal NO synthase levels and activity are unchanged by I/R, and cerebral arterioles show normal responses to exogenous NO donors such as sodium nitroprusside after ischemia.[5,6] Therefore, the primary target of I/R may be the NMDA receptor itself. The acute effect of ischemia on NMDA-induced pial arteriolar vasodilation has been amply demonstrated to be mediated by ROS (Figure 4). Thus, NMDA-induced vascular response has been found to be preserved by ROS scavengers and inhibitors of cyclooxygenase (COX) activity,[4,5,8,24] a major source of ROS after I/R.[28] Our recent observations on the preservation of neural function with K^+ channel openers after hypoxia-ischemia were somewhat at odds with the general scheme of the pathological mechanism of the effect of I/R on NMDA-induced neuronal-vascular sequence. However, our present results may link the beneficial effect of K^+ channel openers on preservation of NMDA-induced vasodilation to reducing oxidative stress on the neurons involved in this response. We speculate that activation of mitoK_{ATP} channels by K^+ channel openers may reduce mitochondrial ROS production.

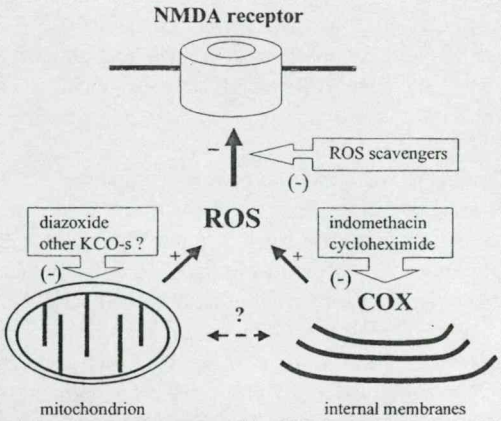

Figure 4. Preservation of NMDA-induced cerebral vasodilation after I/R. The most sensitive component of this neuronal-vascular sequence to I/R may be the NMDA receptor itself. ROS produced by mitochondria and COX seem to play a pivotal role in attenuating the NMDA-induced vasodilation. Thus, pretreatment with ROS scavengers, the COX inhibitor indomethacin, or the protein (including COX) synthesis inhibitor cycloheximide shortly before I/R results in preserved vascular responsiveness to NMDA. NMDA-induced dilation is also preserved by pretreatment with diazoxide, a selective mitoK_{ATP} channel opener, and other K^+ channel openers (KCO-s). Activation of mitoK_{ATP} channels may reduce generation of ROS in reperfusion. Reduction in either COX-derived or mitochondrial ROS production may be sufficient to preserve the vascular response to NMDA after I/R. However, a potentiating interaction between these sources of ROS is conceivable but unknown.

Currently, the physiological role of mitoK_{ATP} channels is still debated and mostly speculative. Briefly, mitoK_{ATP} channels seem to control the activity of the electron transport chain via regulating mitochondrial matrix volume by regulated K^+ uptake. The physiological patterns of activation and inhibition of these channels are largely unknown, but ironically the physiological role of ATP as a regulator is unlikely.[29] In isolated mitochondria, K^+ channel openers induce slight swelling, partially dissipate the transmembrane potential ($\Delta\Psi$, negative inside), but increase the activity of electron transport chain and hence the chemical proton gradient (ΔpH, alkaline inside); thus, the total protonmotive hardly changes.[10–14] However, the activity of numerous important transport mechanisms depends on either $\Delta\Psi$ or ΔpH. One such possibly crucial "metabolite" may be Ca^{2+}. Mitochondria readily uptake Ca^{2+} when intracellular levels increase above a so-called mitochondrial buffer concentration. Ca^{2+} is transported through the mitochondrial inner membrane via the electrogenic Ca^{2+} uniporter down its electrochemical gradient, and thus the rate of this transport is dependent on $\Delta\Psi$.[30] Mitochondrial Ca^{2+} overload substantially influences the recovery of mitochondrial function after ischemic stress: for example, increased mitochondrial Ca^{2+} sequestration has

been demonstrated to increase production of ROS.[31,32] Opening of mitoK$_{ATP}$ channels should decrease mitochondrial Ca^{2+} uptake by decreasing $\Delta\Psi$, and in fact K^+ channel openers induce release of Ca^{2+} from Ca^{2+}-preloaded mitochondria in vitro.[11]

In summary, we conclude that diazoxide in a mitoK$_{ATP}$ channel–selective range dose-dependently preserves neuronal function demonstrated by NMDA-induced arteriolar dilation after I/R. This acute effect of mitoK$_{ATP}$ channel openers may be mediated by decreasing mitochondrial ROS production in the immediate reperfusion. This effect may be important in the protective effect of other nonspecific K^+ channel openers as well. Our findings may offer the development of new therapies to reduce neuronal injury after global hypoxic-ischemic stress in the newborn.

Acknowledgments

This study was supported by grants HL-30260, HL-46558, and HL-50587 from the National Institutes of Health and by grants from the T-026295 OTKA and ETT-T-07614/97 (Hungary). We gratefully acknowledge that aprikalim was a gift from Rhone-Roulenc-Rohrer.

References

1. Busija DW, Leffler CW. Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol.* 1989;257:H1200–H1203.
2. Meng W, Tobin JR, Busija DW. Glutamate-induced cerebral vasodilation is mediated by nitric oxide through *N*-methyl-D-aspartate receptors. *Stroke.* 1995;26:857–862.
3. Faraci FM, Breese KR. Nitric oxide mediates vasodilatation in response to activation of *N*-methyl-D-aspartate receptors in brain. *Circ Res.* 1993; 72:476–480.
4. Bari F, Errico RA, Louis TM, Busija DW. Differential effects of short-term hypoxia and hypercapnia on *N*-methyl-D-aspartate–induced cerebral vasodilatation in piglets. *Stroke.* 1996;27:1634–1639.
5. Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM. Effects of ischemia on cerebrovascular responses to N-methyl-D-aspartate in piglets. *Am J Physiol.* 1996;270:H1225–H1230.
6. Veltkamp R, Domoki F, Bari F, Busija DW. Potassium channel activators protect the *N*-methyl-D-aspartate–induced cerebral vascular dilation after combined hypoxia and ischemia in piglets. *Stroke.* 1998;29:837–843.
7. Hoffman DJ, McGowan JE, Marro PJ, Mishra OP, Delivoria-Papadopoulos M. Hypoxia-induced modification of the N-methyl-D-aspartate receptor in the brain of the newborn piglet. *Neurosci Lett.* 1994;167:156–160.
8. Pourcyrous M, Leffler CW, Bada HS, Korones SB, Busija DW. Brain superoxide anion generation in asphyxiated piglets and the effect of indomethacin at therapeutic dose. *Pediatr Res.* 1993;34:366–369.
9. Inoue I, Nagase H, Kishi K, Higuti T. ATP-sensitive K^+ channel in the mitochondrial inner membrane. *Nature.* 1991;352:244–247.
10. Garlid KD, Paucek P, Yarov-Yarovoy V, Xiaocheng Sun, Schindler PA. The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J Biol Chem.* 1996;271:8796–8799.
11. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol.* 1998;275:H1567–H1576.
12. Garlid KD. Cation transport in mitochondria. *Biochim Biophys Acta.* 1996;1275:123–126.
13. Halestrap AP. Regulation of mitochondrial metabolism through changes in matrix volume. *Biochem Soc Trans.* 1994;22:522–529.
14. Szewczyk A, Czyz A, Wojcik G, Wojczak L, Nalecz M. ATP-regulated K^+ channel in mitochondria: pharmacology and function. *J Bioenerg Biomembr.* 1996;28:147–152.
15. Gross GJ, Fryer R. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ Res.* 1999;84:973–979.
16. Liu Y, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation.* 1998;97:2463–2469.
17. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection. *Circ Res.* 1997;81: 1072–1082.
18. Baines CP, Liu GS, Birincioglu M, Critz SD, Cohen MV, Downey JM. Ischemic preconditioning depends on interaction between mitochondrial K_{ATP} channels and actin cytoskeleton. *Am J Physiol.* 1999;276: H1361–H1368.
19. Beasley TC, Bari F, Thore C, Thrikawala N, Louis TM, Busija DW. Cerebral ischemia/reperfusion increases endothelial nitric oxide synthase levels by an indomethacin-sensitive mechanism. *J Cereb Blood Flow Metab.* 1998;18:88–96.
20. Auchampach J, Grover G, Gross G. Blockade of ischemic preconditioning in dogs by the novel ATP-dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res.* 1992;26:1054–1062.
21. Gross G, Auchampach J. Blockade of the ATP-sensitive potassium channels prevents myocardial preconditioning. *Circ Res.* 1992;70: 223–233.
22. Schulz R, Rose J, Heusch G. Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am J Physiol.* 1994;267:H1341–H1352.
23. Sato T, O'Rourke B, Marban E. Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. *Circ Res.* 1998;83:110–114.
24. Veltkamp R, Domoki F, Bari F, Louis TM, Busija DW. Inhibitors of protein synthesis preserve the *N*-methyl-D-aspartate–induced cerebral arteriolar dilation after ischemia in piglets. *Stroke.* 1999;30:148–152.
25. Rechnerona S, Mela L, Siesjo BK. Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. *Stroke.* 1979;10:437–446.
26. Sims NR. Selective impairment of respiration in mitochondria isolated from brain subregions following transient forebrain ischemia in the rat. *J Neurochem.* 1991;56:1836–1844.
27. Bari F, Louis TM, Busija DW. Kainate-induced cerebrovascular dilation is resistant to ischemia in piglets. *Stroke.* 1997;28:1272–1276.
28. Armstead WM, Mirro R, Busija DW, Leffler CW. Postischemic generation of superoxide anion by newborn pig brain. *Am J Physiol.* 1988; 255:H401–H403.
29. Paucek P, Yarov-Yarovoy V, Xiaocheng S, Garlid KD. Inhibition of the mitochondrial K_{ATP} channel by long chain acyl-CoA esters and activation by guanine nucleotides. *J Biol Chem.* 1996;271:32084–32088.
30. Gunter TE, Gunter KK, Sheu S-S, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. *Am J Physiol.* 1994; 267:C313–C339.
31. Dugan LL, Sensi SL, Canzoniero LMT, Handran SD, Rothman SM, Lin T-S, Goldberg MP, Choi DW. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci.* 1995;15:6377–6388.
32. Dykens JA. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^+: implications for neurodegeneration. *J Neurochem.* 1994;63:584–591.

IV

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^{-4} 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 postischemic 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^{-5} 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 postischemic neuronal damage. Conversely, the postischemic integrity of the neuronal-

See Editorial Comment, page 152

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

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

vascular axis can serve as a parameter for the in vivo study of early effects of experimental interventions on neuronal function.[8]

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.[9–13] 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 vasodilation. 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 in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel and glass cranial window with 3 ports was put into the opening, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic. The closed window was filled with artificial cerebrospinal fluid (aCSF) that was warmed to 37°C and equilibrated with 6% O_2, 6.5% CO_2, and the balance N_2. Arterioles were observed with a microscope (Wild M36) equipped with a television camera (Panasonic), and arteriolar diameter was measured with a video microscaler (IV-550, For-A Co).

Cerebral Ischemia

A 3-mm hole was drilled in the skull with an electric drill with 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^{-5} 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^{-5} mol/L (group 2, n=5) or 10^{-6} 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^{-5} 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 performed. Data analyses were performed on absolute and percentage 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, P_{CO_2} was 25±2 mm Hg, and P_{O_2} was 96±5 mm Hg. One hour after ischemia, pH was 7.41±0.02, P_{CO_2} was 24±2 mm Hg, and P_{O_2} 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^{-6} mol/L) preserved arteriolar responses to the different concentrations of NMDA 1 hour after ischemia (Table, Figure 1, all $P>0.05$). Similarly,

Arteriolar Dilation to NMDA Before and After Ischemia

	Before			1 h After			2 h After			4 h After		
	Baseline	10^{-5} mol/L	10^{-4} mol/L	Baseline	10^{-5} mol/L	10^{-4} mol/L	Baseline	10^{-5} mol/L	10^{-4} mol/L	Baseline	10^{-5} mol/L	10^{-4} mol/L
Control												
Diameter, μm	100±3	110±5	140±2†	102±3	103±2	122±3*†	102±3	104±3	130±3*†	100±2	106±2	134±4†
Δ Diameter, μm		10±2	40±3		1±0*	20±4*		2±1*	28±5		6±1*	34±5
Act-D 10^{-5} mol/L												
Diameter, μm	103±1	110±3	142±4†	104±1	111±3	140±4†	103±1	112±5	141±5†	102±4	111±5	134±6†
Δ Diameter, μm		7±2	39±4		7±2	36±4		10±4	39±4		9±1	32±4
Act-D 10^{-6} mol/L												
Diameter, μm	98±4	104±6	149±15†	99±5	104±6	139±14†	101±5	110±8	146±14†	99±5	106±7	138±11†
Δ Diameter, μm		6±2	51±11		6±2	40±11		8±3	45±9		7±2	40±8
CHX 1.0 mg/kg												
Diameter, μm	101±5	111±5	139±11†	99±6	105±6	133±8†	101±7	106±6	137±8†	99±6	107±6	141±10†
Δ Diameter, μm		10±1	38±8		5±2	34±6		5±2	36±5		8±2	42±7
CHX 0.3 mg/kg												
Diameter, μm	110±5	119±6	153±9†	111±7	120±7	143±10†	111±5	117±5	150±9†	109±5	117±6	155±9†
Δ Diameter, μm		9±2	43±5		8±1	31±7		6±2	39±6		9±1	46±5

Values are mean±SEM. Δ Diameter indicates change in diameter of arteriole.
*Significantly different from respective preischemic value ($P<0.05$).
†Significantly different from respective baseline.

administration of CHX preserved the NMDA-induced vasodilation at 1 hour in a dose-dependent fashion (Table, Figure 2).

In experiments without ischemia, arteriolar dilation to NMDA before and 1 hour after Act-D 10^{-5} mol/L and CHX 1.0 mg/kg was very similar. Arteriolar dilation before and after Act-D was 7±3% versus 8±1% at 10^{-5} mol/L and 37±6% versus 35±4% at 10^{-4} mol/L NMDA. Vasodilation before and after CHX was 7±3% versus 5±2% at 10^{-5} mol/L and 37±6% versus 30±1% at 10^{-4} mol/L NMDA (all $P>0.05$).

Discussion

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

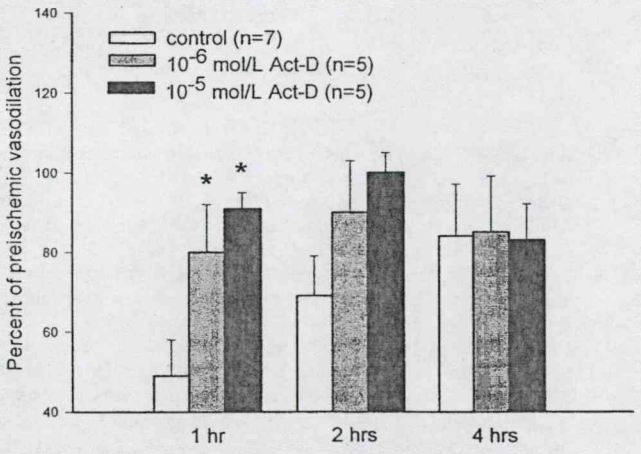

Figure 1. Effects of pretreatment with vehicle or Act-D (10^{-6} or 10^{-5} mol/L) on cerebral arteriolar dilation to NMDA 10^{-4} mol/L at 1, 2, and 4 hours after ischemia. Values are expressed as percentage of preischemic dilation. Values are mean±SEM; n=7 for vehicle, n=5 for each Act-D dose. *Significantly different from vehicle, $P<0.05$.

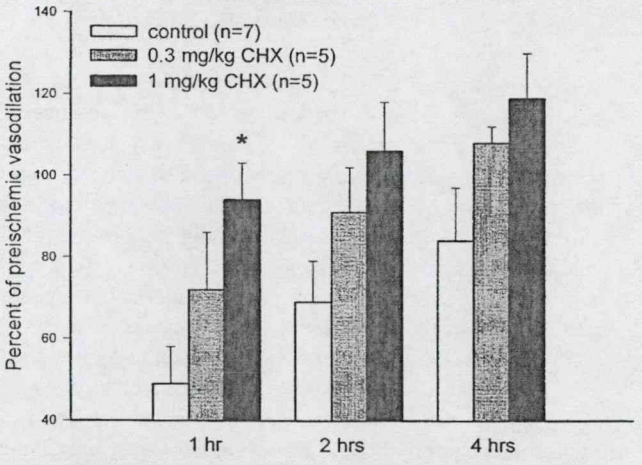

Figure 2. Effects of pretreatment with vehicle or CHX 0.3 or 1 mg/kg on cerebral arteriolar dilation to NMDA 10^{-4} mol/L at 1, 2, and 4 hours after ischemia. Values are expressed as percentage of preischemic dilation. Values are mean±SEM; n=7 for vehicle, n=5 for each CHX dose. *Significantly different from vehicle, $P<0.05$.

steps of the sequence proximal to nitric oxide synthase.[7] In that study, NO synthase (NOS) activity in cerebral cortex as measured by the conversion of L-[^{14}C]citrulline from L-[^{14}C]arginine was unaffected by ischemia.[7] 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.[16] 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.[17,18] Studies in piglets have shown that ischemic and hypoxic exposure can alter NMDA receptor characteristics.[19,20]

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.[7,21,22] Obviously, this suggested a role for free oxygen radicals in the postischemic 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.[23] 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.[21] 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[24] 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[25] demonstrated that CHX blocked the increased production of $PGF_{2\alpha}$ induced by interleukin-1α 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[26] 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,[27] infusion of CHX into the lateral ventricle reduced infarct size in rats. Similarly, Du et al[28] 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[29] 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 postischemic 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

1. Busija DW, Leffler CW. Dilator effects of amino acid neurotransmitters on piglet pial arterioles. *Am J Physiol.* 1989;257:H1200–H1203.
2. Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature.* 1988;336:385–388.
3. Armstead WM, Mirro R, Zuckerman S, Busija DW, Leffler CW. The influence of opioids on local cerebral glucose utilization in newborn pig. *Brain Res.* 1992;571:97–102.
4. Faraci FM, Breese KR. Nitric oxide mediates vasodilation in response to activation of *N*-methyl-D-aspartate receptors in brain. *Circ Res.* 1993;72:476–480.
5. East SJ, Garthwaite J. NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. *Neurosci Lett.* 1991;123:17–19.
6. Busija DW, Meng W. Altered cerebral vascular responsiveness to *N*-methyl-D-aspartate after asphyxia in piglets. *Am J Physiol.* 1993;265:H389–H394.
7. Busija DW, Meng W, Bari F, McGough PS, Errico RA, Tobin JR, Louis TM. Effects of ischemia on cerebrovascular responses to *N*-methyl-D-aspartate in piglets. *Am J Physiol.* 1996;270:H1225–H1230.

8. Veltkamp R, Domoki F, Bari F, Busija DW. Potassium channel activators protect the *N*-methyl-D-aspartate–induced cerebral arteriolar dilation after combined hypoxia and ischemia in piglets. *Stroke*. 1998; 29:837–843.
9. Koistinaho J, Hokfelt T. Altered gene expression in brain ischemia. *Neuroreport*. 1997;8:i-viii.
10. MacManus JP, Linnik MD. Gene expression induced by cerebral ischemia: an apoptotic perspective. *J Cereb Blood Flow Metab*. 1997;17:815–832.
11. Akins PT, Liu PK, Hsu CY. Immediate early gene expression in response to cerebral ischemia: friend or foe? *Stroke*. 1996;27:1682–1687.
12. Hossmann KA. Disturbances of cerebral protein synthesis and ischemic cell death. *Prog Brain Res*. 1996;96:161–177.
13. Kiessling M, Stumm G, Xie Y, Herdegen T, Aguzzi A, Bravo R, Gass P. Differential transcription and translation of immediate early genes in the gerbil hippocampus after transient global ischemia. *J Cereb Blood Flow Metab*. 1993;13:914–924.
14. Shibata M, Mirro R, Armstead WM, Leffler CW. Actinomycin D blocks interleukin-1 alpha-induced pial arteriolar dilation and increased prostanoid production in newborn pigs. *Brain Res Bull*. 1994;33:403–409.
15. Pavlik A, Teisinger J. Effect of cycloheximide administered to rats in early postnatal life: prolonged inhibition of DNA synthesis in the developing brain. *Brain Res*. 1980;192:531–541.
16. Bari F, Louis TM, Busija DW. Kainate-induced cerebrovascular dilation is resistant to ischemia in piglets. *Stroke*. 1997;28:1272–1277.
17. McBain CJ, Mayer ML. *N*-Methyl-D-aspartic acid receptor structure and function. *Physiol Rev*. 1994;74:723–760.
18. Gozlan H, Ben-Ari Y. NMDA receptor redox sites: are they targets for selective neuronal protection? *Trends Pharmacol Sci*. 1995;16:368–374.
19. Marro PJ, Hoffman D, Schneiderman R, Mishra OP, Delivoria-Papadopoulos M. Effect of allopurinol on NMDA receptor modification following recurrent asphyxia in newborn piglets. *Brain Res*. 1998;787:71–77.
20. Graham EM, Apostolou M, Mishra OP, Delivoria-Papadopoulos M. Modification of the *N*-methyl-D-aspartate (NMDA) receptor in the brain of newborn piglets following hyperventilation induced ischemia. *Neurosci Lett*. 1996;218:29–32.
21. Pourcyrous M, Leffler CW, Bada HS, Korones SB, Busija DW. Brain superoxide anion generation in asphyxiated piglets and the effect of indomethacin at therapeutic dose. *Pediatr Res*. 1993;34:366–369.
22. Bari F, Errico RA, Louis TM, Busija DW. Differential effects of short-term hypoxia and hypercapnia on *N*-methyl-D-aspartate–induced cerebral vasodilation in piglets. *Stroke*. 1996;27:1634–1640.
23. Armstead WM, Mirro R, Busija DW, Leffler CW. Postischemic generation of superoxide anion by newborn pig brain. *Am J Physiol*. 1988; 255:H401–H403.
24. Fagan JM, Goldberg AL. Inhibitors of protein and RNA synthesis cause a rapid block in prostaglandin production at the prostaglandin synthase step. *Proc Natl Acad Sci U S A*. 1986;83:2771–2775.
25. Nam MJ, Thore C, Busija DW. Rapid induction of prostaglandin synthesis in piglet astroglial cells by interleukin 1α. *Brain Res Bull*. 1995; 36:215–218.
26. Goto K, Assuage A, Sekiguci K, Lizuka S, Sugimoto A, Aburada M, Hoseya E, Kogure K. Effect of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res*. 1990;534:299–302.
27. Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke*. 1993; 24:2002–2008.
28. Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: a role of apoptosis? *J Cereb Blood Flow Metab*. 1996;16:195–201.
29. Aronowski J, Strong R, Grotta JC. Reperfusion injury: demonstration of brain damage production by reperfusion after transient focal ischemia in rats. *J Cereb Blood Flow Metab*. 1997;17:1048–1056.

V

Cycloheximide rapidly inhibits cortical COX activity and COX-dependent pial arteriolar dilation in piglets

FERENC DOMOKI,[1,4] JAMES V. PERCIACCANTE,[2] ROLAND VELTKAMP,[1,3] GREG ROBINS,[1] FERENC BARI,[1,4] THOMAS M. LOUIS,[5] AND DAVID W. BUSIJA[1]

[1]*Department of Physiology and Pharmacology,* [2]*Department of Pediatrics, and* [3]*Stroke Research Center, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157-1010;* [4]*Department of Physiology, Albert Szent-Györgyi Medical University, Szeged, H-6720 Hungary; and* [5]*Department of Anatomy and Cell Biology, East Carolina University, School of Medicine, Greenville, North Carolina 27858-4353*

Domoki, Ferenc, James V. Perciaccante, Roland Veltkamp, Greg Robins, Ferenc Bari, Thomas M. Louis, and David W. Busija. Cycloheximide rapidly inhibits cortical COX activity and COX-dependent pial arteriolar dilation in piglets. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H1113–H1118, 1999.—We have previously shown that cycloheximide (CHX) preserved neuronal function after global cerebral ischemia in piglets, in a manner similar to indomethacin. To elucidate the mechanism of this protection, we tested the hypothesis that CHX would inhibit cyclooxygenase (COX) activity in the piglet cerebral cortex and vasculature. Pial arteriolar responses to hypercapnia, arterial hypotension, and sodium nitroprusside (SNP) were determined before and 20 min after treatment with CHX (0.3–1 mg/kg iv) using a closed cranial window and intravital microscopy. We also determined baseline and arachidonic acid (AA)-stimulated cortical $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production before and 20–60 min after CHX (1 mg/kg iv) treatment, using ELISA kits. CHX did not affect baseline diameters (~100 μm) but significantly decreased arteriolar dilation to COX-dependent stimuli, such as hypercapnia and hypotension, but not to COX-independent SNP. In the 1 mg/kg CHX-treated group, increases in vascular diameters were reduced from 22 ± 2 to 10 ± 2%, from 49 ± 5 to 31 ± 3% (means ± SE, 5 and 10% CO_2, respectively, $n = 8$), from 12 ± 3 to 3 ± 1%, and from 26 ± 5 to 6 ± 2% (~25 and 40% decreases in blood pressure, respectively, $n = 6$). CHX also inhibited conversion of exogenous AA to both $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$; for example, 20 min after CHX treatment 10 μg/ml AA-stimulated $PGF_{2\alpha}$ concentrations in the artificial cerebrospinal fluid decreased from 14.28 ± 3.04 to 5.90 ± 1.26 ng/ml ($n = 9$). Thus CHX rapidly decreases COX activity in the piglet cerebral cortex. This result may explain in part the preservation of neuronal function of CHX in cerebral ischemia.

cerebral blood flow; arachidonic acid; hypercapnia; arterial hypotension; prostaglandin H synthase

CYCLOOXYGENASE (COX) is the rate-limiting enzyme in the biosynthesis of prostanoids (PGs and thromboxanes). COX-derived metabolites, such as prostanoids and superoxide anions, play an important role in regulating cerebral blood flow in the newborn. For instance, prostanoids contribute to maintaining cerebral blood in hypotensive piglets (17, 18). In addition, prostanoids have a permissive effect on hypercapnia-induced pial arteriolar vasodilation (19, 20).

COX also plays an important role in the pathophysiology of cerebral circulation after ischemic stress. In the piglet cerebral cortex, COX is the source of most of the superoxide radicals detected in the early reperfusion period after total cerebral ischemia (1). Cerebral ischemia-reperfusion results in severe alterations in cerebrovascular dilator responses. For instance, hypoxic/ischemic stress transiently attenuates *N*-methyl-D-aspartate (NMDA)-induced vascular dilation (3–5). This response is neuronally mediated and may play a role in coupling cerebral blood flow to brain metabolism. More importantly, NMDA-induced vasodilation can be used as a sensitive bioassay to assess the functional integrity of the neuronal-vascular axis. After cerebral ischemia, NMDA-induced vasodilation has been shown to be preserved by pretreatment with oxygen free radical scavengers, inhibitors of COX, and most recently, inhibitors of protein synthesis (3–5, 28). However, the mechanism by which inhibitors of protein synthesis [both actinomycin D and cycloheximide (CHX)] preserved the vascular response to NMDA after ischemia has not been elucidated.

In this study we tested the hypothesis that CHX inhibits COX activity in the cerebral cortex and vasculature of newborn pigs. We used the COX-dependent vascular responses to arterial hypercapnia and arterial hypotension as in vivo bioassays to assess the effect of CHX on active COX levels. In addition, we investigated the effect of CHX in the conversion of exogenous arachidonic acid (AA) to $PGF_{2\alpha}$ and prostacyclin.

MATERIALS AND METHODS

Animals. In these experiments newborn piglets of either sex (1- to 7-days old, 1–2 kg body wt, $n = 31$) were used. All procedures were approved by the Institution Animal Care and Use Committee. The animals were anesthetized with thiopental sodium (30–40 mg/kg ip) followed by intravenous injection of α-chloralose (75 mg/kg). Supplemental doses of α-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. The piglets were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range; for instance, in the 1 mg/kg CHX-treated group ($n = 9$) the values were (means ± SE) the following: arterial pH (7.50 ± 0.02), P_{CO_2} (34 ± 2 mmHg), and P_{O_2} (96 ± 5 mmHg).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Body temperature was maintained at 37–38°C by a water-circulating heating pad. The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (diameter 19 mm) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic.

The closed window was filled with artificial cerebrospinal fluid (aCSF), warmed to 37°C, and equilibrated with 6% O_2 and 6.5% CO_2 in balance N_2 to give a pH of 7.33, P_{CO_2} of 46 mmHg, and P_{O_2} of 43 mmHg. The aCSF consisted of (in mM) the following: 132 NaCl, 2.9 KCl, 1.2 $CaCl_2$, 1.4 $MgCl_2$, 24.6 $NaHCO_3$, 6.7 urea, and 3.7 glucose. Diameters of pial arterioles were measured using a microscope (Wild M36, Switzerland) equipped with a video camera (Panasonic, Japan) and a video microscaler (IV-550, For-A-Co. Newton, MA). After surgery, the cranial window was gently perfused with aCSF until a stable baseline was obtained. At the end of the experiments, the animals were killed with an intravenous bolus of KCl while still anesthetized.

Measurement of pial arteriolar responses. Instrumented piglets (n = 22) were divided into three groups: *1*) vehicle-treated control, *2*) animals treated with 0.3 mg/kg CHX, and *3*) animals treated with 1 mg/kg CHX. We examined the responses of cerebral arterioles to arterial hypercapnia, arterial hypotension, and sodium nitroprusside (SNP) before and 20 min after drug treatment. Hypercapnia was elicited by artificially ventilating the animal with a gas mixture containing (5% or 10% CO_2, 21% O_2, balance N_2). Arterial hypotension was induced by withdrawing venous blood to yield ~25 and 40% decreases in mean arterial pressure (MAP), respectively. After the measurements the heparinized blood was reinfused. SNP (10^{-6} and 10^{-5} mol/l) dissolved in aCSF was administered topically through the injection ports of the cranial window onto the brain surface with a single application. Arteriolar diameters were measured continuously for 5 min for each stimulus until steady-state values were obtained. Typically, we obtained data for two different stimuli in each animal. With this protocol we obtained similar arteriolar responses to hypercapnia, hypotension, and SNP as in previous experiences with these challenges. These doses of hypercapnia, hypotension, and SNP were chosen to provide medium and large increases in vascular diameters. Between different stimuli the window was flushed with aCSF, and the arteriolar diameters returned to baseline values. CHX (0.3–1 mg/kg) was dissolved in 3 ml of saline and injected intravenously. Twenty minutes after treatment, challenges of hypercapnia, arterial hypotension, and SNP were repeated according to the procedure described above.

Assessment of cortical PG-synthesizing capacity. We determined cortical conversion of exogenous AA to $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (n = 9). AA (1, 10, and 20 μg/ml) dissolved in aCSF was administered onto the brain surface through the injectable ports of the cranial window. Each dose of AA was applied to the brain surface for 10 min and then the cranial window was gently flushed and the effluent aCSF (~300 μl) was collected and frozen. AA was applied at 1-h intervals twice before and then 20 min and 1 h after CHX (1 mg/kg iv) treatment. Typically, we applied AA three times in each animal. Because the data obtained from these animals did not differ significantly, we combined these data as shown in RESULTS. From the aCSF samples we determined concentrations of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ using ELISA kits (Oxford Biomedical Research, Oxford, MI).

Statistics. Data are expressed as means ± SE. Data were analyzed using repeated measures ANOVA, and one-way ANOVA was used for differences between treatment groups. Pairwise comparisons were made using the Student-Newman-Keuls test where appropriate.

RESULTS

Arterial blood pressure was within normal limits (Table 1) and did not change significantly during hypercapnia. CHX administration caused a transient increase (5–15 mmHg) in MAP, but blood pressure values returned to baseline within 10 min of CHX administration.

Graded hypercapnia induced by ventilating the animals with gas mixtures containing either 5 or 10% CO_2 resulted in a concentration-dependent increase in pial arteriolar diameters in accordance with the elevated P_{CO_2} levels in all groups of animals (Table 1). In the vehicle-treated control group, repeated exposure to high P_{CO_2} levels elicited essentially identical vasodilation in pial arterioles. However, in the groups intravenously injected with either 0.3 or 1.0 mg/kg CHX,

Table 1. *Effect of CHX treatment on pial arteriolar dilation to arterial hypercapnia and hypotension*

		Baseline				5% CO_2 Inhalation				10% CO_2 Inhalation			
		Diameter, μm		P_{CO_2}, mmHg		% Increase		P_{CO_2}, mmHg		% Increase		P_{CO_2}, mmHg	
	n	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Control	5	112 ± 11	113 ± 11	38 ± 1	41 ± 1	27 ± 5	26 ± 5	54 ± 3	56 ± 2	46 ± 5	44 ± 5	76 ± 1	81 ± 2
CHX													
0.3 mg/kg	6	102 ± 2	107 ± 5	33 ± 2	34 ± 2	26 ± 5	15 ± 4*	51 ± 2	46 ± 3	42 ± 8	31 ± 6*	70 ± 3	75 ± 3
1 mg/kg	8	93 ± 3	94 ± 5	34 ± 2	29 ± 2	22 ± 2	10 ± 2*	50 ± 1	48 ± 1	49 ± 5	31 ± 3*	74 ± 2	71 ± 2
		Baseline				Hypotension (*Grade 1*)				Hypotension (*Grade 2*)			
		Diameter, μm		MAP, mmHg		% Increase		MAP, mmHg		% Increase		MAP, mmHg	
	n	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Control	5	108 ± 10	109 ± 8	71 ± 1	71 ± 1	20 ± 4	19 ± 4	53 ± 2	53 ± 2	31 ± 5	33 ± 4	42 ± 3	42 ± 3
CHX													
0.3 mg/kg	6	104 ± 3	106 ± 6	75 ± 5	72 ± 6	10 ± 1	7 ± 1	56 ± 4	54 ± 4	21 ± 4	19 ± 4	46 ± 4	43 ± 3
1 mg/kg	6	96 ± 6	104 ± 12	63 ± 5	59 ± 6	12 ± 3	3 ± 1*	45 ± 2	43 ± 3	26 ± 5	6 ± 2*	35 ± 2	33 ± 3

Values are means ± SE; n = no. of piglets. CHX, cyclohexamide; MAP, mean arterial pressure. *$P < 0.05$, significantly different from respective value before treatment.

vasodilation was significantly reduced to either level of hypercapnia (Table 1, Fig. 1). The attenuation of the response was larger in the group treated with the higher dose of CHX, especially at the lower level of hypercapnia (Fig. 1).

Graded arterial hypotension induced by venous blood withdrawal resulted in a dose-dependent increase in pial arteriolar diameters in accordance with decreased arterial blood pressure levels (Table 1). In the vehicle-treated control group pial arteriolar responses in response to stimulation by arterial hypotension were unaltered (Fig. 1). The vasodilatory response to arterial hypotension was also largely retained in the group treated with 0.3 mg/kg CHX. However, arteriolar responsiveness to arterial hypotension was severely reduced in the animals treated with 1 mg/kg CHX (Table 1, Fig. 1).

SNP ($n = 7$) induced dose-dependent pial arteriolar vasodilation that was unaffected by treatment with 1 mg/kg CHX. Baseline arteriolar diameters were not significantly different before and after CHX treatment (112 ± 6 vs. 118 ± 7 μm, respectively), and the percent changes were 21 ± 4 vs. 19 ± 4% at 10^{-6} mol/l and 38 ± 5 vs. 37 ± 5% at 10^{-5} mol/l.

Topical application of 1, 10, and 20 μg/ml AA onto the brain surface elicited a dose-dependent increase in the aCSF concentrations of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (Figs. 2 and 3). Repeated application of AA resulted in similar changes in aCSF PG levels. CHX (1 mg/kg iv) attenuated baseline as well as AA-stimulated $PGF_{2\alpha}$ levels as early as 20 min after CHX administration (Fig. 2). The inhibition of $PGF_{2\alpha}$ synthesis lasted at least as long as 1 h after CHX administration. Similarly, AA-stimulated 6-keto-$PGF_{1\alpha}$ levels were also significantly reduced 20 min after administration of CHX (Fig. 3).

DISCUSSION

The major new finding in this study is that in vivo CHX rapidly inhibits COX activity in the piglet cerebral cortex and vasculature. More specifically, cerebrovascular reactivity to COX-dependent, vasodilatory stimuli is diminished within 20 min of CHX administration. Similarly, cerebral cortical PG-synthesizing capacity is largely reduced shortly after CHX treatment.

The most likely mechanism of how CHX inhibits COX activity in our experimental model is through its inhibitory effect on protein translation. Although we did not determine the effect of 0.3–1 mg/kg CHX on cortical protein synthesis, similar doses of CHX have been shown previously to be effective in rats (26), and we are unaware of any data suggesting a species difference in response to CHX. Another possible mechanism would be a nonspecific interaction of CHX with COX. However, CHX has been shown not to influence activity of purified COX in vitro (12). Additionally, in the present study, the intact vasodilatory response to the nitric oxide donor SNP suggests that CHX did not have nonspecific inhibitory effects on vascular smooth muscle function. In contrast, CHX did inhibit baseline and AA-stimulated PGE_2 and prostacyclin production in brain, spleen, and muscle slices from rats. This effect of CHX and other protein synthesis inhibitors was found to be proportional to their effect on general protein synthesis inhibition (12). In this study, however, we cannot exclude the remote possibility that CHX could affect other proteins as well that may modulate COX activity. But the potent, rapid decrease in COX activity after CHX treatment may be explained by the biochemical characteristics of COX. COX is thought to be rapidly inactivated by self-produced superoxide anions, such that in an active system, COX

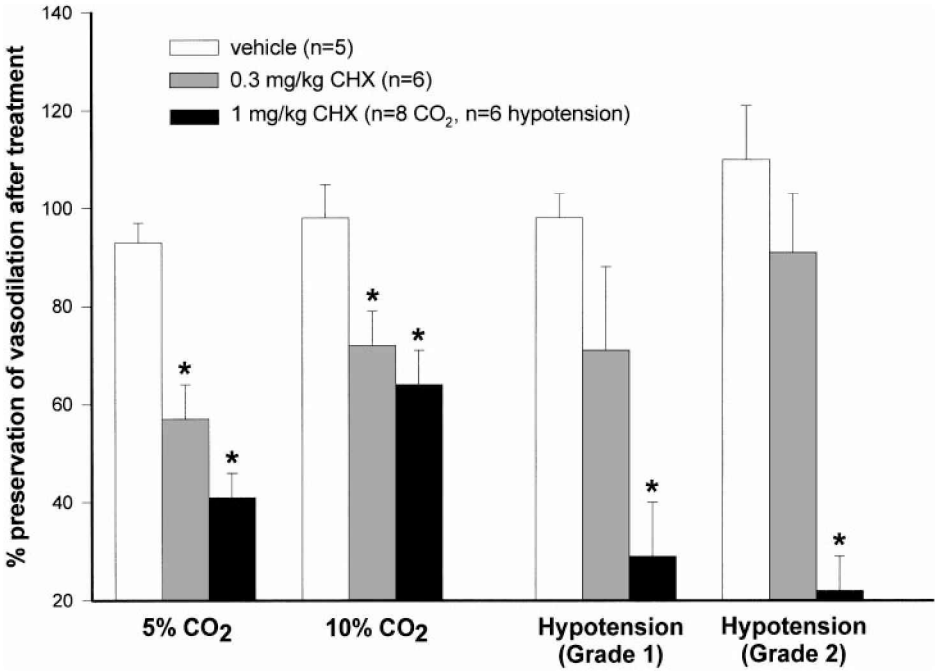

Fig. 1. Effect of cycloheximide (CHX) treatment on cyclooxygenase (COX)-dependent vascular responses. Data are plotted as the percent preservation of vascular dilation to arterial hypercapnia and hypotension after CHX treatment. In the vehicle-treated control group pial arteriolar responses to inhalation of 5–10% CO_2 (5% CO_2 and 10% CO_2, respectively) or graded hypotension [hypotension (*grade 1* and *grade 2*, respectively)] were fully preserved. Treatment with 0.3–1 mg/kg CHX significantly reduced vasodilation to both doses of hypercapnia. Similarly the vascular response to arterial hypotension was also reduced by CHX, and the higher dose of CHX attenuated the response >70%. *Significantly different from control group ($P < 0.05$).

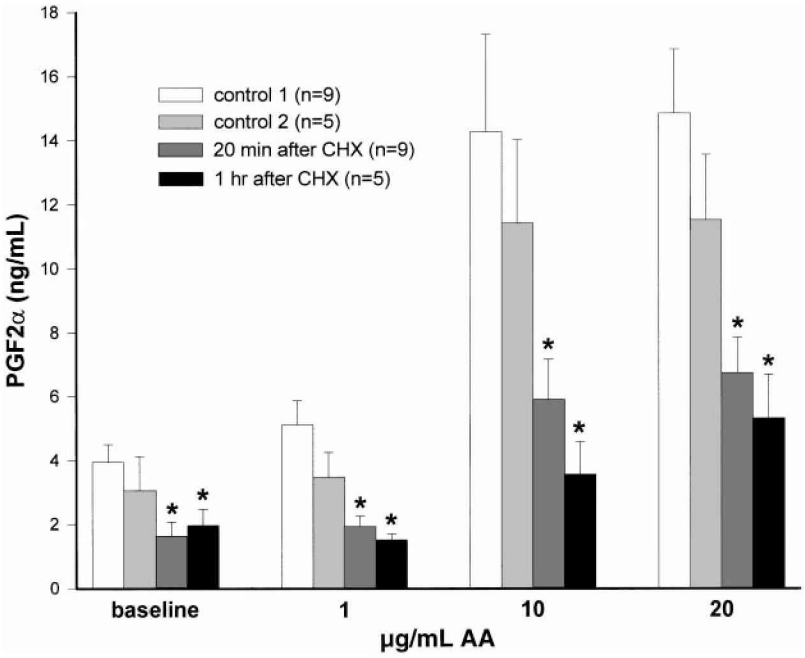

Fig. 2. Effect of CHX treatment on conversion of exogenous arachidonic acid (AA) to $PGF_{2\alpha}$. Topical application of 1–20 μg/ml AA resulted in concentration-dependent, reproducible increases in $PGF_{2\alpha}$ concentrations in the aCSF (*control 1* and *control 2*). In contrast, 20 min after CHX (1 mg/kg iv) treatment, baseline and AA-stimulated $PGF_{2\alpha}$ levels were significantly decreased compared with control values. At 1 h after CHX treatment we obtained similar results, and there was a trend toward further inhibition of $PGF_{2\alpha}$ synthesis. *Significantly different from respective control values ($P < 0.05$).

has a half-life not more than 5–10 min (11). This suggests that maintaining active COX levels would require continuous de novo enzyme synthesis and may explain the rapid attenuation of COX-dependent vascular responses and PG synthesis after CHX administration in the piglet cerebral cortex observed in the present study. Unfortunately, immunoblotting of COX after CHX treatment would probably not yield additional support for this theory. Because COX is rapidly inactivated and degraded, functional and immunoreactive COX levels are not equivalent.

Previous evidence suggests that the PGs required for arteriolar vasodilation to hypercapnia and arterial hypotension are synthesized in the vascular endothelium (18, 20). In the present study, CHX treatment likely affected vascular endothelial cells shown by the attenuation of vascular responses to hypercapnia and arterial hypotension. We also found that 6-keto-$PGF1_{\alpha}$ levels were reduced in the aCSF after CHX treatment, indicating decreased prostacyclin synthesis, further confirming the effect of CHX on cerebrovascular endothelial cells. In contrast, the reduced baseline and AA-stimulated $PGF_{2\alpha}$ levels may represent a more general inhibitory effect of CHX on COX synthesis in both neural and vascular cells.

In our previous study we demonstrated that CHX employed a dose-dependent protective effect on the NMDA-induced vasodilation (28). NMDA-induced vaso-

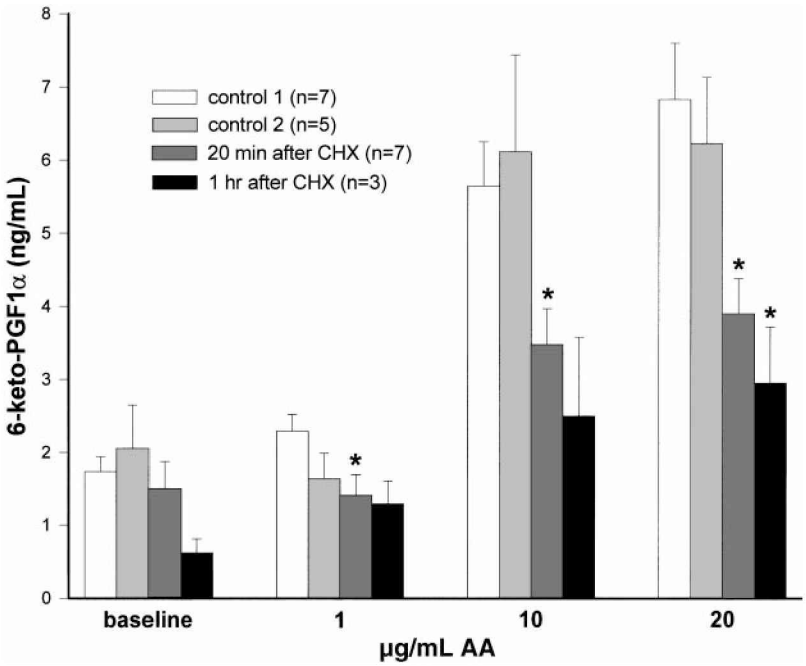

Fig. 3. Effect of CHX treatment on conversion of exogenous AA to 6-keto-$PGF_{1\alpha}$. Topical application of 1–20 μg/ml AA resulted in concentration-dependent, reproducible increases in aCSF concentrations of 6-keto-$PGF_{1\alpha}$ (*control 1* and *control 2*). In contrast, 20 min after CHX (1 mg/kg iv) treatment, AA-stimulated 6-keto-$PGF_{1\alpha}$ levels were significantly decreased compared with control values. At 1 h after CHX treatment we obtained similar results, and there was a trend toward further inhibition of 6-keto-$PGF_{1\alpha}$ synthesis. *Significantly different from respective control values ($P < 0.05$).

dilation is a complex sequence involving the activation of neuronal NMDA receptors, activation of neuronal nitric oxide synthase (nNOS), and pial arteriolar dilation by nitric oxide (13, 22). This response was used as a bioassay to assess the functional integrity of the neuronal-vascular axis after ischemic stress. This response is attenuated by ischemia. The effect of ischemic stress likely affects the events before nNOS activation because nNOS levels and activity as well as vascular dilation to SNP were shown to be unaltered after ischemia (5). COX is an ample source of oxygen radicals in the early reperfusion period and plays a significant role in attenuating the NMDA vascular sequence after ischemia. After ischemic stress, NMDA-induced vasodilation has been shown to be preserved by pretreatment with COX inhibitors and oxygen radical scavengers, clearly indicating the involvement of COX (3–5). In our previous study, 0.3–1 mg/kg CHX was given 15 min before 10 min of global cerebral ischemia. Our present results support the concept that active COX levels could have been reduced before the initiation of ischemia. Thus the acute protective effect of CHX on the NMDA-induced vasodilation may be largely mediated via the inhibition of COX synthesis. We (23, 24) have shown similar results in cultured astroglial cells from piglets and fetal lambs.

CHX was also shown to employ long-term neuroprotective effects in different experimental ischemia models. Pretreatment with CHX was shown to reduce delayed neuronal death after transient focal ischemia (10, 21), and CHX also ameliorated cerebral infarction caused by reperfusion injury after reversible focal ischemia in rats (2). CHX has also been demonstrated to protect CA1 hippocampal neurons after transient global ischemia (14, 25). The mechanism of neuroprotection by CHX in the above-cited studies is not fully understood, but different possible mechanisms have been proposed, including CHX-induced hypothermia, inhibition of apoptosis, and suppression of the postischemic induction of a "noxious/killer protein." However, our present data reveal that pretreatment with protein synthesis inhibitors can result in not only inhibiting the appearance of a noxious/killer protein after ischemia but also in the rapid disappearance of a potentially harmful albeit continuously expressed protein: COX.

At least two distinct isoforms of COX exist (COX-1, COX-2) (9). Originally COX-1 was considered the constitutively expressed isoform, and COX-2 was designated as the inducible isoform. However, in the brain and cerebral blood vessels of newborn pigs, COX-2 but not COX-1 has been identified as the major constitutively expressed isoform (7, 27). COX-2 is an immediate early gene and is readily inducible by a wide variety of stimuli, including ischemic stress in piglet cerebral cortex and blood vessels (6, 8). There is a substantial increase in porcine cortical COX-2 mRNA levels as early as 2 h after ischemic stress, and COX-2 immunoreactivity is also increased within 8 h of cerebral ischemia. However, the short half-life and extremely rapid turnover rate of the COX enzyme may conceal an even more dramatic change in COX expression after ischemic stress. The increased expression of COX-2 may participate in the brain pathology after ischemic stress by increasing the production of oxygen radicals and inflammatory prostanoids. This is an interesting possibility, because overall protein synthesis is assumed to be inhibited even by short periods of cerebral ischemia and reperfusion (16). However, translation of some other immediate early gene mRNAs including heat shock proteins and protooncogenes appears to be increased rapidly after ischemic stress, in contrast to the generally depressed protein synthesis (15). It is quite conceivable that after cerebral ischemia, 1 mg/kg CHX has a greater inhibition on COX synthesis than we have shown with the approaches used in our present study.

In conclusion, to our knowledge we demonstrated for the first time that CHX rapidly inhibits COX activity in vivo in the cerebral cortex, as shown by the attenuation of COX-dependent pial arteriolar responses and decreased cortical metabolism of exogenous AA. This effect of CHX may be responsible for the previously reported early protective effect on neuronal and cerebrovascular function after cerebral ischemia.

This research was supported by National Heart, Lung, and Blood Institute Grants HL-30260, HL-46558, and HL-50587 and in part by T-026295 Országos Tudomanyos Kutatási Alap from the Hungarian Science Foundation.

Address for reprint requests and other correspondence: F. Domoki, Dept. of Physiology and Pharmacology, Wake Forest Univ. School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157-1010 (E-mail: fdomoki@wfubmc.edu).

Received 11 March 1999; accepted in final form 22 April 1999.

REFERENCES

1. **Armstead, W. M., R. Mirro, D. W. Busija, and C. W. Leffler.** Postischemic generation of superoxide anion by newborn pig brain. *Am. J. Physiol.* 255 (*Heart Circ. Physiol.* 24): H401–H403, 1988.
2. **Aronowski, J., R. Strong, and J. C. Grotta.** Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. *J. Cereb. Blood Flow Metab.* 17: 1048–1056, 1997.
3. **Bari, F., R. A. Errico, T. M. Louis, and D. W. Busija.** Differential effects of short-term hypoxia and hypercapnia on *N*-methyl-D-aspartate-induced cerebral vasodilatation in piglets. *Stroke* 27: 1634–1639 1996.
4. **Busija, D. W., and W. Meng.** Altered cerebrovascular responsiveness to *N*-methyl-D-aspartate after asphyxia in piglets. *Am. J. Physiol.* 265 (*Heart Circ. Physiol.* 34): H389–H394, 1993.
5. **Busija, D. W., W. Meng, F. Bari, P. S. McGough, R. A. Errico, J. R. Tobin, and T. M. Louis.** Effects of ischemia on cerebrovascular responses to *N*-methyl-D-aspartate in piglets. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H1225–H1230, 1996.
6. **Busija, D. W., C. Thore, T. Beasley, and F. Bari.** Induction of cyclooxygenase-2 following anoxic stress in piglet cerebral arteries. *Microcirculation* 3: 379–386, 1996.
7. **Degi, R., F. Bari, T. C. Beasley, N. Thrikawala, C. Thore, T. M. Louis, and D. W. Busija.** Regional distribution of prostaglandin H synthase-2 and neuronal nitric oxide synthase in piglet brain. *Pediatr. Res.* 43: 683–689, 1998.
8. **Degi, R., F. Bari, N. Thrikawala, T. C. Beasley, C. Thore, T. M. Louis, and D. W. Busija.** Effects of anoxic stress on prostaglandin H synthase isoforms in piglet brain. *Brain Res. Dev. Brain Res.* 107: 265–276, 1998.

9. **DeWitt, D. L.** Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim. Biophys. Acta* 1083: 121–134, 1991.
10. **Du, C., R. Hu, C. A. Csernansky, C. Y Hsu, and D. W. Choi.** Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis?. *J. Cereb. Blood Flow Metab.* 16: 195–201, 1996.
11. **Egan, R. W., P. H. Gale, E. M. Baptista, K. L. Kennicott, W. J. VandenHeuvel, R. W. Walker, P. E. Fagerness, and F. A. Kuehl, Jr.** Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. *J. Biol. Chem.* 256: 7352–7361, 1981.
12. **Fagan, J. M., and A. L. Goldberg.** Inhibitors of protein and RNA synthesis cause a rapid block in prostaglandin production at the prostaglandin synthase step. *Proc. Natl. Acad. Sci. USA* 83: 2771–2775, 1986.
13. **Faraci, F. M., and K. R. Breese.** Nitric oxide mediates vasodilatation in response to activation of *N*-methyl-D-aspartate receptors in brain. *Circ. Res.* 72: 476–480, 1993.
14. **Goto, K., A. Ishige, K. Sekiguchi, S. Iizuka, A. Sugimoto, M. Yuzurihara, M. Aburada, E Hosoya, and K. Kogure.** Effects of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res.* 534: 299–302, 1990.
15. **Koistinaho, J., and T. Hokfelt.** Altered gene expression in brain ischemia. *Neuroreport* 8: i-viii, 1997.
16. **Krause, G. S., and B. R. Tiffany.** Suppression of protein synthesis in the reperfused brain. *Stroke* 24: 747–755, 1993.
17. **Leffler, C. W., D. W. Busija, D. G. Beasley, and A. M. Fletcher.** Maintenance of cerebral circulation during hemorrhagic hypotension in newborn pigs: role of prostanoids. *Circ. Res.* 59: 562–567, 1986.
18. **Leffler, C. W., and D. W. Busija.** Prostanoids and pial arteriolar diameter in hypotensive newborn pigs. *Am. J. Physiol.* 252 (*Heart Circ. Physiol.* 21): H687–H691, 1987.
19. **Leffler, C. W., R. Mirro, L. J. Pharris, and M. Shibata.** Permissive role of prostacyclin in cerebral vasodilation to hypercapnia in newborn pigs. *Am. J. Physiol.* 267 (*Heart Circ. Physiol.* 36): H285–H291, 1994.
20. **Leffler, C. W., R. Mirro, D. R. Shanklin, W. M. Armstead, and M. Shibata.** Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in newborn pigs. *Am. J. Physiol.* 266 (*Heart Circ. Physiol.* 35): H623–H630, 1994.
21. **Linnik, M. D., R. H. Zobrist, and M. D. Hatfield.** Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24: 2002–2008, 1993.
22. **Meng, W., J. R. Tobin, and D. W. Busija.** Glutamate-induced cerebral vasodilation is mediated by nitric oxide through *N*-methyl-D-aspartate receptors. *Stroke* 26: 857–862, 1995.
23. **Nam, M. J., C. Thore, and D. W. Busija.** Rapid induction of prostaglandin synthesis in piglet astroglial cells by interleukin 1. *Brain Res. Bull.* 36: 215–218, 1995.
24. **Nam, M. J., C. Thore, and D. W. Busija.** Role of protein synthesis in interleukin 1-induced prostaglandin production in ovine astroglia. *Prost. Leuk. Ess. Fatty Acids* 53: 69–72, 1995.
25. **Papas, S., V. Crepel, D. Hasboun, I. Jorquera, P. Chinestra, and Y. Ben-Ari.** Cycloheximide reduces the effects of anoxic insult in vivo and in vitro. *Eur. J. Neurosci.* 4: 758–765, 1992.
26. **Pavlik, A., and J. Teisinger.** Effect of cycloheximide administered to rats in early postnatal life: prolonged inhibition of DNA synthesis in the developing brain. *Brain Res.* 92: 531–541, 1980.
27. **Peri, K. G., P. Hardy, D. Y. Li, D. R. Varma, and S. Chemtob.** Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J. Biol. Chem.* 270: 24615–24620, 1995.
28. **Veltkamp, R., F. Domoki, F. Bari, T. M. Louis, and D. W. Busija.** Inhibitors of protein synthesis preserve the *N*-methyl-D-aspartate-induced cerebral arteriolar dilation after ischemia in piglets. *Stroke* 30: 148–152, 1999.