Effects of hypothermia on neuronal-vascular function after cerebral ischemia in piglets

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Perciaccante, James V., Ferenc Domoki, Michelle Puskar, and David W. Busija. Effects of hypothermia on neuronal-vascular function after cerebral ischemia in piglets. Am J Physiol Regul Integr Comp Physiol 283: R1362–R1367, 2002; 10.1152/ajpregu.00134.2002.—We determined whether cerebral arteriolar dilation to N-methyl-D-aspartate (NMDA), a response dependent on stimulation of cortical neurons and inhibited by anoxic stress, would be preserved by hypothermia during and following ischemia. Pial arteriolar diameters in anesthetized piglets were determined via intravital microscopy. Arteriolar responses to NMDA (10, 50, and 100 μmol/l) were measured before and 1 h after 10 min of global ischemia. Piglets were exposed to either total body or selective brain cooling (33–34°C). Arteriolar dilation to lower doses or to 100 μmol/l NMDA was not affected by hypothermia alone (51 ± 3 vs. 46 ± 7%, normothermia vs. hypothermia; n = 7) in nonischemic animals. However, arteriolar responses to 100 μmol/l NMDA were clearly attenuated after ischemia despite body cooling during ischemia (53 ± 3 vs. 32 ± 6%; n = 8), hypothermia during ischemia and early reperfusion (49 ± 10 vs. 20 ± 3%; n = 8), or selective brain cooling (48 ± 5 vs. 20 ± 5%; n = 10). In contrast, pretreatment with indomethacin resulted in complete preservation of NMDA-induced vasodilation after ischemia. Thus, hypothermia fails to protect against neuronal dysfunction during ischemia.

N-methyl-D-aspartate; cerebral circulation; cerebral arteries; neonate

IN NEWBORN PIGS, CEREBRAL arterioles dilate to glutamate and its receptor subtype-specific analog N-methyl-D-aspartate (NMDA) via a multistep process, involving the activation of NMDA receptors, neuronal production, and release of nitric oxide (NO), and subsequent relaxation of smooth muscle (10, 16, 25). Because glutamate is the predominant excitatory neurotransmitter in the central nervous system, it has been suggested that this sequence of events may represent one important mechanism for coupling neuronal activity with local blood flow (3, 11, 16). However, NMDA-induced arteriolar dilation is highly susceptible to hypoxic or ischemic insults. Thus, even short periods of hypoxia or ischemia dramatically reduce arteriolar dilator responses to NMDA in a dose- and time-dependent manner (4, 5, 12, 13). The mechanisms of impairment of NMDA-induced dilation to hypoxia or ischemia are complex. However, vascular responses to NO remain intact, indicating that the attenuation of NMDA vascular responsiveness that follows hypoxia or ischemia is due to effects from the insult that occurs at the level of the neurons (13, 16). These local neuronal effects appear to involve events related to the production of superoxide anion and other reactive oxygen species (ROS) via the activated cyclooxygenase (COX) pathway and from damaged mitochondria (2, 4, 5, 16, 17, 26, 29).

Indomethacin, which decreases ROS production in newborn piglet brain after ischemia (2) and asphyxia (26), has been shown to preserve the NMDA response after asphyxia (12), ischemia (18), and hypoxia (4). Similar protective effects have been obtained following administration of superoxide dismutase (4) or the selective blockade of COX-2. However, it is unknown whether nonpharmacological manipulations can similarly preserve NMDA-induced arteriolar dilation after ischemia.

Local or systemic hypothermia has been shown to be an effective procedure for the attenuation of ischemic damage to neurons in experimental animals (19, 21, 23). Additionally, hypothermia is an obligatory protective procedure during heart and upper body vascular surgery in children and adults (8, 27, 28) and is being used to treat asphyxiated babies. The precise mechanisms of hypothermic neuroprotection are unknown, but they undoubtedly involve many different factors. For example, hypothermia has been shown to decrease the release of potentially damaging excitotoxic neurotransmitters in the penumbra and ROS formation during and after ischemia in experimental animals (9, 14) and to cause generalized reductions in enzymatic activity (23) possibly including COX in brain (1).
Whether hypothermia is able to preserve NMDA-induced arteriolar dilation in the cerebral circulation has never been examined.

The purpose of this study was to study the effects of hypothermia on ischemia and reperfusion injury in piglets. We tested the hypothesis that hypothermia would preserve the normal arteriolar dilation to NMDA during reperfusion despite the preceding ischemic insult. Specifically, we examined whether whole body hypothermia or local brain cooling could counteract the effects of ischemia. As a positive control for these experiments, we also determined if indomethacin was able to preserve the NMDA response after ischemia.

**MATERIALS AND METHODS**

**Surgical Preparation**

We used anesthetized and ventilated newborn piglets of either sex with weights between 1.5 and 2.5 kg. The Institutional Animal Care and Use Committee approved all procedures. Anesthesia was induced with thiopental sodium (30 to 40 mg/kg ip) and maintained with α-chloralose (50 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 allow for the administration of drugs and fluid, respectively. The piglets were intubated via tracheotomy and mechanically ventilated with room air. The ventilation rate (~20 breaths/min) and tidal volume (~15 ml/kg) were adjusted to maintain blood gases in the normal physiological range.

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 craniotomy, 19 mm in diameter, was then made in the left parietal bone. The dura was cut and reflected over the cranium. A stainless steel cranial window with three needle ports was placed in 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% oxygen, 6.5% carbon dioxide, balance nitrogen to give pH ~ 7.34. Pco2 = 48 mmHg, and Po2 = 46 mmHg. The aCSF consisted of the following (mmol/l): 132 NaCl, 2.9 KCl, 1.2 CaCl2, 1.4 MgCl2, 24.6 NaHCO3, 6.7 urea, and 3.7 glucose. Diameters of pial arterioles were measured with a microscope (Wild M36) equipped with a video camera (Panasonic) and a video microscaler (IV-550, For-A-Co). In each animal, an arteriole of ~100 μm was selected for study.

**Cerebral Ischemia**

A 3-mm hole was cut in the left frontal cranium using an electric drill with a toothless bit. Once the dura was exposed, a hollow brass bolt was inserted and fixed into place with cyanoacrylate ester and dental acrylic. Cerebral ischemia was induced by infusion of aCSF to raise intracranial pressure above arterial blood pressure. We confirmed the presence of ischemia by observing a cessation of blood flow in the pial vessels that were visible under the cranial window. We previously showed using microspheres that there is complete cerebral ischemia with this method (7). Venous blood was withdrawn as needed during ischemia to maintain mean arterial blood pressure near normal values. At the end of the 10-min period of ischemia, the infusion tube was clamped and intracranial pressure was allowed to return to preischemic levels. The heparinized blood was reinfused intravascularly. After ischemia, the animals were allowed to recover for a period of 1 h. This 1-h time period was chosen because we previously showed the greatest attenuation of cerebral vasodilation to NMDA occurs at this time.

**Thermoregulation**

Core body temperature was maintained with the use of an adjustable warm water blanket and a flexible heating pad. We inserted a rectal temperature probe for continuous monitoring of body temperature in all of the piglets. Before the start of the hypothermia protocols, body temperature was maintained at ~38°C. For those piglets that received selective brain cooling, a temperature probe was inserted into the deep cortex of the parietal lobe opposite the cranial window via a second hollow brass intracranial bolt. Systemic cooling was performed by removing the external heating sources and wrapping the piglets in a cold-water blanket until their body temperature reached 33–34°C at which time the cooling blanket was removed. We chose this level of hypothermia because it is the lowest temperature that piglets can be exposed to without having major depressive effects on arterial blood pressure.

We showed previously that rectal and cerebral cortex temperatures are almost identical at this level of hypothermia (11). However, in the piglets from the previous study, the dura, skull, and scalp were intact. Therefore, to assess the cortical temperature under normothermic and hypothermic conditions in piglets with a cranial window in place, a needle temperature probe was inserted into the cortex at the level of the NMDA receptor-containing neurons while another temperature probe was inserted into the rectum. At baseline conditions, brain temperature was 37.6 ± 0.6°C and rectal temperature was 37.8 ± 0.2°C (n = 5, not significant). When body temperature was lowered, brain temperature was 33.3 ± 0.2°C and rectal temperature was 33.3 ± 0.2°C (n = 5, not significant). Therefore, there was no significant difference in temperature between these two locations.

For the piglets that were in the selective brain cooling group, we perfused the cranial window with aCSF chilled to 4°C, while maintaining normal body temperature by adjusting the warm-water blanket and heating pad. Unfortunately, because of technical limitations, we could not continue perfusing the window with chilled aCSF during the posts ischemic period. Opening the stopcocks attached to the needles of the cranial window in the first 15 min of reperfusion typically results in the brain swelling and touching the cranial window.

**Experimental Protocols**

Piglets were divided into six study groups. In each group, we first obtained stable baseline measurements of pial arterioles that are ~100 μm in diameter. Then we measured responses to NMDA at concentrations of 10, 50, and 100 μmol/l. Ascending doses of NMDA were given sequentially in all cases. NMDA was dissolved in aCSF, and a single application for each concentration was administered topically through the injection ports of the cranial window onto the brain surface. Arteriolar diameters were measured continuously for 5 to 7 min after application, which is sufficient for maximal responses during normothermic and hypothermic conditions (unpublished observations). After application of all three doses, the window was flushed with aCSF and arteriolar diameters returned to baseline values. All animals...
were normothermic during this initial period except the piglets in the prolonged hypothermia group. These animals in group 3 were hypothermic throughout the experiment protocol.

*Group 1: nonischemic control animals.* In this group, we examined the influence of body temperature on pial arteriolar response to topical application of NMDA in the absence of ischemia. After measuring the response to NMDA under normothermic conditions (−38°C), we cooled these piglets to −33−34°C over 30 min (n = 7). NMDA was reapplied 1 h after the initial application to determine the effects of hypothermia on NMDA-induced dilator responses. In another group of animals (n = 3), NMDA responses were examined during normothermia and after rewarming following hypothermia. Animals were rewarmed over 30 min by applying a warm-water blanket and flexible heating pad. The interval between the two applications of NMDA in this group was ∼60 min.

We also examined arteriolar dilator responses to repeated applications of NMDA in normothermic animals (n = 9). These animals were maintained at normal body temperature and dilator responses to NMDA were examined twice. The time interval between the two challenges with NMDA was 1 h.

*Group 2: systemic hypothermia during ischemia.* These animals were studied to determine if whole body hypothermia during ischemia would preserve NMDA-induced dilation. Piglets in this group (n = 8) were normothermic during the initial application of NMDA, hypothermic during ischemia, rewarmed during reperfusion, and normothermic during the posts ischemic NMDA application. These piglets were made hypothermic just before ischemia. Once their core body temperature reached 33−34°C, the cooling blanket was removed and the animals were exposed to 10 min of global ischemia. They were rewarmed during the 1-h recovery period by reapplying the warm-water blanket and flexible heating pad. The animals were normothermic by 30 min after ischemia. Measurements of NMDA responses were made after 1 h of reperfusion.

*Group 3: prolonged systemic hypothermia during ischemia and reperfusion.* These animals were studied to determine if prolonging hypothermia into the posts ischemic reperfusion phase would protect NMDA-induced vasodilation. Piglets in this group (n = 8) were made hypothermic (33−34°C) before the initial application of NMDA and kept hypothermic throughout ischemia, reperfusion, and reapplication of NMDA. After the initial application of NMDA, the piglets were exposed to 10 min of global ischemia. Repeated measurements of NMDA responses were made after 1 h of reperfusion.

*Group 4: selective brain cooling during ischemia.* These animals were studied to determine if the direct cooling of the surface of the brain would protect dilator responses to NMDA. Direct cooling would be expected to negate possible systemic effects of hypothermia. In piglets of this group (n = 11), the brain was cooled before ischemia by perfusing aCSF chilled to 4°C over the surface of the brain continuously until the temperature of the deep cortex (5−10 mm from the surface) was 33−34°C on the side contralateral to the window. During ischemia, chilled aCSF was used to increase intracranial pressure and provide further cerebral cooling. Normal rectal temperatures were maintained. After the cessation of ischemia, deep cortical temperatures typically ranged between 23 and 27°C. During reperfusion, the temperature of the deep cortex gradually returned to normothermic values. Although we could not continue to perfuse the window with cold aCSF because of technical difficulties, as described above, the deep cortical temperature was below normothermic values especially during the early posts ischemic period. NMDA-induced changes in arteriolar diameter were measured after 1 h of reperfusion.

*Group 5: normothermic ischemia and reperfusion.* These animals were studied to provide comparative data concerning effects of ischemia on NMDA-induced arteriolar dilation. Piglets in this group (n = 8) were normothermic (−38°C) during the entire protocol, and arteriolar dilator responses to NMDA were examined before and 60 min after ischemia.

*Group 6: indomethacin-pretreated normothermic animals.* These animals were studied as a positive control to determine whether indomethacin pretreatment was able to preserve normal NMDA-induced dilation after ischemia (n = 6). To extend our previous finding that indomethacin preserved NMDA-induced vasodilation during less severe insults, piglets in this group were pretreated with 5 mg/kg iv of indomethacin 20 min before ischemia. This dose of indomethacin is sufficient to inhibit COX and to prevent anoxia-induced increases in superoxide anion production (18, 26). Measurements of NMDA responses were made before the indomethacin infusion and were compared with the response to NMDA after 1 h of reperfusion. Normothermia (−38°C) was maintained throughout the experiment.

**Statistical Analysis**

Data are expressed as means ± SE. Pial arteriolar diameter and blood pressure data were analyzed with repeated-measures ANOVA. Pairwise comparisons were made using the Student-Newman-Keuls method. P values <0.05 were considered statistically significant.

**RESULTS**

*Group 1*

Hypothermia alone or hypothermia plus rewarming in the absence of ischemia did not significantly change the effect of NMDA on pial vasodilation. Percent dilations from baseline during normothermia (38.1 ± 0.1°C) and hypothermia (33.6 ± 0.1°C) were 7 ± 1 and 6 ± 1% to 10 μmol/l NMDA, 25 ± 7 and 22 ± 7% to 50 μmol/l NMDA, and 51 ± 3 and 46 ± 7% to 100 μmol/l NMDA, respectively. Arterial blood pressure was 70 ± 2 mmHg and baseline diameter was 93 ± 4 μm during normothermia. During hypothermia, arterial blood pressure was 61 ± 2 mmHg and baseline diameter was 94 ± 4 μm.

For other animals undergoing cooling and rewarming, diameter changes were 5 ± 1, 24 ± 18, and 47 ± 13% at 10, 50, and 100 μmol/l, respectively. Baseline diameter for this group was 104 ± 8 μm.

NMDA-induced dilation was repeatable in normothermic animals. In normothermic animals (38.5 ± 0.3°C), NMDA dilated by 10 ± 2, 22 ± 5, and 42 ± 5% during the first application and by 9 ± 2, 29 ± 7, and 41 ± 6% during the second application of the three doses of NMDA. Arterial blood pressure was 64 ± 5 mmHg and baseline arteriolar diameter was 101 ± 3 μm during the first application, and during the second application, arterial blood pressure was 66 ± 5 mmHg and arteriolar diameter was 101 ± 3 μm.
Fig. 1. Effects of ischemia on N-methyl-D-aspartate (NMDA)-induced pial arteriolar dilation. Data are presented as percent preservation of preischemia responses compared with posts ischemia responses to 100 μmol/l NMDA. In all 3 hypothermia groups (groups 2-4) and in the normothermia group (group 5), NMDA-induced dilation was substantially reduced by ischemia. Also, there were no differences among responses to NMDA in these 4 groups. Similar results were seen with 50 μmol/l NMDA (data not shown). However, administration of indomethacin preserved dilator responses to NMDA after ischemia (group 6). *P < 0.05 compared with the response before ischemia and compared with the indomethacin-treated group. NS refers to the lack of statistical significance among groups 2-5.

Group 2

Brief, mild whole body hypothermia did not preserve the response to NMDA after ischemia (Fig. 1). Rectal temperature was 37.5 ± 0.3°C during the application of NMDA before hypothermia, 33.5 ± 0.1°C during ischemia, and 37.6 ± 0.2°C during the second application of NMDA following ischemia. Changes in diameter of pial arterioles in response to NMDA application were no different at the lowest concentration, 4 ± 2 vs. 4 ± 2%. However, the vascular responses to the middle and highest concentration were significantly reduced 37 ± 7 vs. 14 ± 5% and 53 ± 3 vs. 32 ± 6%, respectively (P < 0.05). Before ischemia, arterial blood pressure was 64 ± 2 mmHg and baseline arteriolar diameter was 101 ± 4 μm, and after ischemia, arterial blood pressure was 64 ± 4 mmHg and baseline diameter was 100 ± 5 μm.

Group 3

Prolonging hypothermia into the reperfusion phase also failed to preserve the dilator response to NMDA. Rectal temperature was 33.4 ± 0.1°C before ischemia, 33.8 ± 0.5°C during ischemia, and 33.3 ± 0.2°C after ischemia. Vascular responses to NMDA were modestly reduced at the 10-μmol/l concentration of NMDA, 10 ± 2 vs. 7 ± 2%. However, a significant reduction occurred at the higher concentrations. Percent dilation from baseline to NMDA (preischemia vs. posts ischemia) was 39 ± 9 vs. 15 ± 3% at 50 μmol/l (P < 0.05) and 49 ± 10 vs. 20 ± 3% at 100 μmol/l (P < 0.05). Baseline arteriolar diameter was 90 ± 5 and 96 ± 4 μm before and after ischemia, and arterial blood pressure was 61 ± 3 and 58 ± 2 mmHg before and after ischemia.

Group 4

Similarly, local cooling of the surface of the brain did not preserve NMDA-induced arteriolar dilation. Vascular responses to 10, 50, and 100 μmol/l NMDA (before vs. 1 h after ischemia with selective hypothermia) were 10 ± 3 vs. 5 ± 2%, 31 ± 5 vs. 8 ± 5% (P < 0.05), and 48 ± 5 vs. 20 ± 5% (P < 0.05), respectively. Before ischemia, baseline arteriolar diameter was 103 ± 7 μm and arterial blood pressure was 76 ± 5 mmHg. After ischemia, arteriolar diameter was 108 ± 6 μm and arterial blood pressure was 62 ± 4 mmHg.

Group 5

Under normothermic conditions (37.7 ± 0.1°C), 10 min of cerebral ischemia (37.8 ± 0.1°C) followed by 1 h of reperfusion (37.6 ± 0.2°C) significantly reduced pial arteriolar responses to NMDA. Arteriolar dilation to 10, 50, and 100 μmol/l NMDA was reduced from 6 ± 2, 28 ± 5, and 40 ± 6% preischemia to 2 ± 1, 9 ± 3 (P < 0.05), and 13 ± 3% (P < 0.05) posts ischemia. Before ischemia, baseline arteriolar diameter was 100 ± 3 μm and arterial blood pressure was 56 ± 4 mmHg. After ischemia, arteriolar diameter was 103 ± 4 μm and arterial blood pressure was 56 ± 4 mmHg.

Group 6

Pretreatment with indomethacin in normothermic animals completely preserved NMDA-induced vasodilation after cerebral ischemia and 1 h of reperfusion. The responses to NMDA at 10, 50, and 100 μmol/l (preischemia vs. 1 h after ischemia) were 4 ± 1 vs. 8 ± 3%, 14 ± 5 vs. 23 ± 7%, and 33 ± 6 vs. 33 ± 6%, respectively. Baseline arteriolar diameter was 105 ± 5 μm and arterial blood pressure was 59 ± 3 mmHg before ischemia, and diameter was 100 ± 8 μm and arterial blood pressure was 59 ± 3 mmHg after ischemia.

DISCUSSION

There are two major findings of this study. First, cerebral vascular responsiveness to an excitatory neurotransmitter is intact despite the reduced metabolic rate during hypothermia. Dilator responses to NMDA, which were dose dependent and reversible during hypothermia, were virtually identical in magnitude to responses during normothermia. Second, hypothermia fails to preserve cerebral vascular responses to NMDA after ischemia. Therefore, nonspecific suppression of the brain metabolic rate during ischemia fails to inhibit the cellular mechanisms responsible for disruption of this neurally initiated vascular response. These results suggest that protective effects of hypothermia may not be most apparent in the immediate
postischemic period, and neuroprotection may be mechanism selective rather than generalized.

We measured the preservation of NMDA-induced vasodilation after global cerebral ischemia and reperfusion as an assessment of neuronal function after ischemia. An advantage of this approach is that we can repeatedly assess neuronal function in a minimally invasive manner in the same animal under different conditions. Because cerebral resistance vessels do not themselves possess NMDA receptors linked to vasmotor responses (6, 20, 31), pial arteriolar dilation to NMDA requires initial activation of receptors on the surface of neurons with subsequent synthesis, diffusion, and actions of NO. Additionally, recent studies by our laboratory directly document the involvement of neurally derived NO in the mediation of dilator responses (16). In previous studies, we showed that cerebrovascular responses to exogenous NO remain intact after 10 min of ischemia (13) or 15 min of hypoxia (4), thereby further indicating the neuronal basis for this response. In addition, cortical NO synthase (NOS) activity is not affected by ischemia (13). Therefore, impairment of vasodilation after cerebral ischemia is not due to decreased NOS activity or reduced vascular responsiveness to NO but to a modulation of the response to NMDA at the neuronal level. It seems likely that NMDA receptors are a primary target of ROS derived from COX metabolism of arachidonic acid as well as from damaged mitochondria after ischemia (22, 24, 30). Our studies showing that administration of structurally different inhibitors of COX (indomethacin and NS398) (4, 12, 18), as well as superoxide dismutase (4), is able to preserve NMDA-induced dilation following a variety of hypoxic/ischemic conditions provide support for the concept that superoxide formation via the COX pathway is a pivotal event in neuronal dysfunction under these conditions.

On the basis of these earlier results and previous findings that indicate a protective effect of hypothermia against neuronal injury in several experimental models of brain injury (15, 23, 32), it seemed reasonable to hypothesize that hypothermia would preserve normal NMDA-induced dilation. Thus, it was surprising to us that ischemia-related decreases in arteriolar dilator responses were similar in hypothermia and normothermia animals. Furthermore, it was unexpected that dilator mechanisms involved in NMDA-induced dilation were fully functional in nonischemic but hypothermic animals. Therefore, our results indicate that despite generalized decreases in metabolic rate and enzymatic activity during hypothermia (1, 11, 23), cortical neurons are still responsive to activation of NMDA receptors, and this vascular response is affected by ischemic insult. Although we did not further explore mechanisms of impaired dilator responses to NMDA, there is no reason to think that they are different during hypothermia compared with normothermia. The differences between our results and those of others showing a protective effect of hypothermia may be in the duration of the experiment and the target of investigation. For example, our observation period after ischemia was limited to 1 h, rather than to hours or days. In addition, we assessed neuronal function and vasoactivity rather than neuronal cell death.

Another surprising finding was the absence of a change in pial arteriolar diameter when the piglets were cooled to 33–34°C. We previously showed that at a rectal temperature of 33.9°C, temperature of the superficial cortex was almost identical to this value, and cortical blood flow and metabolic rate were both reduced by ~40% (11). Furthermore, direct measurements of cortical temperature in animals equipped with a cranial window in the current study show a similar relationship between rectal and brain temperatures. The most likely explanation is that under these circumstances, hypothermia-induced arteriolar constriction occurred predominantly at the level of the intraparenchymal small pial arterioles. Unfortunately, we were unable to examine responsiveness of intraparenchymal arterioles in the current study because of technical limitations. Nonetheless, as we showed previously with arterial hypercapnia and with the current study using topical NMDA, arteriolar responsiveness is intact despite hypothermia.

In summary, hypothermia does not preserve NMDA-induced vasodilation in newborn pig pial arterioles after ischemia. These findings suggest that hypothermia alone may not be fully effective in protecting against brain injury in asphyxiated babies and should be used in conjunction with pharmacological agents directed against production and actions of ROS.

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