Uncomplicated Rapid Posthypothermic Rewarming Alters Cerebrovascular Responsiveness

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Background and Purpose—Recently, we focused on the cerebrovascular protective effects of moderate hypothermia after traumatic brain injury, noting that the efficacy of posttraumatic hypothermia is related to the rate of posthypothermic rewarming. In the current communication, we revisit the use of hypothermia with varying degrees of rewarming to ascertain whether, in the normal cerebral vasculature, varying rates of rewarming can differentially affect cerebrovascular responsiveness.

Methods—Pentobarbital-anesthetized rats equipped with a cranial window were randomized to 3 groups. In 1 group, a 1-hour period of hypothermia (32°C) followed by slow rewarming (over 90 minutes) was used. In the remaining 2 groups, either a 1- or 2-hour period of hypothermia was followed by rapid rewarming (within 30 minutes). Vasoreactivity to hypercapnia and acetylcholine was assessed before, during, and after hypothermia. Additionally, the vascular responses to sodium nitroprusside (SNP) and pinacidil, a K_{ATP} channel opener, were also examined.

Results—Hypothermia itself generated modest vasodilation and reduced vasoreactivity to all utilized agents. The slow rewarming group showed restoration of normal vascular responsivity. In contrast, hypothermia followed by rapid rewarming was associated with continued impaired responsiveness to acetylcholine and arterial hypercapnia. These abnormalities persisted even with the use of more prolonged (2-hour) hypothermia. Furthermore, posthypothermic rapid rewarming impaired the dilator responses of SNP and pinacidil.

Conclusions—Posthypothermic rapid rewarming caused cerebral vascular abnormalities, including a diminished response to acetylcholine, hypercapnia, pinacidil, and SNP. Our data with acetylcholine and SNP suggest that rapid rewarming most likely causes abnormality at both the vascular smooth muscle and endothelial levels. (*Stroke*. 2004;35:601-606.)

Key Words: cerebrovascular circulation ∎ hypothermia ∎ rewarming ∎ rats

W e previously reported that rapid rewarming after moderate hypothermia led to an exacerbation of axonal injury and altered pial arteriolar relaxation in response to hypercapnia and acetylcholine in traumatically brain-injured animals.[1,2] In fact, in terms of the acetylcholine responsiveness, posthypothermic rapid rewarming reduced the vascular response more than that seen with normothermia alone, further highlighting the damaging consequences of rapid posthypothermic rewarming. These relatively adverse consequences of rapid posthypothermic rewarming also appear consistent with other recent reports advocating the use of slow rewarming as the appropriate approach after hypothermic treatment.[3–5] To better understand, however, how posthypothermic rewarming exerts its damaging consequences, the use of concomitant trauma, ischemia, and/or other forms of injury introduces another subset of problems. Accordingly, to more fully investigate the effects of rapid posthypothermic rewarming, in this study we have chosen to use normal animals equipped with cranial windows for direct observation of pial microcirculation over the parietal cortex. During hypothermia and subsequent rapid rewarming, cerebral vascular responsiveness was tested against various known vasodilators to probe for differential responses to address potential mechanistic issues.

Materials and Methods

Thirty-five male Sprague-Dawley rats aged 3 to 4 months (Charles River Laboratories) were prepared in accordance with our institution's policies on animal use and care. After administration of sodium pentobarbital anesthesia (55 mg/kg IP), each animal's femoral vein and artery were cannulated. After completion of tracheotomy, the lungs were mechanically ventilated with room air, and each animal received pancuronium bromide (3 mg/kg IV). Arterial blood samples were taken before each measurement of pial arteriolar diameter. During hypothermia, alpha-stat management was used. Ventilation was adjusted so that $PaCO_2$ ranged from 35 to 40 mm Hg at the resting state. This $PaCO_2$ level, unless modified for pial vessel assessment, was maintained throughout each experiment

by changing the respirator setting. Both the temporalis muscle and rectal temperatures were monitored and maintained at 37°C throughout all surgical procedures.

Cranial Window Installation

The microcirculation of right parietal cortex was visualized through a cranial window. The cranial window and the technique for its implantation have been described previously.[2,6] Briefly, a midline sagittal scalp incision was made over the parietal bones, and the skull was exposed via blunt dissection. A craniotomy (2×4 mm) was made in the right parietal bone. The cranial window was implanted over the craniotomy after dural incision. The space under the window was filled with artificial cerebrospinal fluid (CSF). The window itself had 3 openings, 2 of which could be used as inlet and outlet for vehicle or drug infusion into the CSF bathing the pial vessels. The third was connected to a pressure transducer for measurement of intracranial pressure. Intracranial pressure was set at 5 mm Hg via connection to a fluid column set at a predetermined height. The pH of bathing CSF was adjusted to approximately 7.35 by equilibration with a gas mixture containing 6% O_2 and 6% CO_2 balanced with N_2. The caliber of pial arterioles was measured with a Vickers' image-splitting device.

Vasoreactivity to Hypercapnia

After an equilibration period, the diameters of several clearly delineated pial vessels were measured while the animals breathed room air. Subsequently, changes in $PaCO_2$ were induced by ventilating the animal with gases containing 3% or 5% CO_2 in air. Each CO_2 level was maintained for at least 7 minutes before measurements were initiated. The vessel diameter was again measured until a steady state was established.

Vasoreactivity to Acetylcholine

Acetylcholine (Sigma) was dissolved in artificial CSF to reach a final concentration of either 0.1 or 10 μmol/L. Vessel diameters were measured between 2 and 4 minutes after topical application of each concentration of acetylcholine.

Vasoreactivity to Sodium Nitroprusside and Pinacidil

Sodium nitroprusside (SNP) (Sigma) was dissolved in artificial CSF to reach a final concentration of either 0.5 or 1 μmol/L. Pinacidil (Sigma), a K_{ATP} channel opener, was dissolved in artificial CSF to reach a final concentration of either 1 or 2 μmol/L. Pinacidil stock solution was made in ethanol and diluted in CSF. The final concentration of ethanol was 0.5%, which has been shown to have no effect on K_{ATP} channels.[7] Responses to topically applied drugs were determined 2 to 4 minutes after each application.

Experimental Design

All animals were examined for their baseline cerebrovascular responses to hypercapnia or the chosen vasoactive agents. Temporalis muscle temperature was used as a substitute for brain temperature.[8] Hypothermia was induced by ice packs placed around the body, and rewarming was achieved by heat lamp and heating pad, as described previously.[2] When the temperature reached 32°C, this time was designated the starting point on which all subsequent measurements were based. Temperatures were maintained at 32°C for either 1 or 2 hours. After the hypothermic period, the animals were rewarmed to 37°C over either a 90-minute period (slow rewarming) or a 30-minute period (rapid rewarming) according to protocols for controlled rewarming described in detail elsewhere.[1] The cranial window was shielded from light except for the period of observation.

Group 1

After a 1-hour period of hypothermia, the animals were rewarmed slowly to 37°C. Twenty vessels were assessed (4 vessels per animal) in response to elevated CO_2 (n=5) and varying concentrations of acetylcholine (n=5). Cerebrovascular responsivity was tested before hypothermia as well as at 40, 180, and 240 minutes after a temperature of 32°C was reached.

Group 2A

After a 1-hour period of hypothermia, the animals were rapidly rewarmed to 37°C within 30 minutes. Cerebrovascular responsivity was tested with hypercapnia (n=5) or acetylcholine (n=5) before hypothermia as well as at 40, 120, 180, and 240 minutes after a temperature of 32°C was reached.

Group 2B

This subgroup was prepared to examine pial vasodilatory effects of pinacidil and SNP. The hypothermic and response test paradigms were the same as for group 2A. Twenty vessels from 5 animals were assessed in response to varying concentrations of pinacidil and SNP. The interval required for the assessment typically spanned 5 minutes, with the diameter allowed to return to resting levels before the next drug administration.

Group 3

After a 2-hour period of hypothermia, the animals were rapidly rewarmed to 37°C within 30 minutes. Cerebrovascular responsivity was tested with hypercapnia (n=5) or acetylcholine (n=5) before hypothermia as well as at 40, 180, and 240 minutes after a temperature of 32°C was reached.

Statistical Analysis

Data are expressed as mean±SD. Data were analyzed with the use of either 1-way ANOVA or a generalized linear model of multivariate procedure for repeated measurements. The generalized linear model multivariate procedure for repeated measurements was used for the comparison of time-dependent arterial diameter changes in response to hypercapnia or acetylcholine among the 3 groups. When the F value was significant, pairwise comparisons were made with the Bonferroni test. Differences were considered to be statistically significant at $P<0.05$.

Results

Physiological Observations

Blood gases and pH values were taken before each measurement of pial arteriolar diameter. These values are shown in Table 1. During hypothermia, the PaO_2 showed significant increases; however, these values returned to control levels after rewarming. In all groups, the $PaCO_2$ was maintained at 35 to 40 mm Hg during the resting state, with elevation to 50 and 60 mm Hg via the use of inhaled 3% and 5% CO_2, respectively. Similar elevations of $PaCO_2$ were observed over the entire period of their use. Physiological data obtained during assessment of the cerebrovascular responses to the chosen vasoactive agents were within normal limits (Table 2). Finally, use of the chosen hypothermic protocols allowed attainment of a controlled temporal pattern of body cooling and rewarming (Figure 1).

Resting Vessel Diameters

The mean vessel diameters for each group at control levels ranged from 41.8±10.7 to 42.4±7.4 μm, with no statistical significance. During hypothermia, pial vessels typically dilated. Group 1 maintained its initial resting diameter at all time points; however, in groups 2 and 3 the vessel diameters increased significantly in comparison to control diameters (Figure 2).

TABLE 1. Physiological Data in the Hypercapnia Tests

	Control			40 min			120 min (Group 2)	180 min			240 min		
	Resting	3% CO_2	5% CO_2	Resting	3% CO_2	5% CO_2		Resting	3% CO_2	5% CO_2	Resting	3% CO_2	5% CO_2
MABP, mm Hg													
Group 1	118±2	112±2	117±2	109±4	109±6	109±3	109±4 (resting)	103±5	112±5	108±4	104±5	113±3	113±2
Group 2	113±0	119±3	117±4	113±3	103±2	104±3	106±5 (3%)	106±5	111±6	108±6	103±4	97±6	102±9
Group 3	115±2	117±3	120±1	112±2	116±2	110±3	113±6 (5%)	110±3	104±6	108±3	106±2	106±4	110±3
Pao₂, mm Hg													
Group 1	82±3	80±2	87±2	113±2*	118±3*	128±2*	72±3 (resting)	76±4	89±3	89±3	84±4	91±4	98±5
Group 2	76±3	79±3	86±2	105±9*	109±8*	123±7*	88±1 (3%)	83±2	87±2	93±3	85±4	93±2	99±3
Group 3	84±5	94±3	98±4	132±5*	133±5*	139±4*	94±3 (5%)	74±4	90±7	89±9	88±5	98±2	99±4
Paco₂, mm Hg													
Group 1	38±1	52±1	66±1	35±1	52±1	67±0	38±0 (resting)	38±1	51±1	64±1	39±1	51±1	67±1
Group 2	35±0	50±1	65±0	35±2	51±1	66±1	51±1 (3%)	36±1	51±1	64±1	37±1	50±1	65±1
Group 3	37±2	50±1	62±1	34±2	54±2	69±2	63±2 (5%)	37±1	49±2	64±3	36±1	51±1	64±1
pH													
Group 1	7.5±0	7.4±0	7.3±0	7.5±0	7.4±0	7.3±0	7.4±0 (resting)	7.5±0	7.4±0	7.3±0	7.4±0	7.3±0	7.3±0
Group 2	7.5±0	7.4±0	7.3±0	7.5±0	7.4±0	7.3±0	7.4±0 (3%)	7.4±0	7.3±0	7.3±0	7.4±0	7.3±0	7.2±0
Group 3	7.4±0	7.4±0	7.3±0	7.5±0	7.3±0	7.3±0	7.3±0 (5%)	7.4±0	7.3±0	7.3±0	7.4±0	7.3±0	7.3±0

*$P<0.05$ indicates significant differences in comparison to corresponding control values.
MABP indicates mean arterial blood pressure.
All physiological data are expressed as mean±SEM.

Responses to Arterial Hypercapnia

The vasodilator response to CO_2 was significantly reduced during hypothermia. After completion of rewarming, group 1 showed a restoration of normal responsivity to hypercapnia, while in groups 2 and 3 the responses were diminished significantly in comparison to the corresponding control responses (Figure 3).

TABLE 2. Physiological Data in the ACh, Pinacidil, and SNP Tests

	Control	40 min	120 min	180 min	240 min
MABP, mm Hg					
Group 1	120±2	109±5		115±2	111±4
Group 2	114±3	104±6	104±4	104±3	110±5
Group 3	117±2	113±1		111±4	106±3
Pao₂, mm Hg					
Group 1	80±1	108±9*		75±5	81±5
Group 2	78±4	117±7*	80±2	86±3	84±3
Group 3	91±5	133±5*		73±4	88±5
Paco₂, mm Hg					
Group 1	39±1	36±1		41±2	41±2
Group 2	38±1	37±2	37±1	38±1	37±1
Group 3	37±1	35±2		37±1	36±1
pH					
Group 1	7.5±0	7.5±0		7.4±0	7.4±0
Group 2	7.5±0	7.5±0	7.4±0	7.4±0	7.4±0
Group 3	7.5±0	7.5±0		7.4±0	7.4±0

*$P<0.05$ indicates significant differences in comparison to corresponding control values.
MABP indicates mean arterial blood pressure.
All physiological data are expressed as mean±SEM.

Response to Acetylcholine

During hypothermia, all animals showed reduced vasoreactivity to acetylcholine. After the completion of rewarming, group 1 showed a restoration of normal acetylcholine responsivity. Group 2 showed a normal responsivity after completion of rewarming until 120 minutes, when the vasoreactivity diminished dramatically. These reductions were significant at both the 180- and 240-minute time points in comparison to the control groups. Group 3 also showed significantly reduced responsiveness (Figure 4).

Response to Pinacidil and SNP

During hypothermia, both pinacidil- and SNP-induced vasodilations were significantly reduced. On completion of rapid rewarming, these reduced responses were not fully restored, with these reductions achieving significance in comparison to each corresponding control value (Figure 5).
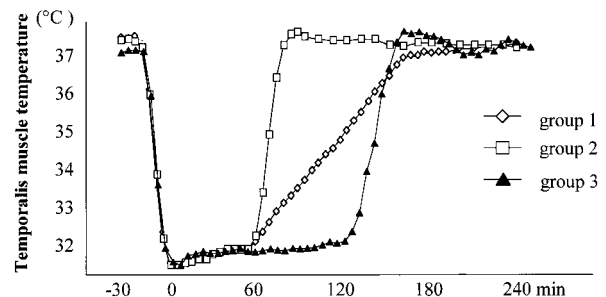

Figure 1. Changes of mean temporalis muscle temperature. In group 1, after 1 hour of hypothermia the animals were rewarmed to normothermic levels over a 90-minute period. In groups 2 and 3, after a 1- or 2-hour hypothermic period, the animals were rewarmed to normothermic levels within 30 minutes.
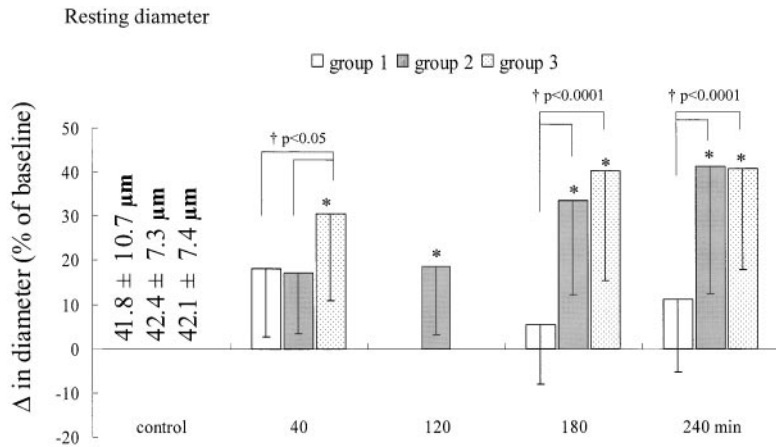
Resting diameter

Figure 2. In all groups, the diameter of pial arterioles revealed a progressive increase during hypothermia. Pial vessels in group 1 returned to their initial size; however, both rapid-rewarming groups showed significant vascular dilation. *$P < 0.05$ compared with corresponding control value.

Discussion

The data from this study show that moderate hypothermia induces mild to moderate cerebral arteriolar dilation and significantly inhibits the response of cerebral arterioles to known dilators such as acetylcholine and hypercapnia. On cessation of hypothermia, vessel caliber returned to control values only in animals that were rewarmed slowly. In contrast, cerebral dilation and vascular abnormalities persisted in those animals subjected to rapid posthypothermic rewarming, even in animals exposed to prolonged hypothermia (2 hours). Furthermore, rapid posthypothermic rewarming inhibited cerebral arteriolar dilation to pinacidil, a known ATP-sensitive K^+ channel opener, and SNP, an endothelium-independent dilator.

The mechanisms of hypothermic protection are likely multifactorial, with contemporary thought now advocating that hypothermia prevents secondary brain damage by slowing or preventing the failure of various energy parameters, preserving brain ATP levels, while reducing the brain's demand for oxygen and glucose.[9] In this study we found that

hypothermia of 32°C caused mild to moderate dilation in most cerebral arterioles, similar to observations made by others in isolated cerebral vessels from experimental animals and humans.[10,11] In contrast, vasoconstriction in response to hypothermia at 30°C has been reported[12]; however, in that study the space under the window underwent continuous perfusion with artificial CSF, washing out endogenous vasoactive factors. Recently, Mustafa and Thulesius,[13] who reported cooling-induced carotid artery dilation, speculated that local carotid cooling may have therapeutic potential by increasing the brain's blood supply. Cerebral vasorelaxation on cooling may lead to a loss of resting tone. Thus, the vessels become passively relaxed, possibly increasing cerebral blood flow. The caveat here, however, is that increased arterial dilation does not in all cases translate into increased cerebral blood flow.

In addition to vasodilation, hypothermia in our study also reduced the vascular responsiveness to known dilators, including acetylcholine, SNP, pinacidil, and hypercapnia. Irikura et al[12] reported that hypothermia at 30°C inhibited cerebral autoregulation to hypotension, whereas the response
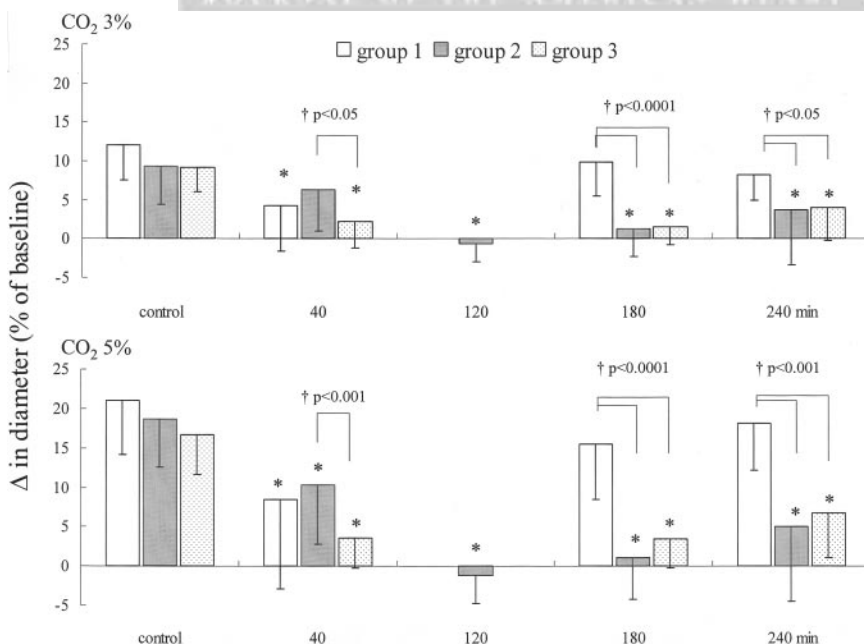

Figure 3. Hypercapnia-induced vasodilation was significantly reduced during hypothermia. Although group 1 returned to initial levels of responsivity after rewarming, groups 2 and 3 showed severely reduced hypercapnia-induced relaxation after rewarming. Although the reduced responsivity persisted through the 240-minute time point, it showed slight recovery with continued survival (group 2; 5% CO_2 induced the following percent increase in dilation: 120→240 minutes, $-1.2 \pm 3.5\% \rightarrow 5.1 \pm 9.6\%$ [$P < 0.05$]). *$P < 0.05$ compared with corresponding control value.
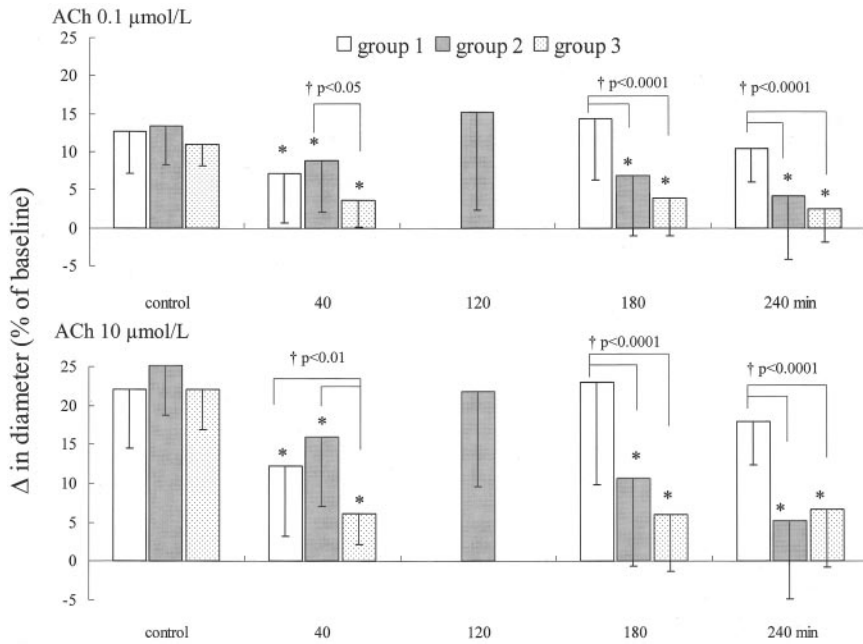
Figure 4. Acetylcholine (ACh)-induced vasodilation was significantly reduced during hypothermia. Group 1 showed complete restoration of responsivity after rewarming. Groups 2 and 3 showed severely reduced acetylcholine-induced relaxation after rewarming, and these abnormalities deteriorated with time. In group 2, at 120 minutes, immediately after completion of the rapid rewarming, acetylcholine-induced relaxation had not deteriorated; however, with continued survival the reactivity ultimately deteriorated. *$P<0.05$ compared with corresponding control value.

to hypercapnia remained intact. It is not clear, however, whether the continuous perfusion of CSF over the exposed brain surface in their preparation contributed to this difference.

Inoue et al[14] reported that hypothermia significantly attenuated nitroglycerin-induced cerebral dilation in cats, while the dilator response to cromakalim, a K_{ATP} channel opener, was enhanced. In contrast, Saito et al[15] demonstrated in isolated guinea pig aorta that cromakalim-induced vasodilation was greatly reduced during hypothermia at 23°C. It is not clear whether differences in species played a role.

The assessment of any hypothermic treatment would be incomplete in terms of its clinical implications without consideration of the consequences of rewarming. In the present study the animals were rewarmed either slowly over

90 minutes or rapidly within 30 minutes. We found that while hypothermia rendered the cerebral arterioles dysfunctional, normal responsiveness to known vasodilators was restored on slow rewarming. This is consistent with previous findings from our laboratory showing that slow posthypothermic rewarming reduced the number of traumatically injured axons, whereas rapid rewarming exacerbated axonal damage in brain-injured animals.[1] Taken together, it is unlikely that hypothermia itself or hypothermia followed by slow rewarming causes permanent vascular impairment.

It should be noted that rapid rewarming after prolonged hypothermia of 2 hours resulted in no improvement in vascular responsiveness. The fact that cerebral blood vessels dilated further after onset of rapid rewarming suggests that tissue injury perturbation most likely occurred during or
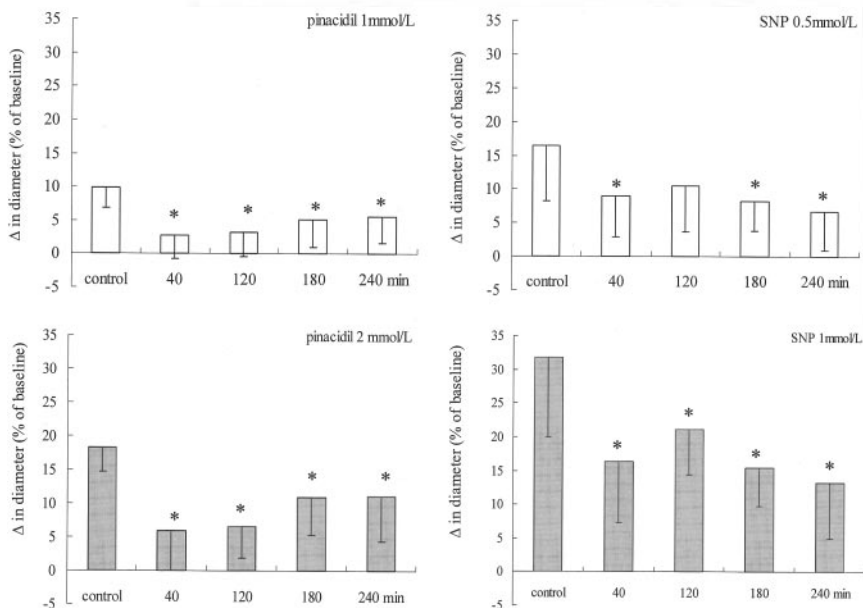

Figure 5. Pinacidil-induced vasodilation was significantly reduced after hypothermia/rewarming. Although the reduced responsiveness persisted after rewarming, significant response improvement was observed from 40 to 240 minutes (6.0±6.0%→11.1±6.8% [2 μmol/L]; $P<0.05$, 1-way ANOVA followed by Bonferroni test). SNP-induced vasodilation was significantly reduced during hypothermia. After completion of rapid rewarming, this reduced responsiveness persisted. *$P<0.05$ compared with corresponding control value.

immediately after rapid rewarming. It has been posited that rapid rewarming causes an increase in the cerebral metabolic rate for oxygen temporarily unmatched by cerebral blood flow.[16]

In the present study the cerebral dilator response to acetylcholine remained significantly inhibited for 4 hours after onset of hypothermia. While the vasoactive response of acetylcholine depends on a healthy endothelium, SNP does not. As a nitric oxide donor, SNP directly affects the vascular smooth muscle, activating guanylate cyclase to lead to increased cGMP, resulting in vascular smooth muscle relaxation. The currently observed failure to respond to acetylcholine and SNP strongly suggests that rapid posthypothermic rewarming caused impairment of the vascular smooth muscle as well as the endothelium. It is known that guanylate cyclase contributes to the regulation of resting tone.[17,18] Furthermore, since potassium channels are purportedly silent at basal conditions,[19] the inhibition of guanylate cyclase most likely explains the sustained vasodilation observed in animals subjected to rapid posthypothermic rewarming.

In addition to the aforementioned investigations, we also examined another major mechanism for the cerebral arteriolar relaxation, namely, the activation of K_{ATP} channels,[20] with pinacidil, a K_{ATP} channel opener, used to elicit vasodilation. As shown, the response to pinacidil was significantly reduced; however, slight improvement was observed with time. Although our tests with pinacidil and hypercapnia were performed in separate animal groups, the pattern of vascular responses to pinacidil paralleled those responses seen with hypercapnia.

In summary, posthypothermic rapid rewarming triggers direct perturbation of the cerebral microcirculation. It not only nullifies the potential protective effect of hypothermic treatment but also causes vascular abnormalities at both endothelial and vascular smooth muscle levels, even in the uninjured brain. These findings may have long-term implications for patients managed with hypothermia, calling for continued preclinical and clinical evaluation.

Acknowledgment

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