Effect of profound hypothermia during circulatory arrest on neurologic injury and apoptotic repressor protein Bcl-2 expression in an acute porcine model

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Objectives: We reported that the neocortex and hippocampus are selectively vulnerable to injury in an acute porcine model of hypothermic circulatory arrest at 18°C. We hypothesize that further cooling to 10°C could reduce neurologic injury in these regions. To further elucidate the mechanisms of neurologic injury and protection, we assessed the expression of the anti-apoptotic protein Bcl-2.

Methods: Twelve piglets underwent 75 minutes of hypothermic circulatory arrest at 18°C (n = 6) and 10°C (n = 6). After gradual rewarming and reperfusion, animals were put to death and brains were perfusion-fixed and cryopreserved. Regional patterns of neuronal apoptosis after hypothermic circulatory arrest were characterized by in situ DNA fragmentation with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) histochemistry. Bcl-2 protein expression was characterized with immunohistochemistry. Statistical comparisons were made by *t* test, analysis of variance, and Mann-Whitney *U* test, as appropriate.

Results: Concentrations of TUNEL(+) cells were significantly lower after profound hypothermia at 10°C compared with 18°C hypothermia in the sensory and motor neocortex and hippocampus (*t* test, $P < .0001$; $P < .006$; $P < .006$, respectively). Positive Bcl-2 immunostaining was observed only in the motor and sensory neocortex and hippocampus after 18°C hypothermic circulatory arrest. Profound cooling to 10°C resulted in a significant increase in Bcl-2 immunostaining in the motor and sensory cortex as compared with 18°C (Mann-Whitney *U* test, $P < .05$).

Conclusions: Deep hypothermia at 10°C protects the neocortex and hippocampus from insult during hypothermic circulatory arrest as suggested by significantly reduced TUNEL(+) staining in these areas. Although a concomitant increase in Bcl-2 expression was observed in the neocortex at 10°C, it remains unclear whether profound hypothermia deters from neuronal injury by activation of the anti-apoptotic protein Bcl-2.

Experimental studies have demonstrated that prolonged hypothermic circulatory arrest (HCA) can lead to neuronal cell death, probably as a consequence of a number of different pathways triggered by ischemia.¹⁻³ Cerebral ischemia can lead to neuronal injury by the process of apoptosis, as well as by necrosis, with a series of steps existing between the initial ischemic insult and neuronal death. Within this

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Abbreviations and Acronyms

ANOVA	= analysis of variance
CPB	= cardiopulmonary bypass
HCA	= hypothermic circulatory arrest
PLSD	= protected least significant difference
TUNEL	= terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

cascade, several proteins that facilitate neuronal survival compete with molecules that contribute to cell death. Ultimately, the final balance between cell survival-promoting proteins versus cell death-promoting proteins determines the fate of the cell.⁴ The Bcl-2 family of proteins plays an important role in this cell survival–cell death decision.^{5,6}

HCA has been used for some 40 years as a means of interrupting normal perfusion of the brain and preventing subsequent cerebral ischemic injury during various cardiovascular surgical procedures. Neuroprotection appears to be effectively achieved by hypothermia during HCA, although the mechanisms underlying this effect remain to be elucidated. Hypothermia acts by reducing cerebral metabolic activity and oxygen demand, preventing the release of neurotransmitters, and delaying the onset of fatal biochemical cascade.^{7–9} Although reduced, brain metabolism is not adequately suppressed and remains relatively high in conventional HCA protocols at 18°C.⁷ In a previous report, we¹⁰ characterized acute brain injury after HCA in a juvenile pig model. We found that after 75 minutes of HCA at 18°C, there were increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive staining cells indicating DNA fragmentation, especially in the neocortex and hippocampus, with the absence of morphologic evidence of apoptosis. We hypothesized that these findings were compatible with the early activation of the apoptotic pathway.

In light of evidence suggesting that the cascade of events leading to apoptosis may be inhibited in the earlier stages,^{1,11} the present study was undertaken to assess whether profound cooling to 10°C can reduce neurologic injury during 75 minutes of HCA in an acute porcine model compared with less profound cooling (18°C). To further elucidate the mechanisms of neurologic injury and protection, we assessed the expression of the anti-apoptotic protein Bcl-2.

Materials and Methods

Sixteen male juvenile pigs from a commercial farm, 2.5 to 3.5 months of age and weighing 30 to 35 kg, were used for this study. The animals were divided into three groups: group A (n = 6) underwent HCA at 18°C for 75 minutes, group B (n = 6) underwent HCA at 10°C for 75 minutes, and group C (n = 4) served as normal controls. All animals were treated in accordance with the “Principles of Laboratory Animal Care,” as described by the National Society for

Medical Research, and the “Guide for the Care and Use of Laboratory Animals” of the Institute of Laboratory Resources, National Research Council. The protocol used in this study was also approved by the Animal Care and Use Committee of the University of Ioannina.

Animal Preparation

Preparation and surgery were performed as previously described.¹⁰ In brief, catheters were inserted in an ear vein and the left femoral artery for monitoring purposes and withdrawal of blood samples. Anesthesia was induced with intramuscular ketamine hydrochloride (15 mg/kg), atropine (0.05 mg/kg), and midazolam (Dormicum; 0.1 mg/kg) and was maintained with intravenous fentanyl (50–200 µg/kg), midazolam, and 1% to 2% isoflurane. Paralysis was achieved with an intravenous bolus of rocuronium (0.6 mg/kg) and was maintained with 20% of the total dose every 30 minutes.

Animals were ventilated mechanically with 100% oxygen, after endotracheal intubation. Ventilator rate and tidal volume were adjusted to maintain the P_{aCO_2} tension at 40 mm Hg. Hematocrit values during cardiopulmonary bypass (CPB) were maintained between 13% and 23%. A temperature probe was placed in the rectum, and brain temperature was determined with bilateral tympanic membrane probes. Urine output was collected through a bladder catheter (Foley 8F–10F). Arterial pressure, end-expired carbon dioxide, electrocardiogram, and blood gases (ABL Radiometer Medical A/S DK-2700, Copenhagen, Denmark) were monitored.

CPB and HCA

As previously described, the chest was opened via a right thoracotomy in the fourth intercostal space.¹⁰ After administration of intravenous heparin (300 IU/kg), cannulas were advanced to the ascending aorta (16F arterial cannula) and to the right atrium (single 26F cannula). Nonpulsatile CPB was initiated at a flow rate of 100 mL · kg⁻¹ · min⁻¹ and then adjusted to maintain a minimum arterial pressure of 50 mm Hg. To avoid distention of the left ventricle during CPB, we inserted a 10F vent catheter via the superior pulmonary vein. The lungs were allowed to collapse after CPB was initiated. The CPB circuit was primed with a bloodless solution consisting of 1000 mL lactated Ringer's solution, 50 mL mannitol, and 5000 IU heparin. Sodium bicarbonate was added to adjust the pH to 7.4, as necessary.

CPB was continued for an average 58 or 106 minutes, to reach a deep brain temperature of 18°C or 10°C, respectively. Myocardial protection was afforded by applying iced saline (4°C) topically during the 75-minute interval of HCA. When the tympanic membrane temperature reached 18°C or 10°C, bypass was discontinued, the blood was drained into the oxygenator reservoir, and circulatory arrest was maintained for 75 minutes. Ice bags were positioned around the head to maintain the brain temperature during HCA. At the end of the arrest, bypass was initiated again with gradual rewarming to a rectal temperature of approximately 35°C to 36°C. A temperature gradient exceeding 10°C between the perfusate and the core temperature was avoided. A temperature of 36°C was reached after an average of 83 or 104 minutes of reperfusion for animals treated with 18°C or 10°C HCA, respectively. Systemic pressure was maintained above 60 mm Hg during reperfusion. Measurements of hemodynamics (heart rate, mean arterial pressure), arterial blood gases, hematocrit, glucose, as well as temperatures were recorded at 5 time points during the experiment: (1) baseline at 37°C and before CPB; (2) at the initiation

of CPB; (3) during CPB, while cooling to a brain temperature of 18°C or 10°C just before HCA; (4) during rewarming; and (5) at the end of CPB.

Histologic Preparation and Evaluation

At the end of the experiment (approximately 170 minutes after the onset of circulatory arrest), brains were perfused in situ with chilled saline solution 0.9% (1 L) followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline solution (1 L, pH 7.4). The descending aorta was crossclamped to avoid significant loss of perfusion solution to the lower body. The brains were removed en toto, immersed in 4% paraformaldehyde, and stored at 4°C in phosphate-buffered saline solution. Control animals ($n = 4$) received no intervention and were put to death for histologic analysis.

All brains were bisected in the sagittal plane. Tissue blocks from the left hemisphere were cut to encompass brain regions known for their vulnerability to hypoxia and ischemia. Brain regions evaluated included the precentral gyrus (motor neocortex), the postcentral gyrus (sensory neocortex), hippocampus, cerebellum, thalamus, and anterior ventral medulla. Tissue blocks were dehydrated in ethanol and xylene and embedded in paraffin. Serial 8- μ m sections were cut from each tissue block and were mounted onto slides. Hematoxylin and eosin was used to characterize cell damage morphologically.

Neuronal apoptosis was characterized by in situ DNA fragmentation with TUNEL histochemistry. The TUNEL assay was performed as described elsewhere⁴ with the Apop Tag in situ Apoptosis Detection Kit–Peroxidase (Oncor, Gaithersburg, Md). Each assay included positive and negative controls. All slides were evaluated by a neuroanatomist in a blind fashion. Cell damage was categorized as either necrotic or apoptotic according to classic morphologic criteria in sections prepared with hematoxylin and eosin, as previously described.¹⁰

TUNEL(+) cells were identified by a red-stained, condensed nucleus with apoptotic bodies, along with a diminutive or absent cytoplasm. To describe the extent of apoptosis in the various brain regions, we used a semiquantitative scoring system.¹¹ Each slide was scored on a scale of 0 to 5, as follows: grade 0, no TUNEL(+) cells; grade 1, less than 10% TUNEL(+) cells; grade 2, 10% to 25% TUNEL(+) cells; grade 3, 25% to 50% TUNEL(+) cells; grade 4, 50% to 75% TUNEL(+) cells; and grade 5, greater than 75% TUNEL(+) cells. Scores from histologic evaluation and TUNEL assays were averaged from 4 to 8 slides from every region in each animal.

Bcl-2 Immunohistochemistry

Cryosections were fixed in 75% acetone and 25% ethanol for 10 minutes, then treated with 10 μ g/mL proteinase K (Dako Corporation, Carpinteria, Calif) for 15 minutes and 0.5% Triton X-0.03% H₂O₂-0, 1% body surface area for 20 minutes. Sections were incubated in 1.5% normal goat serum for 1 hour, then incubated overnight at 4°C in specific first Bcl-2 antibody (Dako). Slides were then incubated for 1 hour at room temperature in appropriate biotinylated secondary immunoglobulin G preabsorbed to normal serum. After being washed in phosphate-buffered saline solution, the sections were incubated for 1 hour at room temperature in 2% avidin-biotin complex, followed by 3,3'-diaminobenzidine as the chromogen. Negative controls consisted of sections incubated without the antibodies. Thymus was used as a positive control.

The number of immunopositive cells in 4 to 5 fields was counted by an investigator blinded to the treatment groups. Staining intensities were graded according to the number of positive cells counted with a 4-grade scale: (1) negative: 0 cells stained; (2) weakly positive: 1 to 5 cells stained; (3) positive: 6 to 15 cells stained; and (4) moderately positive: more than 15 cells stained.

Statistical Analysis

Values are expressed as mean $\pm$ standard deviation (SD) unless indicated otherwise. When appropriate, differences between two groups were assessed by the unpaired 2-tailed t test. Differences among groups in TUNEL histochemistry studies were compared by analysis of variance (ANOVA) followed by the Fisher protected least significant difference (PLSD) post hoc analysis. Differences between groups in Bcl-2 immunohistochemistry studies were assessed with the Mann-Whitney U rank sum test for noncontinuous data.

Results

Physiologic and Metabolic Parameters

All experimental animals survived the surgical protocol and HCA, as described above. All animals used in this study were male and were housed for at least 3 days in the Animal Housing Facilities of the University of Ioannina. Mean ($\pm$ SD) preoperative body weights for animals treated with 18°C and 10°C HCA and normal controls were 30.7 ± 3.7 , 34.2 ± 3.1 , and 31.3 ± 3.0 kg, respectively. Respective mean ($\pm$ SD) ages were 70.5 ± 7.7 , 86.2 ± 5.6 , and 74.8 ± 3.8 days.

The mean duration ($\pm$ SD) of CPB cooling for animals with 18°C versus 10°C HCA was 57.5 ± 17.3 and 105.8 ± 21.8 minutes, respectively (t test; $P \leq .002$). The mean duration ($\pm$ SD) of CPB warming for animals with 18°C versus 10°C HCA was 82.5 ± 10.4 and 104.2 ± 19.8 minutes, respectively (t test; $P \leq .05$). Perioperative physiologic variables are shown in Table 1. Although there were some minor variations, no apparent clinically relevant hemodynamic differences were observed between treatment groups. Lactate levels were significantly higher after HCA at 10°C compared with 18°C during rewarming. Po₂ levels were significantly lower in 18°C HCA animals than in 10°C during cooling, and hematocrit levels dropped to a similar degree in all experimental animals during the procedure.

Histologic Evaluation

None of the treatment animals undergoing HCA or the controls in this short-term protocol demonstrated histologic evidence of neuronal injury in any of the brain regions assessed by hematoxylin and eosin staining.

Acute Neuronal Injury—TUNEL Assay for DNA Fragmentation

18°C HCA. HCA for 75 minutes at 18°C resulted in significantly higher TUNEL(+) scores compared with normal controls in all brain regions examined. Compatible with our previous findings, a significantly higher concentration of TUNEL(+) cells were observed in the sensory cortex, motor

TABLE 1. Physiologic variables

Variable	Baseline	Initial CPB	Cooling	Warming	End CPB
Brain temperature (°C)					
18°C	36.5 ± 0.4	34.1 ± 1.8	18.0 ± 0.0	25.8 ± 3.2	36.5 ± 0.8
10°C	36.5 ± 0.4	33.2 ± 1.6	10.0 ± 0.0	28.2 ± 3.1	36.9 ± 0.2
MAP (mm Hg)					
18°C	114.0 ± 14.9	57.2 ± 16.3	55.2 ± 8.1	67.8 ± 15.7	68.3 ± 25.7
10°C	118.7 ± 13.0	59.7 ± 10.1	54.0 ± 3.4	69.4 ± 16.5	85.0 ± 8.9
Arterial pH					
18°C	7.40 ± 0.12	7.26 ± 0.19	7.26 ± 0.12	7.20 ± 0.07	7.35 ± 0.14
10°C	7.34 ± 0.13	7.32 ± 0.11	7.28 ± 0.11	7.32 ± 0.08	7.38 ± 0.13
Po ₂ (mm Hg)					
18°C	409.9 ± 67.8	751.4 ± 202.8	787.1 ± 319.06*	429.0 ± 126.9	424.6 ± 112.1
10°C	378.4 ± 118.3	689.5 ± 45.5	1066.0 ± 122.8	562.8 ± 123.4	459.4 ± 45.4
Pco ₂ (mm Hg)					
18°C	51.32 ± 19.0	73.7 ± 37.8	67.2 ± 31.3	69.3 ± 14.4	38.7 ± 18.7
10°C	58.1 ± 23.3	60.0 ± 17.7	58.2 ± 12.4	44.0 ± 12.9	31.7 ± 9.0
Hematocrit (%)					
18°C	26.4 ± 3.8†	16.4 ± 3.4	15.51 ± 3.3	16.6 ± 3.9	15.5 ± 3.1
10°C	26.0 ± 3.8†	18.5 ± 3.0	18.6 ± 3.7	19.2 ± 3.5	19.2 ± 3.0
Lactate (mmol/L)					
18°C	2.7 ± 1.8	4.5 ± 2.1	5.6 ± 2.5	6.3 ± 1.7*	11.0 ± 4.3
10°C	3.1 ± 1.03	4.4 ± 1.6	8.3 ± 3.0	11.6 ± 2.8	11.9 ± 3.5

All values are expressed as mean ± SD. * $P \leq .05$ between animals treated with HCA at 18°C versus 10°C (unpaired 2-tailed t test). † $P \leq .05$ between sample times (ANOVA followed by Fisher PSLD).

cortex, and hippocampus than in the cerebellum, thalamus, and medulla ($P \leq .05$; ANOVA followed by Fisher PSLD) (Figure 1, Table 2)

10°C HCA. HCA for 75 minutes at 10°C also resulted in significantly higher TUNEL(+) scores compared with normal controls in all brain regions assessed, although scores were generally lower than those observed at 18°C HCA. TUNEL(+) staining was elevated in the motor and sensory neocortex of animals treated with 10°C HCA compared with controls ($P \leq .04$ and $P \leq .002$, respectively). In these regions,

Figure 1. Photomicrograph showing apoptosis in the brain after HCA in a short-term model. Cluster of TUNEL(+) apoptotic neurons (nucleus red stained) are interspersed among normal neurons in the anteroventral medulla. (Original magnification $\times 400$.)

positive staining cells were primarily located in the superficial gray matter. Normal neurons were found interspersed. TUNEL(+) staining was higher in 10°C-treated animals than in controls in the hippocampus ($P \leq .005$) and was dispersed throughout the CA1-3, CA4, and the dentate gyrus. TUNEL(+) staining was also significantly higher in the anteroventral medulla, thalamus, and cerebellum in animals treated at 10°C compared with controls ($P \leq .001$, $P \leq .002$, and $P \leq .004$, respectively). In the cerebellum, TUNEL(+) cells were located in the deep layer of the cerebellar gray matter.

In contrast with our findings at 18°C HCA,¹⁰ animals treated with 75 minutes of HCA at 10°C HCA showed no differences in tissue-specific vulnerabilities among the neural

TABLE 2. TUNEL scores in brain regions of animals treated with HCA at 18°C or 10°C compared with controls

Brain region	18°C	10°C	Control
Motor cortex	3.28 ± 0.32*	1.79 ± 0.38‡	0.50 ± 0.22
Sensory cortex	3.88 ± 0.13†	1.60 ± 0.31¶	0.14 ± 0.14
Hippocampus	2.67 ± 0.36*	1.39 ± 0.24§	0.17 ± 0.17
Cerebellum	2.13 ± 0.48	1.82 ± 0.23§	0.71 ± 0.18
Medulla	2.00 ± 0.41	2.08 ± 0.23¶	0.57 ± 0.20
Thalamus	2.33 ± 0.67	1.54 ± 0.31¶	0.00 ± 0.00

All values are expressed as mean ± SE. * $P \leq .006$ and † $P \leq .0001$ compared with values from animals treated with 10°C HCA. ‡ $P \leq .05$; § $P \leq .005$; ¶ $P \leq .002$ compared with normal control levels.

Figure 2. Photomicrographs of brain tissue sections after TUNEL histochemistry: A, B, and C, the precentral gyrus (motor neocortex); D, E, and F, the hippocampus. Photomicrographs A and D are from HCA at 18°C HCA; B and E are from 10°C HCA; and C and F are from normal controls. Note elevated TUNEL(+) staining at 18°C compared with 10°C and the lack of any staining in normal controls. (Original magnification $\times 400$.)

regions assessed. ($P \geq .05$; ANOVA followed by Fisher PLSD) (Figure 2).

Neural Protection

Region-specific differences. The mean number of TUNEL(+) cells in serial sections from the sensory cortex, motor cortex, and hippocampus showed a significant reduc-

tion when cooled to 10°C as compared with 18°C (t test $P < .0001$, $P < .006$, and $P < .006$, respectively). Although levels were lower at 10°C, sections from the thalamus, anteroventral medulla, and cerebellum failed to show any significant reduction in TUNEL(+) staining when animals were treated with deep hypothermia at 10°C as compared with 18°C (Figure 3).

Figure 3. Differences in mean TUNEL(+) scores between HCA at 18°C (solid bars) and profound cooling at 10°C (open bars). Whiskers indicate $\pm$ SE. (* $P \leq .006$ and ** $P \leq .0001$.)

Figure 4. Photomicrographs of brain tissue sections after Bcl-2 immunohistochemistry: A, B, and C, the precentral gyrus (motor neocortex); D, E, and F, the post-central gyrus (sensory cortex). Photomicrographs A and D are from HCA at 18°C HCA; B and E are from 10°C HCA; and C and F are from normal controls. (Original magnification $\times 400$.)

Bcl-2 immunostaining. Positive Bcl-2 immunostaining was observed in the motor and sensory neocortex and hippocampus after HCA at 18°C and at 10°C. Bcl-2 immunostaining was absent in the cerebellum, thalamus, and medulla. Profound cooling to 10°C resulted in a significant increase in Bcl-2 expression in the neocortex compared with that observed at 18°C ($P \leq .05$ Mann-Whitney U). No significant change was observed in Bcl-2 expression in the hippocampus after profound cooling to 10°C compared with 18°C HCA (Figure 4, Table 3).

Discussion

Hypothermic metabolic suppression remains a major protection strategy for the brain during intervals of circulatory arrest required for complex aortic reconstruction, among other surgical procedures.⁷ Concern about brain intolerance to anoxia has limited ischemic intervals despite cooling to 18°C. This has prompted a search for improved ways of implementing HCA in the hope that the duration of arrest can be tolerated without apparent ill effects.

Previously, we assessed acute neuronal injury in various regions of the brain after HCA at 18°C in a short-term porcine animal model. We reported that neurons in the sensory and

motor neocortex, as well as in the hippocampus, were selectively vulnerable to cell injury acutely after 75 minutes of HCA, as determined by a positive TUNEL reaction for DNA fragmentation.¹⁰ Although nerve cell populations in the cerebellum, thalamus, and ventral medulla also showed cell injury, the percentage of TUNEL(+) cells in these areas was significantly less than that observed in the primary motor and sensory cortex and in the hippocampus.¹⁰ These findings were compatible with those reported in models of long-term HCA.^{1,2,12} Taken together, it appears that hypoxia-ischemia results in variable injury to selected regions of the brain, rather than global injury.^{10,13} Selective vulnerability occurs in both the adult and neonatal brain and reflects heightened sensitivity of specific neuron groups to injury.¹² It should be noted that although TUNEL testing is a hallmark for apoptosis, it shows poor sensitivity and specificity, inasmuch as the TUNEL assay is unable to distinguish DNA fragmentation associated with apoptotic versus necrotic cell death.

In the present study, we found that profound hypothermia at 10°C during HCA resulted in a significant reduction in neurologic injury as indicated by TUNEL(+) staining in these selectively vulnerable brain regions. TUNEL(+) staining was significantly reduced at 10°C in the motor and sensory cortex and the hippocampus compared with 18°C HCA, indicating increased cerebral protection in these areas. These findings are compatible with previous reports that profound hypothermia results in a better neurologic outcome than conventional HCA methods.¹³ Although this study does not elucidate the mechanisms, it does affirm that profound hypothermia exerts a neuroprotective effect. It is noteworthy that the magnitude of the tissue-specific vulnerabilities to insult among the neural regions is less if not altogether

TABLE 3. Regional Bcl-2 immunoreactivity in neocortex and hippocampus

Brain region	18°C	10°C	Control
Motor cortex	1.67 $\pm$ 0.33	2.5 $\pm$ 0.22*	1.8 $\pm$ 0.25
Sensory cortex	0.83 $\pm$ 0.31	1.8 $\pm$ 0.31*	1.8 $\pm$ 0.63
Hippocampus	1 $\pm$ 0.52	1 $\pm$ 0.45	1 $\pm$ 0.41

All values are expressed as mean $\pm$ SE. * $P \leq .05$ compared with values from animals treated with 18°C.

absent at 10°C. This is compatible with the findings of Laptook and colleagues,¹⁴ which showed less protection of the hippocampus, thalamus, and striatum with hypothermia compared with other nerve cell populations.

Delayed cell death via apoptotic pathways is of special interest because of the potential for intervention. Although questions remain regarding its specificity and sensitivity, a hallmark of apoptosis is the fragmentation of DNA into smaller ordered oligonucleosomes with 3'-OH end groups, detectable with *in situ* labeling (TUNEL).¹⁵⁻¹⁷ Recent studies using a variety of methods have noted multiple different patterns of apoptotic cell damage in brains after HCA.^{1,12,18} Most previous studies use long-term animal models and investigate the extent of brain injury at a later time, resulting in a potential underestimation of the contribution of apoptotic mechanisms to the cerebral sequelae after HCA.^{1,3} Several authors have expressed concern regarding the temporal pattern of brain damage and apoptosis after HCA.^{1,12,13} In an effort to evaluate the time course of cerebral injury, Hagl and colleagues¹ put animals to death at 6, 24, 48, and 72 hours and at 7, 10, and 12 days after HCA. The authors reported that the brain already exhibited serious brain injury at 6 hours after HCA. For the most part, previous reports using a long-term model, although able to assess behavioral outcome, express concern about missing the optimal time for detection of apoptosis.¹³ To our knowledge, the only other short-term model is that of Ye and colleagues,¹⁹ who used a much longer insult (120 minutes) and a temperature intermediate (15°C) to that used in the present study. As a result of these previous reports, we selected a very early time point (80-100 minutes after HCA). At this time point we found not morphologic evidence of apoptosis, but significantly greater levels of TUNEL(+) cells in the brain regions assessed, suggesting that damaged cells are being shunted into apoptosis.

Although there is a consensus about the benefits of profound hypothermia, the optimal temperature for maximizing cerebral protection has yet to be identified. Moreover, the exact mechanism of cerebral protection during hypothermia is not clear. It is assumed that, at least in part, protection is achieved secondary to metabolic suppression.⁷ In this regard, cerebral oxygen metabolism has been found to be significantly reduced with profound hypothermia at 8°C, whereas at 18°C it remains as high as 24% of baseline, suggesting a less complete cerebral protection at the latter temperature.⁸ As further lowering of metabolic rate is achieved with profound cooling, we hypothesize that better cerebral protection is also achieved. Despite this apparent benefit, deep hypothermia has not only been associated with side effects, such as coagulation disorders, but also results in an increase in the time necessary for prolonged CPB owing to the time needed for rewarming. As the temperature decreases, the rate of venous return to the oxygenator pump decreases, as a result of the trapping of blood in areas of capillary stasis. Although low-molecular-weight dextran is

able to lessen this effect, limited clinical experience indicates that hypothermia can be associated with hemodynamic instability, cardiac arrhythmias, increased serum lipase and amylase levels, thrombocytopenia, and decreased total white blood cell counts.^{20,21}

The majority of reports use the classic 90-minute HCA, 20°C model, which results in more severe cerebral injury than that usually observed clinically, where HCA is carried out for shorter intervals.^{1,11-13} In the present short-term model, animals were treated with HCA for 75 minutes and were evaluated after approximately 80 to 100 minutes of reperfusion. We found no morphologic evidence of apoptosis, but significantly greater levels of TUNEL(+) cells in the brain regions assessed. It has been suggested that subtle injury results in a greater proportion of damaged cells being shunted into apoptosis, as compared with necrosis. Thus, long-term models may have underestimated the contribution of apoptosis to the cerebral sequelae after HCA.¹ The observation that TUNEL-labeled cells may eventually, but not necessarily, progress into morphologically distinct apoptotic cells also confirms the idea that different morphologic characteristics may reflect different stages of the same death process.²² A wide variety of stimuli can initiate the apoptotic cascade. After an appropriate stimulus, the first stage or the decision phase is initiated. This is referred to as the genetic control point of cell death, which appears to be regulated by the Bcl-2 family of genes. This is followed by the "execution phase," which is responsible for the morphologic changes of apoptosis.¹⁸ Cellular disruption results from activation of the caspases family. Inasmuch as we lack clear morphologic evidence of apoptosis, we hypothesize that our findings indicate an early point of activation of the apoptotic pathway (decision phase).

This hypothesis is supported by our findings in Bcl-2 expression. The Bcl-2 family of proteins are important for the regulation of apoptosis during the "decision phase."¹⁸ An increase of Bcl-2 has been suggested as an internal protective mechanism against apoptotic cell death, where Bcl-2 is persistently expressed in neurons that survive in ischemia.^{4,14} In the present study, brain regions that were selectively vulnerable to neurologic injury, particularly the neocortex and hippocampus, showed higher levels of Bcl-2 expression after HCA at 18°C compared with other brain regions (thalamus, cerebellum, and medulla). Moreover, profound hypothermia at 10°C resulted in a significant decrease in TUNEL(+) staining in these brain regions. Although a concomitant increase in Bcl-2 expression was observed in the neocortex, it remains unclear whether profound hypothermia deters from neuronal injury by activation of anti-apoptotic protein Bcl-2 expression.

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Discussion

Dr Frank L. Hanley (Stanford, Calif). Dr Ananiadou, you and your colleagues have designed and executed an excellent study that convincingly shows in this particular porcine model that increased levels of hypothermia from 18°C down to 10°C during 75 minutes of HCA

results in less DNA fragmentation and greater expression of the anti-apoptotic protein Bcl-2 in the vulnerable neocortex and hippocampus.

The results of this nicely designed study stand on their own. They are very clear and I would have no argument with what you have shown. However, when it comes to the suggested mechanism and causality that you have implied, there is a little less clarity. That is where I would like to focus just one comment and question and see what you think about it.

You have stated very specifically that reduced temperature activates Bcl-2, implying a proposed specific protective effect of lower temperature itself. This would obviously have extremely important implications for how we manage patients clinically and in patients in whom deep HCA is used. However, is it really the lower temperature that activates this anti-apoptotic mechanism or is it simply that the lower temperature reduces the overall ischemic insult and it is this lesser ischemic insult itself that allows for the increased Bcl-2 activity? Clearly, the ischemic insult is related to two things, the length of circulatory arrest and the temperature at which that length of circulatory arrest exists. The combination of the length and the temperature define the ischemic insult in rough terms.

What I am really asking is, have you looked at this in another way? Would an insult of less length of circulatory arrest, say 50 minutes rather than 75, with a temperature of only 18°C result in increased Bcl-2 activity? Or alternatively, if you increased the ischemic time, the circulatory arrest time to, say, 90 minutes at 10°C, would that not increase Bcl-2 activity, getting more to the mechanism of what actually is increasing your protective anti-apoptotic protein expression?

Dr Ananiadou. What has changed in this protocol, in the two groups, is not the duration of ischemia; it is only the temperature. That is why we hypothesize that the lower temperature is the mechanism that causes increased Bcl-2 immunoreactivity. It is the only parameter to change in this model. The arrest time is the same, 75 minutes. Thus the ischemic interval is the same. The ischemic insult is the same. The protective mechanism that induces the increase of Bcl-2 immunoreactivity is the lowering temperature. That is what I think.

Dr Hanley. I understand. But I think there is a fine point here that is very, very important. The ischemic insult is the same, 75 minutes, there is no question, but the injury induced by an ischemic insult may be very different at different temperatures. It is the injury to the neuron that is critical here.

If you are arguing that temperature in and of itself directly, mechanistically increases Bcl-2, then if we are going to do a circulatory arrest operation and have only 20 minutes of circulatory arrest, it would mean we should go down to 10°C. However, if we might need to do circulatory arrest for 40 or 50 or 60 minutes, knowing whether going down to 10°C is more important in that setting than in the shorter setting is critical to how we would manage our patients.

You are implying that the temperature of 10°C in and of itself is always going to be helpful, whereas I am saying it does not seem that clear to me from your model that that is actually the case. Rather, it is the level of neuroinjury that is a combination of arrest time and the lower temperature that may well be what is important.

Dr Ananiadou. It is believed that hypothermia alters the pattern of sensitivity of cells. That is why we have different damage. But, on the whole, I agree with you; profound hypothermia is not the only parameter.