Proteomics of cerebral injury in a neonatal model of cardiopulmonary bypass with deep hypothermic circulatory arrest

Amir M. Sheikh, MBBS, MRCS,^a Cindy Barrett, MD,^b Nestor Villamizar, MD,^c Oscar Alzate, PhD,^d Sara Miller, PhD,^e John Shelburne, MD, PhD,^f Andrew Lodge, MD,^a Jeffrey Lawson, MD, PhD,^c and James Jagers, MD^a

Dr Sheikh

Supplemental material is available online.

Objective: Concern over neurologic injury limits safe duration of deep hypothermic circulatory arrest (DHCA) in surgery for congenital cardiac disease. Proteomics is a novel and powerful technique to study global protein changes in a given protein system. Using a neonatal model of cardiopulmonary bypass with DHCA, we sought to characterize the protein changes associated with DHCA brain injury.

Methods: Ten neonatal piglets were randomized to cardiopulmonary bypass with DHCA or sham operation. DHCA animals underwent induction of bypass ($100 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), cooling to 18°C , then DHCA for 60 minutes. Animals were rewarmed to normothermia, weaned from bypass, and harvested after 30 minutes off bypass. Sham animals underwent sternotomy without further instrumentation. Plasma samples were taken before bypass and before harvest. Proteins differentially expressed in the cerebral neocortex between the 2 groups were determined by 2-dimensional differential gel electrophoresis using fluorescent cyanine dyes and mass spectrometry. A second group of 4 piglets were similarly randomized and, after the experiment, tissues underwent perfusion-fixation for histologic examination.

Results: Cardiopulmonary bypass with DHCA caused extensive histologic and ultrastructural cerebral injury. Proteomic analysis of cerebral cortex found 10 protein spots to be differentially expressed; 9 were identified by mass spectrometry to represent 6 proteins, including apolipoprotein A-1, neurofilament-M protein, and enolase. Decreased expression of plasma apolipoprotein A-1 was found in DHCA.

Conclusions: The acute protein changes associated with cerebral injury in a neonatal model of cardiopulmonary bypass with DHCA have been characterized. These may direct further research aimed at attenuating injury seen from cardiopulmonary bypass with DHCA.

From the Departments of Pediatric Cardiac Surgery,^a Pediatric Critical Care,^b and Surgery,^c the Neuroproteomics Center and the Department of Neurobiology,^d and the Department of Pathology,^e Duke University Medical Center, Durham, NC, and the Department of Pathology,^f Veterans Affairs Hospital, Durham, NC.

Dr Sheikh was partly supported by a St Jude Medical Scholarship.

Read at the Eighty-sixth Annual Meeting of The American Association for Thoracic Surgery, Philadelphia, Pa, April 29–May 3, 2006.

Dr Sheikh was the recipient of the C. Walton Lillehei Resident Research Award, presented at the Eighty-sixth Annual Meeting of The American Association for Thoracic Surgery.

Received for publication April 27, 2006; revisions received July 11, 2006; accepted for publication July 13, 2006.

Address for reprints: Amir M. Sheikh, MBBS, MRCS, 34 Thornhill Rd, Flat 4, Plymouth, PL3 5NE, United Kingdom (E-mail: amsheikh10@hotmail.com).

J Thorac Cardiovasc Surg 2006;132:820-8

0022-5223/\$32.00

Copyright © 2006 by The American Association for Thoracic Surgery

doi:10.1016/j.jtcvs.2006.07.002

Abbreviations and Acronyms

apoA1	= apolipoprotein A-1
CHMP4b	= charged multivesicular body protein 4b
CPB	= cardiopulmonary bypass
CSF	= cerebrospinal fluid
DHCA	= deep hypothermic circulatory arrest
IPG	= immobilized pH gradient
MALDI	= matrix-assisted laser desorption ionization
MS	= mass spectrometry
MW	= molecular weight
NFH	= heavy-chain neurofilament protein
NFL	= light-chain neurofilament protein
NFM	= medium-chain neurofilament protein
NSE	= neuron-specific enolase
TOF	= time of flight

Deep hypothermic circulatory arrest (DHCA) remains an important technique in the repair of complex congenital cardiac defects. Despite advances in neuroprotective strategies, concerns continue over brain injury sustained during DHCA that may manifest early or late. These may range from overt deficits such as seizures and choreoathetosis to more subtle neurodevelopmental ones such as impaired psychomotor function.^{1,2} Selective vulnerability of brain regions is seen; neocortex and hippocampus have been found to be most vulnerable.^{2,3} Different processes contribute to the neuronal injury seen with DHCA: ischemia and reperfusion, inflammatory response to cardiopulmonary bypass (CPB), hypothermia, and rewarming. The molecular mechanisms responsible for the neuronal injury are still being elucidated with a hope of leading to successful neuroprotective strategies.^{4,5}

Proteomics is a rapidly evolving set of technologies that allows protein expression to be examined globally in any given system. With the use of 2-dimensional gel electrophoresis and appropriate protein labeling, thousands of proteins can be resolved and relative protein abundances determined. Proteins whose level of expression are found to change in the experimental or disease states can be excised from gels and, through sensitive and accurate mass spectrometric analysis, be identified.⁶ Proteins that have never been previously characterized may, in this way, be discerned. Differential gel electrophoresis is a form of proteomic analysis that uses covalent labeling of proteins with 3 fluorescent cyanine ester protein dyes (Cy2, Cy3, and Cy5), which are detected by their independent excitation and emission spectra. This allows experimental and control animal samples to be fractionated concurrently, along with an internal control, on each gel. As well as being highly sensitive, the use of the internal control eliminates intergel variations and allows gels to be standardized accurately to one another, thereby greatly enhancing statistical accuracy.⁷

We undertook this novel approach in a preliminary attempt to elucidate the molecular basis for neonatal cerebral injury in CPB with DHCA at the protein level. Employing differential gel electrophoresis proteomic analysis, we sought to determine the protein changes that occur acutely in neonatal cerebral cortex after CPB with DHCA, by examining all the protein changes globally for those that underwent significant change in expression. This characterization may uncover novel proteins in the pathogenesis of brain injury and could lead to new potential molecular targets in neuroprotection. For this initial study we controlled for anesthesia and surgical instrumentation.

Methods**Animal Preparation**

All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, revised in 1996. The experimental protocols for the individual studies were approved by Duke University's Institutional Animal Care and Use Committee.

The study was accomplished in 2 stages. In the first stage, the proteomic changes in the brain were characterized after 1 hour of DHCA. Ten Yorkshire 1-week-old piglets (weighing 2.5-3.0 kg) were randomized to CPB with DHCA or to a sham operation consisting of sternotomy to serve as controls. Piglets were sedated with intramuscular acepromazine (1.1 mg/kg), followed by intravenous ketamine (22 mg/kg) administered via a cannula inserted into the marginal vein of the ear. Orotracheal intubation was performed with a 3.0-mm endotracheal tube and ventilation was administered (Bird 8400ST Ventilator, Bird Products Corporation, San Diego, Calif) at 25 breaths/min, tidal volume 50 mL, administering isoflurane at 2% and oxygen at 40%, adjusted appropriately to maintain arterial blood oxygen tension at 200-300 mm Hg and carbon dioxide tension at 35-45 mm Hg. A recirculating water mattress and heating lamps were used to maintain animal temperature at 36°C to 37°C. The femoral artery was cannulated with a 20F-gauge cannula. Esophageal temperature, blood oxygen saturations, electrocardiogram, and arterial waveform were monitored continuously.

CPB and DHCA

Cardiac exposure was through a median sternotomy. Systemic heparin was administered to both groups (300 IU/kg). Sham-operated animals had no further instrumentation, and temperature was maintained at 37°C. DHCA animals were cannulated for CPB. A 10F pediatric arterial cannula (DLP Inc, Grand Rapids, Mich) was placed into the ascending aorta, with a 20F single-stage venous cannula inserted into the right atrium through polypropylene purse-string sutures. CPB was instituted at a flow of 100 mL · kg⁻¹ · min⁻¹. The pump-oxygenator system consisted of a nonpulsatile roller pump (Stockert Shiley, Irvine, Calif) and a hollow-fiber membrane oxygenator (Minimax PLUS; Medtronic Inc, Minneapolis, Minn). No arterial filter was used. The circuit was primed with 500 mL of heparinized fresh blood from a donor pig. Ringer's lactate and sodium bicarbonate solutions were added to the prime to achieve a hematocrit value of 0.25 and a pH of 7.4

at 37°C. The temperature of the perfusate was controlled with the integral heat exchanger in the venous reservoir of the oxygenator and a water bath system (BIO-CAL 370; Medtronic BioMedicus, Eden Prairie, Minn).

Piglets were actively cooled to a core temperature of 18°C over 20 minutes and maintained at full CPB flow at 18°C for another 10 minutes to ensure uniform cooling. Ice cold saline slush was placed in the pericardial cavity to help prevent rewarming of the animal, and ice was packed around the head. Cardioplegia was not used. Blood gases were managed throughout cooling and rewarming according to the alpha-stat strategy. DHCA was established by clamping the arterial and venous cannulas. After 60 minutes of DHCA, perfusion was re-established at 100 mL · kg⁻¹ · min⁻¹ and piglets were rewarmed to 37°C over 30 minutes. On reaching normothermia, CPB was continued for 15 minutes more to ensure all animals were fully rewarmed and allow temperature stabilization. Animals were weaned from CPB and maintained off bypass for an additional 30 minutes. No animal required inotropic or vasopressor support during this time. In summary, the timeline for DHCA piglets was as follows:

30-minute CPB cooling phase → 1 hour DHCA
→ 45 minutes of CPB rewarming → 30 minutes off CPB.

Sham-operated animals were maintained anesthetized with the chest open for an equal duration to the DHCA animals.

Blood samples were collected in ethylenediaminetetraacetic acid tubes after heparinization (baseline sample) and just before tissue harvest (postprocedure sample), centrifuged at 2000 rpm for 10 minutes, and the plasma snap frozen in liquid nitrogen. For brain harvest, piglets were turned prone. The skull vault was accessed by lifting the skin and cranium completely (Stryker autopsy 810 saw; Thermo Electron Corporation, Waltham, Mass). The brain was carefully excised and washed in saline before a 5 × 5 × 5-mm block of cortex was excised from the medial end of the precentral gyrus of the right cerebrum and snap frozen in liquid nitrogen before storage in a freezer at -80°C for subsequent proteomic analysis. The precentral gyrus was chosen because it is readily and reproducibly identifiable in the piglet.

Preparation for Electron Microscopy: Perfusion Fixation of the Brain

In the second stage of the study, brain histologic and ultrastructural changes associated with DHCA were examined as a correlate to the protein changes. Four piglets were randomized to DHCA or sham operation, as described. At the conclusion of the study, animals for electron microscopic studies underwent perfusion tissue fixation with Karnovsky fixative, a hyperosmolar combined formaldehyde-glutaraldehyde fixative, delivered via the aortic cannula. Sham-operated animals were therefore also cannulated before tissue harvest. Karnovsky fixative was prepared as previously described.⁸ After exsanguination of the animal via the venous cannula, 1 L of heparinized saline (10,000 IU heparin sulfate in 1 L normal saline) was infused via the aortic cannula to flush the circulation, followed by 1 L of Karnovsky fixative, using a pressure bag to achieve a systemic arterial pressure of 50 mm Hg. The brain specimen was then harvested as described. The samples were stored in Karnovsky fixative.

Proteomics Analysis

Protein separation: two-dimensional electrophoresis. Fifty milligrams of piglet neocortex was homogenized in 1 mL of 25-mmol/L DHPC lysis buffer (Appendix E1). Ultrasound sonication, to enhance membrane lysis and solubilization of membrane proteins, was also used. Plasma samples (15 µL) were prepared with albumin and immunoglobulin G removal kit (GE Healthcare, Piscataway, NJ). The resulting pellet was resuspended in lysis buffer (Appendix E1).

For brain tissue, each gel consisted of 3 samples run concurrently: 1 DHCA animal sample, 1 control animal sample, and 1 internal control. Five gels were therefore prepared. To create the internal control, we pooled equivalent amounts of all the brain samples in the experiment. Labeling with the fluorescent cyanine dyes was as follows: internal control, Cy2 (blue); experimental animal, Cy3 (green); control animal, Cy5 (red). For blood samples, 5 gels were prepared; each gel consisted of both baseline sample (Cy3) and postprocedure sample (Cy5) for the same animal and internal control (mix of all plasma samples, Cy2) loaded in each gel.

Total protein, 120 µg, from each sample was labeled with 400 pmol/L of Cy dyes for 30 minutes at 4°C in the dark. The internal control sample was labeled similarly after combining 60 µg from each animal brain lysate or of plasma to prepare a single solution. The labeling reaction was stopped by adding 1 µL (10 mmol/L stock) lysine (Sigma Aldrich, St Louis, Mo). The samples were complemented with equal amounts of 2X buffer (Appendix E1) for 15 minutes. The 3 samples (Cy2, Cy3, Cy5) to be run on a single gel were then combined (250 µL) and applied to immobilized pH gradient (IPG) strips (13 cm, pI ranges 4-7 for brain and 3-10 for plasma; Immobiline DryStrip; GE Healthcare). After active rehydration (30 V for 14 hours), IPG strips underwent isoelectric focusing with an Ettan IPGphor II IEF system (GE Healthcare) for a total of 28 kVh. After equilibration with dithiothreitol followed by iodoacetamide, the IPG strips were placed on 12% homogeneous polyacrylamide gels (4% stacking) for the second dimension. Each sodium dodecylsulfate-polyacrylamide gel electrophoresis was run at 9 mA for 16 hours in a Hoefer SE-600 system (Hoefer Scientific Instruments, San Francisco, Calif).

Gel scanning and statistical analysis. The Cy2, Cy3, and Cy5 components of each gel were scanned with mutually exclusive excitation/emission wavelengths (Typhoon 9410 Variable Mode Imager, GE Healthcare). A visible protein stain, Coomassie Brilliant Blue G-250 stain (BioRad, Hercules, Calif), was applied to allow visual inspection of the protein spots. DeCyder 5.0 (GE Healthcare) differential analysis software was used for protein spot detection, quantitation, spot matching, and statistical analysis. All gels were standardized with the internal control (Cy2) images. Statistical analysis was performed by the unpaired Student *t* test for the brain tissue, comparing the standardized volume of each gel spot in the DHCA animals against the corresponding spot in the sham-operated animals. A paired *t* test was used for blood analysis, comparing postprocedure spots of each DHCA animal to its own baseline spots. A robotic spot cutter (Ettan Spot Picker; GE Healthcare) excised 2-mm gel plugs from the selected protein spots for mass spectrometry (MS) analysis.

Protein identification: MS. The identity of the proteins in the selected spots was determined using MS. Matrix-assisted laser

desorption ionization (MALDI) time of flight (TOF) MS was performed in conjunction with tandem MS (MS/MS; Michael Hooker Proteomics Core Facility, University of North Carolina, Chapel Hill, NC). In brief, MALDI-MS and MALDI-MS/MS data were acquired using an ABI Voyager 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Inc, Foster City, Calif). The 8 most intense peaks with a signal-to-noise ratio greater than 25 were selected automatically for MS/MS analysis. The peptide mass fingerprinting and sequence tag data from the TOF/TOF were evaluated with GPS Explorer scores (Applied Biosystems). MS/MS spectra were submitted to the National Center for Biotechnology Information database to generate ion scores via the Mascot search engine (Matrix Science Inc, Boston, Mass).⁹

Brain tissue histologic and ultrastructural examination.

Karnovsky-fixed brain tissue blocks were bisected and prepared separately for light and transmission electron microscopic examination. Light microscopy specimens were dehydrated through ethanol and xylene baths and embedded in paraffin wax. Five-micrometer sections were cut and stained with hematoxylin and eosin. For electron microscopy, tissue was dissected for the neocortex and prepared as has been previously described.⁸ A minimum of 6 grids were prepared per animal with 1 or 2 sections per grid. They were carbon coated in an Edwards AUTO 306 vacuum evaporator (BOC Edwards, Wilmington, Mass) and viewed in a Philips CM 12 electron microscope (FEI Company, Hillsboro, Ore). With blinding to the perfusion protocol, each grid was viewed in its entirety, and representative areas were photographed and reviewed.

Statistical Analysis

Perioperative variables were analyzed by repeated-measures analysis of variance and the unpaired Student *t* test. For proteomic analysis, statistical analysis was performed with DeCyder 5.0 software described above.

Results

There were no operative deaths. Mean arterial blood pressure increased in the DHCA animals after arrest (40 ± 5.5 to 54 ± 16.8 mm Hg), while decreasing in the controls (44 ± 3.5 to 37 ± 3 mm Hg), reflecting a hyperdynamic circulation after DHCA. There were no other significant differences in perioperative variables between the 2 groups (Table E1).

Brain Proteomics

A mean of 2160 ± 123 spots were detected. Figure 1 shows a representative gel. Eight protein spots were found to have statistically significant ($P < .05$) differential expression between the groups (Table 1). A further 2 spots achieved borderline statistical significance. Nine of these total 10 spots were successfully and unambiguously identified by MS. Five different proteins were found to account for the 8 statistically significant spots; apolipoprotein A-1 (apoA1) precursor and neurofilament triplet M protein (fragment) were both responsible for 2 spots each. The other proteins of significance were neuron-specific enolase (NSE), charged

Figure 1. Example of a 2-dimensional differential gel electrophoresis of brain neocortex. The Cy2 (internal control) image is shown including identification numbers for the protein spots of interest. A typical 3-dimensional representation of a protein gel spot, as analyzed and displayed by DeCyder software, is shown below the gel, taking apoA1 as an example. The Cy5 (sham) protein image is on the left and the Cy3 (DHCA) image on the right. It can be seen there is a decrease of apoA1 peptide in the DHCA animal.

multivesicular body protein 4b (CHMP4b), and a hypothetical chaperone protein. The hypothetical protein represents a theoretical protein product yet to be characterized but the existence of which has been deduced from the genomic database. The 2 gel spots that achieved borderline significance ($P = .057$ and $P = .069$) were identified as the same protein, protein disulfide-isomerase. Of these total 9 protein spots that were successfully identified, the level of expression was found to increase in 5 (neurofilament triplet M protein fragment [2 spots], hypothetical chaperone protein, protein disulfide-isomerase [2 spots]), increases ranging from 17% to 77%, and to decrease in 4 (apoA1 [2 spots], NSE, and CHMP4b), decreases ranging from 15 to 47%.

Observed molecular weight (MW) of the proteins of interest were also calculated as part of the DeCyder analysis. Both apoA1 peptides had observed MWs half of the

TABLE 1. Cerebral neocortex proteomics analysis

Gel spot number	Protein identification	Protein accession number*	Average ratio change†	Percent change‡
2124	Apolipoprotein A-1 precursor	A46018	-1.59 ± 0.36	-37%
2132	Apolipoprotein A-1 precursor	A46018	-1.89 ± 0.39	-47%
1294	Neurofilament triplet M protein fragment	QFPGM	1.77 ± 0.31	+77%
1306	Neurofilament triplet M protein fragment	QFPGM	1.52 ± 0.33	+52%
1437	Neuron-specific enolase	NOHUG	-1.18 ± 0.29	-15%
1754	Charged multivesicular body protein 4b (CHMP4b)	AAH11429	-1.39 ± 0.32	-28%
1215	Hypothetical protein chaperone	Q9BU08	1.59 ± 0.33	+59%
1239	Protein disulfide-isomerase	CAD88075	1.37 ± 0.47	+37%
1248	Protein disulfide-isomerase	S68363	1.17 ± 0.27	+17%

Protein spots depicted were statistically significant by the Student unpaired *t* test ($P < .05$) comparing deep hypothermic circulatory arrest protein spot volumes to corresponding control spot volumes after standardization to the internal control (except protein disulfide-isomerase, where the 2 spots were $P = .057$ and $.069$). *The protein accession number is obtained from public databases, such as Protein Information Resource (<http://pir.georgetown.edu/>) or Swissprot (<http://us.expasy.org>). †Average ratio change, also known as fold-change, calculated by DeCyder, are given as mean $\pm$ standard error. Values are displayed in the range of $-\infty$ to -1 for decreases in expression and $+1$ to $+\infty$ for increases in expression (no values between -1 and $+1$). Therefore, a ratio of 2.0 is a 2-fold increase and -2.0 is a 2-fold decrease. ‡The average ratio change has been converted into percentage change. For a positive ratio change of x , this is calculated as $(x - 1) \cdot 100$ to give percentage increase; for a negative ratio change of x , this is calculated as $[1 - (1/x)] \cdot 100$ to give the percentage decrease.

parent precursor protein (apoA1 precursor): 15.4 kDa and 15.3 kDa (theoretical 30.3 kDa). This suggests a post-translational modification whereby these 2 proteins of interest represent fragments of the apoA1 precursor. The expression of both of these fragments, as mentioned above, was reduced in DHCA animals. The other proteins had similar MWs to their predicted values (with theoretical MWs from protein databases): neurofilament triplet M protein fragment 51.8 kDa and 53.8 kDa (59.9 kDa); enolase 44.7 kDa (47.1 kDa); CHMP4b 28.8 kDa (24.9 kDa), and protein disulfide-isomerase 57.3 kDa and 56.8 kDa (56.7 kDa). The hypothetical chaperone protein had an MW of 58.9 kDa (theoretical MW 56.7 kDa).

Plasma Proteomics

Two spots were found to have statistically significant expression change after DHCA. Both of these spots were identified as apoA1 precursor and showed a mean 1.89-fold (47%) decrease in level of expression (2.01 ± 0.39 and 1.75 ± 0.31 -fold decrease; database accession ID: A46018).

Histology

Light microscopy. Representative light micrographs are shown in Figure 2. Control piglets showed a normal neuropil architecture and healthy cells. DHCA piglets displayed extensive destruction of the neuropil. Vacuolations in the neuropil were widespread, with loss of neuronal cells, axons, as well as glial and astrocyte cells. Both neuronal and glial cells exhibited swollen and karyolytic nuclei. Capillary endothelial cells at the surface of the brain appeared intact, but endothelial cell nuclei of deeper capillaries were swollen. Perivascular edema was noted around these capillaries.

Electron microscopy. Healthy cellular ultrastructure was seen in control piglets. DHCA animals displayed extensive

cellular and ultrastructural injury. Axons and dendrites appeared swollen in the DHCA animals (Figure 2). Both neurons and glia exhibited marked swelling of the nuclei and cytoplasmic organelles. Almost universally, mitochondria showed high-amplitude swelling in the DHCA piglets. Some mitochondria also displayed flocculent densities; these are indicative of severe injury. Ribosomal scattering, a pattern wherein ribosomes, which are normally attached to endoplasmic reticulum, become dispersed in the cytoplasm, was present. Plasma membrane breaks were visible in some cells; this is indicative of irreversible cellular injury. Capillary endothelial cells were intact, but some endothelial cells exhibited nuclear swelling.

Discussion

Brain injury after DHCA is multifactorial^{10,11}; global ischemia, reperfusion, inflammatory and embolic effects of CPB, hypothermia, and rewarming all play a role, which makes determination of key responsible mechanisms difficult. Cerebral apoptosis as well as necrosis is seen after DHCA.^{3,12} Much work has been done to elucidate the key molecular mechanisms and thereby identify suitable targets for neuroprotection: for example, mitogen-activated protein kinases,⁴ nitric oxide,¹³ hypoxia inducible factor,⁵ thromboxane A₂-receptor,¹⁴ and apoptotic proteins such as caspase-3 and cytochrome c.¹² However, no pharmacologic agents have as yet been shown to have a beneficial effect on neurodevelopment, and the search for novel therapeutic targets continues.

In a neonatal model of CPB with DHCA, which exhibits demonstrable histologic and ultrastructural damage to the cerebral neocortex, we have characterized the associated acute protein changes. The light and electron microscopic findings are consistent with previous DHCA work,⁸ in

Figure 2. All panels are of piglet neocortex. Panels A, C, and E are representative control samples. Panels B, D, F are representative experimental samples. Panels A and B are 100 $\times$ magnification light micrographs (stained with hematoxylin and eosin) of neocortex; micron bar represents 50 μ m. Panel A shows intact, normal neuropil, nuclei (N), and vasculature (V). Panel B shows evidence of swelling of the neuropil, including swollen and karyolytic nuclei, and pericellular and perivascular edema. Panels C and D are transmission electron micrographs at 3000 $\times$ magnification; micron bar represents 1 μ m. Panel C shows intact axons with normal mitochondria (M), as well as an intact plasma membrane (arrow). In contrast, in panel D the architecture is disrupted by swollen mitochondria and organelles. There is loss of plasma membrane, indicative of irreversible cell injury. Panels E and F are transmission electron micrographs at 5000 $\times$ magnification; micron bar represents 1 μ m. Panel E shows a normal nucleus (N), mitochondria (M), endoplasmic reticulum (ER), and an intact plasma membrane (PM). In contrast, panel F exhibits karyolysis, chromatin clumping, and edema. Mitochondria appear swollen. Flocculent deposits are present in mitochondria (arrow); this also denotes irreversible cell injury. DHCA, deep hypothermic circulatory arrest.

which functional impairment of the brain was also demonstrated. Five of the 6 proteins identified in this study have never previously been linked to cerebral injury with DHCA; this indicates the value of proteomics analysis to discover novel proteins of interest. Protein expression changes may reflect de novo protein synthesis or post-translational mod-

ifications, for example, phosphorylation, glycosylation, and cleavage by proteases. The acuteness of this study must preclude de novo synthesis from gene transcription through to mRNA translation, and expression changes in this study must be post-translational.

ApoA1 is the predominant protein found in high-density lipoproteins and contains amphipathic α -helical segments responsible for surfactant properties of the particles.¹⁵ There are, however, increasing reports linking apoA1 to roles outside of blood cholesterol metabolism. It has been suggested as a biomarker for colorectal cancer and may have a potential anti-inflammatory role by modulating T-cell and macrophage interaction.^{16,17} Brain cholesterol homeostasis for development and maintenance is met entirely by cholesterol synthesis within the central nervous system.¹⁸ ApoA1 has been identified in human brain and its down-regulation has been found in human adult brain when compared with fetal, through proteomic studies using MALDI-MS for protein identification.¹⁹ In a proteomics study of Alzheimer disease, cerebrospinal fluid (CSF) levels of 2 isoforms of apoA1 were reduced in Alzheimer patients.²⁰ Although its role is not clear, apoA1 has been clearly linked to neurologic damage. Saito and associates²¹ demonstrated marked increase in CSF apoA1 concentrations in polio-infected macaques, the level of which correlated with the degree of neurologic impairment, as well as the level of neuronal histologic injury. No changes in serum concentration were found, suggesting neurologic production of apoA1. In studying CSF lipoproteins after human traumatic brain injury, Kay and colleagues¹⁸ found CSF levels of apolipoprotein E fell significantly, and although CSF levels of apoA1 remained unchanged, the apoA1-containing particles became much smaller, implying remodeling of the lipoprotein particles in the central nervous system. This again suggests a role of lipoproteins in central nervous system injury.

A recent study by Yang and coworkers,²² using proteomic methods, found increased expression of apoA1 in hippocampus of patients with drug-resistant mesial temporal lobe epilepsy. Using immunohistochemistry, they suggested that apoA1 was mainly found to be extravascular and concluded that extravasated apoA1 secondary to impaired blood-brain barrier function was likely responsible for the increased expression and as such extravasated plasma protein can confound proteomic brain studies. Yang and coworkers²² examined a very select group, patients with severe refractory epilepsy who underwent neurosurgical intervention, and compared them with control brain tissue obtained via autopsy, with postmortem delays of 6 to 11 hours. Such delay will undoubtedly have changed the protein milieu in tissue as sensitive as brain. Neurosurgical trauma at the time of tissue harvest, as well as a control group in which protein expression was certain to be abnormal, makes interpretation of their results difficult. The authors do not explain why

increased expression of many other plasma proteins was also not seen in the brain tissue. Our study has found that DHCA is associated with a decrease in 2 polypeptides that arise from apoA1 precursor protein. Their decrease may reflect degradation by a protease, and their subsequent loss may contribute to the cerebral cellular injury. Both of these peptide fragments had MWs of 15 kDa, which is half of that of the intact protein. What the significance of these proteins is and their potential role in the pathogenesis of cerebral injury is not answered in this work and requires further studies.

Plasma apoA1 levels fell with DHCA. As post-DHCA hematocrit values were not significantly different from baseline, the fall in apoA1 concentration does not reflect hemodilution. ApoA1 has been described as a “negative” acute phase protein, levels falling with inflammatory conditions such as rheumatoid arthritis.²³ After DHCA, diminished apoA1 plasma levels may serve as a biomarker of cerebral injury.

Neurofilament proteins are the most important of the cytoskeletal proteins of neuronal axons.²⁴ Classified as type IV intermediate filaments, these heavily phosphorylated polymer proteins comprise 3 neurotriplet proteins: light (NFL), medium (NFM), and heavy chains (NFH). There are many reports in the literature linking neurofilament proteins to neurologic disease states such as Alzheimer disease and multiple sclerosis.²⁵ CSF levels of NFL in cardiac arrest survivors has been correlated to poor long-term outcome.²⁶ To our knowledge, NFM has never been linked to DHCA. We found 2 fragments of NFM, with MWs of 52 and 54 kDa, and a mean increase in level of 66%. Identified in the protein databases as recognized fragments of NFM (theoretical MW 59.9 kDa), the actual parent NFM protein is 160 kDa. It is likely that these represent products of protease activity. In vivo, neurofilament degradation has been found to be mediated by calmodulin. Identification of the putative protease and its antagonist may lead to novel neuroprotective agents.

NSE is a cytosolic enzyme, a homodimer that catalyses conversion of 2-phospho-D-glycerate to phosphoenolpyruvate in the glycolysis pathway. NSE has been described extensively in the literature as a marker of brain injury,²⁷ including in the setting of cardiac surgery. NSE is also found in erythrocytes and platelets; hemolysis during cardiac surgery may confound interpretation of NSE after cardiac surgery. Nevertheless, serum NSE levels have been correlated with neurocognitive outcome in adults undergoing coronary artery bypass.²⁸ We found decreased expression of NSE with DHCA. This probably reflects leakage out of neuronal cells.

Protein disulfide-isomerase is an endoplasmic reticulum enzyme that not only catalyzes formation and/or rearrangement of protein disulfide bridges, but is also a chaperone,

namely a protein that catalyzes the folding of other proteins into their correct tertiary structures.²⁹ Chaperones also play an important role of preventing the aggregation of misfolded proteins. Up-regulation of this protein in glial cells appears to confer resistance to ischemia-induced apoptosis.³⁰ We found a 27% increase in the expression of this protein, perhaps reflecting reactionary protective cellular mechanisms to the ischemia of DHCA. Methods to induce up-regulation of this protein may be neuroprotective. We also found a hypothetical chaperone protein that had a 59% increase in level. This protein needs further characterization.

CHMP4b showed a 28% fall in expression with DHCA. The CHMP family is a group of proteins that are involved with intracellular vesicle transport³¹ and lead to the formation of “endosomal sorting complex required for transport” complexes. These complexes traffic the endosomal cargo of multivesicle bodies, a specific morphologic form of endosome, and comprising transmembrane proteins, to lysosomes for degradation. It is unclear at present what role this plays in cerebral injury with DHCA.

Traditionally, scientific research entails surmising a hypothesis and testing it. However, current knowledge will limit hypothesis postulation. By taking a global view without presupposing for any proteins, we discerned novel proteins. This is the first study to link the proteins found (with the exception of NSE) to cerebral injury after DHCA and opens the door to further studies to elucidate the potential roles played by these proteins in the pathogenesis of DHCA cerebral injury. This may lead to novel neuroprotection strategies in DHCA. A limitation of this study is that actual changes in protein expression have not been revealed, for example, what post-translational modifications take place or what proteases have been activated to cause peptide cleavage. This study does not delineate cause or effect for the identified proteins. This will need further work. There are several pathophysiologic processes active in the DHCA groups compared with the controls. As an initial study, we attempted to exclude any effects resulting from anesthesia and surgical trauma, thereby examining changes resulting from the cumulative processes of DHCA, namely, CPB, hypothermia, rewarming, global ischemia, and reperfusion. Studies can be undertaken to further dissect the protein changes associated with each of these pathophysiologies.

In summary, we have characterized the acute protein expression changes seen in a clinically relevant model of DHCA, in which marked cerebral injury was demonstrated. Novel proteins have been identified that may play a role in the pathogenesis of cerebral injury such as apoA1 and could serve as new targets for neuroprotection. Diminishing apoA1 plasma levels may serve as a biomarker for cerebral injury.

We thank George Quick, Mike Lowe, and Elaine Parker for their expertise in preparing the animal experiments; Cristina Osorio, Michael Herbstreith, and Dong Ning He (Duke Neuroproteomics) for the 2-dimensional gel work; the Michael Hooker Proteomics Center (University of North Carolina at Chapel Hill) for the mass spectrometry; Damian Craig for his statistical and bioengineering help; and Susan Reeves for help in preparing the photomicrographs.

References

1. Clancy RR, McGaurn SA, Wernovsky G, Gaynor JW, Spray TL, Norwood WI, et al. Risk of seizures in survivors of newborn heart surgery using deep hypothermic circulatory arrest. *Pediatrics*. 2003; 111:592-601.
2. Bellinger DC, Wypij D, Kuban KC, Rappaport LA, Hickey PR, Wernovsky G, et al. Developmental and neurological status of children at 4 years of age after heart surgery with hypothermic circulatory arrest or low-flow cardiopulmonary bypass. *Circulation*. 1999;100:526-32.
3. Kurth CD, Priestley M, Golden J, McCann J, Raghupathi R. Regional patterns of neuronal death after deep hypothermic circulatory arrest in newborn pigs. *J Thorac Cardiovasc Surg*. 1999;118:1068-77.
4. Aharon AS, Mulloy MR, Drinkwater DC Jr, Lao OB, Johnson MD, Thunder M, et al. Cerebral activation of mitogen-activated protein kinases after circulatory arrest and low flow cardiopulmonary bypass. *Eur J Cardiothorac Surg*. 2004;26:912-9.
5. Kerendi F, Halkos ME, Kin H, Corvera JS, Brat DJ, Wagner MB, et al. Upregulation of hypoxia inducible factor is associated with attenuation of neuronal injury in neonatal piglets undergoing deep hypothermic circulatory arrest. *J Thorac Cardiovasc Surg*. 2005;130: 1079-85.
6. Marouga R, David S, Hawkins E. The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal Bioanal Chem*. 2005;382:669-78.
7. Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, et al. Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics*. 2004;4:793-811.
8. Langley SM, Chai PJ, Miller SE, Mault JR, Jagers JJ, Tsui SS, et al. Intermittent perfusion protects the brain during deep hypothermic circulatory arrest. *Ann Thorac Surg*. 1999;68:4-12.
9. Parker CE, Warren MR, Loisele DR, Dicheva NN, Scarlett CO, Borchers CH. Identification of components of protein complexes. *Methods Mol Biol*. 2005;301:117-51.
10. Amir G, Ramamoorthy C, Riemer RK, Reddy VM, Hanley FL. Neonatal brain protection and deep hypothermic circulatory arrest: pathophysiology of ischemic neuronal injury and protective strategies. *Ann Thorac Surg*. 2005;80:1955-64.
11. Scallan MJ. Cerebral injury during paediatric heart surgery: perfusion issues. *Perfusion*. 2004;19:221-8.
12. Ditsworth D, Priestley MA, Loepke AW, Ramamoorthy C, McCann J, Staple L, et al. Apoptotic neuronal death following deep hypothermic circulatory arrest in piglets. *Anesthesiology*. 2003;98:1119-27.
13. Tsui SS, Kirshbom PM, Davies MJ, Jacobs MT, Greeley WJ, Kern FH, et al. Nitric oxide production affects cerebral perfusion and metabolism after deep hypothermic circulatory arrest. *Ann Thorac Surg*. 1996;61:1699-707.
14. Tsui SS, Kirshbom PM, Davies MJ, Jacobs MT, Kern FH, Gaynor JW, et al. Thromboxane A2-receptor blockade improves cerebral protection for deep hypothermic circulatory arrest. *Eur J Cardiothorac Surg*. 1997;12:228-35.
15. Harr SD, Uint L, Hollister R, Hyman BT, Mendez AJ. Brain expression of apolipoproteins E, J, and A-I in Alzheimer's disease. *J Neurochem*. 1996;66:2429-35.
16. Engwegen JY, Helgason HH, Cats A, Harris N, Bonfrer JM, Schellens JH, et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. *World J Gastroenterol*. 2006;12:1536-44.
17. Hyka N, Dayer JM, Modoux C, Kohno T, Edwards CK 3rd, Roux-Lombard P, et al. Apolipoprotein A-I inhibits the production of interleukin-1beta and tumor necrosis factor-alpha by blocking contact-mediated activation of monocytes by T lymphocytes. *Blood*. 2001;97: 2381-9.
18. Kay AD, Day SP, Kerr M, Nicoll JA, Packard CJ, Caslake MJ. Remodeling of cerebrospinal fluid lipoprotein particles after human traumatic brain injury. *J Neurotrauma*. 2003;20:717-23.
19. Chen W, Ji J, Xu X, He S, Ru B. Proteomic comparison between human young and old brains by two-dimensional gel electrophoresis and identification of proteins. *Int J Dev Neurosci*. 2003;21:209-16.
20. Puchades M, Hansson SF, Nilsson CL, Andreassen N, Blennow K, Davidsson P. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. *Brain Res Mol Brain Res*. 2003;118: 140-6.
21. Saito K, Seishima M, Heyes MP, Song H, Fujigaki S, Maeda S, et al. Marked increases in concentrations of apolipoprotein in the cerebrospinal fluid of poliovirus-infected macaques: relations between apolipoprotein concentrations and severity of brain injury. *Biochem J*. 1997;321:145-9.
22. Yang JW, Czech T, Gelpi E, Lubec G. Extravasation of plasma proteins can confound interpretation of proteomic studies of brain: a lesson from apo A-I in mesial temporal lobe epilepsy. *Brain Res Mol Brain Res*. 2005;139:348-56.
23. Park YB, Lee SK, Lee WK, Suh CH, Lee CW, Lee CH, et al. Lipid profiles in untreated patients with rheumatoid arthritis. *J Rheumatol*. 1999;26:1701-4.
24. Kupina NC, Detloff MR, Bobrowski WF, Snyder BJ, Hall ED. Cytoskeletal protein degradation and neurodegeneration evolves differently in males and females following experimental head injury. *Exp Neurol*. 2003;180:55-73.
25. Petzold A. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. *J Neurol Sci*. 2005;233:183-98.
26. Rosen H, Karlsson JE, Rosengren L. CSF levels of neurofilament is a valuable predictor of long-term outcome after cardiac arrest. *J Neurol Sci*. 2004;221:19-24.
27. Kofke WA, Konitzer P, Meng QC, Guo J, Cheung A. The effect of apolipoprotein E genotype on neuron specific enolase and S-100beta levels after cardiac surgery. *Anesth Analg*. 2004;99:1323-5.
28. Rasmussen LS, Christiansen M, Eliassen K, Sander-Jensen K, Moller JT. Biochemical markers for brain damage after cardiac surgery—time profile and correlation with cognitive dysfunction. *Acta Anaesthesiol Scand*. 2002;46:547-51.
29. Wilkinson B, Gilbert HF. Protein disulfide isomerase. *Biochim Biophys Acta*. 2004;1699:35-44.
30. Ko HS, Uehara T, Nomura Y. Role of ubiquitin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. *J Biol Chem*. 2002;277:35386-92.
31. Katoh K, Shibata H, Hattori K, Maki M. CHMP4b is a major binding partner of the ALG-2-interacting protein Alix among the three CHMP4 isoforms. *Arch Biochem Biophys*. 2004;421:159-65.

Discussion

Dr John G. Byrne (Nashville, Tenn). How do you see this used as a tool clinically going forward in 3 to 5 years?

Dr Sheikh. The importance of this study is in trying to establish the pathogenesis of brain injury at a molecular level. This being a very preliminary study; we have to be careful about overreaching our conclusions and taking it to a clinical application. However, we have taken a very old problem that has been extensively studied, namely DHCA in CPB, and without presupposing for any proteins, we have actually examined globally for all the protein changes that are taking place simultaneously. This has allowed us to therefore identify proteins that have not been previously thought about or linked to circulatory arrest. Further studies to elucidate the role of these proteins may help in determining or coming up with neuroprotective strategies, particularly pharmaco-

logic neuroprotective strategies, which are currently lacking in DHCA.

Dr Marc Ruel (*Ottawa, Ontario, Canada*). As you know, with proteomics, as with other genomic methods, there is a huge potential for false negatives and false positives. Can you tell us how you went around that? For instance, how many proteins did you test for in the assay that you used?

Also, some of the changes in translation levels of your proteins are rather modest, in my opinion, such as 15% and 28%. One would expect higher fold-changes than this. Were these changes found in all animals (I think your N was 7 or 8 per group) before you actually called the change a significantly positive one?

Finally, with MS you tested for positive findings, but did you also validate some proteins that showed no change to see whether this lack of change was real?

Dr. Sheikh. First of all, the number of proteins is actually picked up by the software analysis of the gel images themselves. We found somewhere in the region of just over 2000 proteins.

The Student unpaired *t* test was used to identify cerebral protein spots that underwent significant change between our control and experimental groups. Proteins found to be statistically significant showed a consistent change in all or nearly all animals.

In relation to the MS, we did not look at negative proteins; however, the data from MS yields several scores. We received 3 scores in total for each protein, and the level of the scores is indicative of how accurate the identification is from MS. Importantly, for 9 of the 10 spots that we found, which went on to be

identified as 6 proteins, the MS scores were unambiguous, and therefore we are satisfied that they are indeed the true protein identifications. The tenth protein spot yielded ambiguous scores, and therefore we have not included this protein in our conclusions.

Dr Beat H. Walpoth (*Geneva, Switzerland*). I have a question concerning your model. I am not too sure whether your control group is adequate for such sensitive assays, since the pig shows profound changes even in normothermic CPB.

Dr Sheikh. The choice of the control group was slightly difficult because this is the first time this sort of study has being undertaken in this particular problem. We were seeking to have a control group whereby we would have sufficient difference so that we would actually identify protein changes. To this end, a control group could be anywhere between a native piglet with no anesthesia or sternotomy or heparinization to a piglet that undergoes full CPB but does not undergo the 1 hour of DHCA. In fact, our future work is actually looking specifically at the 1 hour of DHCA.

Does that answer your question?

Dr Walpoth. That answers the question. So, it is not specific for hypothermia at the moment?

Dr Sheikh. Yes. What we were also seeking to do was to have a clinically relevant model and use the proteomic technique to see how it works in a clinically relevant model. Therefore, we do accept that there are several pathologic processes going on there: CPB, hypothermia, rewarming, as well as ischemia/reperfusion.

Appendix E1

25 mmol/L DHCPC lysis buffer: 7 mol/L urea, 2 mol/L thiourea, 15 mmol/L DHPC (1,2-diheptanoyl-*sn*-glycero-3-phosphatidylcholine), 0.5% Triton X-100, 30 mmol/L Tris, 5 μ L protease inhibitor solution (1 tablet of Complete [Roche Diagnostics Corporation, Indianapolis, Ind] in 1 mL Tris).

Lysis buffer: 8 mol/L urea, 4% CHAPS (3[3-cholaminopropyl diethylammonio]-propane sulfonate), 20 mmol/L Tris, pH 8.0.

2X buffer: 8 mol/L urea, 4% w/v CHAPS, 20 mg/mL dithiothreitol, 2% v/v Pharmalyte (GE Healthcare) (pI 4-7 for brain samples, and 3-10 for plasma samples).

TABLE E1. Perioperative variables

	Control (n = 5)	DHCA (n = 5)
Weight (kg)	3.2 ± 0.2	3.3 ± 0.8
Mean arterial blood pressure (mm Hg)*		
Before arrest	44 ± 3.5	40 ± 5.5
After arrest	37 ± 3.0	54 ± 16.8
Pearrest temperature (°C)	37.1 ± 0.8	36.4 ± 0.6
Arterial Pco ₂ (mm Hg)		
Before arrest	34 ± 15	36 ± 8.1
After arrest	40 ± 3.0	41.2 ± 8.8
Hematocrit (%)		
Before CPB	24 ± 3.4	25 ± 3.7
After CPB	23 ± 5.9	27 ± 7

Repeated-measures analysis of variance was applied to blood pressure, arterial carbon dioxide tension (*Pco₂*) and hematocrit. Student unpaired *t* test was applied to weight and prearrest temperature. Values are means ± standard error. *Mean arterial pressure showed significant group-intervention effect (*P* = .03), decreasing with time in controls, but increasing after DHCA in experimental piglets. All other parameters were not statistically significant.