Apoptotic cell death in the hippocampus due to prolonged hypothermic circulatory arrest: comparison of cyclosporine A and cycloheximide on neuron survival

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Abstract

Objective: To determine whether cyclosporine A (CsA) or cycloheximide (CHX) can reduce neuronal apoptosis in the hippocampus in a chronic animal model of hypothermic circulatory arrest (HCA). Methods: Twenty-eight pigs (28–33 kg) underwent 90 min of HCA at 20°C. In a blinded study, animals were randomized to placebo (n = 12), 5 mg/kg CsA (n = 8), or 1 mg/kg CHX (n = 8). After elective sacrifice 7 days postoperatively, brains were perfusion-fixed and the left hippocampus was examined for evidence of neuronal cell death. An in situ double-labeling method was used on cryosections to unequivocally identify apoptotic nuclei by the simultaneous visualization of DNA fragmentation and apoptotic chromatin condensation. Sections were also examined by immunocytochemistry for upregulation of the pro-apoptotic proteins Bax, activated caspase 3, and glyceraldehyde-3-phosphate dehydrogenase. Results: Apoptotic nuclear degradation was clearly present in the CA1, CA2 and CA3 subregions of the hippocampus after HCA. However, there was also morphological evidence for an accompanying necrotic-like cell death. There was no significant difference between the number of apoptotic nuclei observed in CSA-treated animals, mean value 4.4 ± 1.63 SEM or CHX-treated animals, mean value 4.0 ± 1.92 SEM, and age-matched control HCA pigs, mean value 4.85 ± 1.69 SEM, (P > 0.10). Conclusions: The data clearly demonstrate apoptotic cell death in pigs after HCA by simultaneously demonstrating in situ end labeling (TUNEL reaction) and apoptotic chromatin condensation using a nucleic acid-binding dye. Since CsA shows promising neuroprotective effects in behavioral studies, and since the peak of HCA-induced apoptosis occurs earlier than 7 days, further studies will be required to determine whether CsA can improve neuronal survival in the first few days after HCA. CHX was not effective in reducing apoptosis in this model. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Hippocampus; Global ischemia; Hypothermic circulatory arrest; Cyclosporine A; Pig model

1. Introduction

The global ischemia that is attendant upon hypothermic circulatory arrest (HCA) initiates a series of events that ultimately lead to neuronal death and loss of function in both adult and infant patients undergoing cardiac surgery. Specific regions of the brain like the striatum, hippocampus, cerebellum, amygdala, lateral hypothalamic nucleus and 3rd–5th layers of the neocortex appear to be especially vulnerable to ischemic cell loss [1,2]. Data from patient studies and experimental animal models of HCA indicate that there are differences with respect to vulnerability of neuronal populations that vary with age as well as the duration and temperature of HCA [3]. The four-vessel and two-vessel occlusion models of global ischemia in rats have shown that both apoptotic and necrotic cell death may occur in the hippocampus and other vulnerable regions [3]. The pig has become an increasingly popular model for cardiopulmonary bypass with HCA, in part because of similarities with human anatomy. Transient ischemia by carotid occlusion in 1-day-old piglets [4] or 90 min of HCA in 3–10 day old neonates [5] have both demonstrated terminal dUTP nick-end-labeling (TUNEL) positive nuclei in the hippocampus, indicative of DNA fragmentation. It was not determined in these studies what contribution developmentally programmed cell death made to the number of observed TUNEL positive nuclei, although it was noted that TUNEL alone could not provide an unequivocal identification of apoptosis.

Apoptotic DNA fragmentation is produced by endonu-
cleavage digestion of DNA, creating single- and/or double-strand breaks producing high molecular weight DNA fragments of about 50–300 kb [6]. DNA digestion usually, but not always, continues to the final production of low molecular weight, oligonucleosomal-size DNA fragments (180–200 bp) [7] that can be visualized as a ‘ladder’ pattern by gel electrophoresis. It was initially believed that in situ end labeling (ISEL) could specifically detect this final, double-strand break between oligonucleosomes. It is now clear that ISEL/TUNEL methods also detect the single- or double-strand breaks that can occur in necrotic cell death [8], or with degenerative changes that result from a prolonged post-mortem interval.

We have developed a double-labeling method, which uses ISEL to detect DNA fragmentation and concomitant staining with the nucleic acid-binding dye YOYO-1 to allow visualization of apoptotic chromatin condensation in the same nucleus [9]. Apoptotic chromatin condensation is an independent event from DNA fragmentation and requires the presence of ATP [10] and specific initiating proteins like the caspase-activated acinus protein [11]. Chromatin condensation therefore provides a unique and independent marker of apoptosis, yet occurs in the degradative phase of the apoptotic pathway with a similar frequency to DNA fragmentation [12].

Some but not all apoptotic pathways rely on new protein synthesis to remove cells. The juvenile pig offers the advantage that developmentally programmed cell death is unlikely to contribute to the apoptotic nuclei observed in the hippocampus after HCA. Since it is not known what apoptotic pathways may be invoked in post-mitotic neurons in the pig brain, cycloheximide (CHX), a protein synthesis inhibitor, was tested along with cyclosporine A (CsA). In vitro studies have shown that CsA can block apoptosis by keeping the permeability transition pore closed and thus prevent a decline in mitochondrial membrane potential [13]. CsA has been shown to enhance neuron survival in the hippocampus in the rat two-vessel occlusion model of global ischemia [14]. Encouraged by initial findings in a pilot study (data not shown), a blinded study was carried out to compare the effects of CsA and CHX on apoptotic cell death in the pig hippocampus 7 days post-HCA. This time point was chosen to allow for neurobehavioral evaluation in the same animals: the results of this aspect of the study are presented in the accompanying report.

Although ISEL/YOYO staining can provide reliable identification of apoptotic nuclei, it does not provide information on which specific apoptotic pathway may be involved. Therefore, sections were examined for immuno-cytochemical evidence of Bax, activated caspase 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upregulation and nuclear accumulation: these three proteins are known to participate in mitochondrially-dependent apoptotic pathways [15]. Determining which effector proteins participate in apoptosis may contribute to the development of new therapeutic treatments that could limit neuronal loss and the ensuing functional deficits that can be observed following HCA.

2. Methods and materials

2.1. Study design

A blinded study was initiated to compare the effects of CsA and CHX on apoptosis in the hippocampus at 7 days after hypothermic circulatory arrest (HCA). Young but mature female Yorkshire pigs (Th. D. Morris, Inc., Reistertown, NY, USA) were exposed to HCA at 20°C for 90 min. Pigs ranged between 3 and 4 months of age and weighed 28–33 kg. Pigs were randomly assigned to 5 mg/kg CsA (Novartis Pharmaceuticals Co., East Hanover, NJ, USA) or 1 mg/kg CHX (Sigma–Aldrich Company, St. Louis, MO, USA). CsA was administered intravenously before and after HCA, as well as subcutaneously for 7 days post-operatively. CHX was given as a single intravenous bolus after HCA and weaning from CPB. Further details can be found in the accompanying report by Hagl et al. [16]. All animals received 1 g of methylprednisone immediately before commencing HCA. All animals received humane care in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ published by the National Institute of Health (NIH Publication No. 88-23, revised 1985). Protocols for all experiments were approved by the Mt. Sinai Institutional Animal Care and Use Committee.

2.2. Perioperative management, anesthesia, and operative protocol

Each animal underwent preoperative behavioral assessment, intraoperative hemodynamic and metabolic monitoring, measurement of intracranial pressure, and recording of quantitative EEG and cervical as well as cortical somato-sensory evoked potentials (SSEP) both before and at several points during recovery from HCA. Early behavioral recovery was scored by observers blinded to the experimental protocol, and the daily neurological/behavioral evaluation was carried out until elective sacrifice 7 days after HCA.

2.3. Perfusion-fixation protocol and cryosectioning

Prior to perfusion, pigs were pre-treated with intramuscular ketamine (15 mg/kg) and atropine (0.03 mg/kg), and anesthetized with intravenous pentobarbital (20 mg/kg). After endotracheal intubation, the pigs were ventilated mechanically and isoflurane was used to maintain deep anesthesia. Paralysis was achieved with intravenous pancuronium (0.1 mg/kg). Heparin (300 IU/kg) was administered intravenously prior to insertion of an 18F cannula into the ascending aorta. Immediately before perfusion-fixation, the descending aorta was cross-clamped to avoid significant loss of perfusion solution to the lower body, and the head was packed in ice.
Perfusion was started with 500 ml cold saline with a perfusion pressure of 120–150 cm H2O; the fluid was allowed to drain passively via an incision in the right atrial appendage. One litre of ice-cold (4°C) 10% buffered formaldehyde (Fisher Chemicals, Fair Lawn, NJ, USA) was then administered over a period of 10 min; a second litre of fixative was infused over 45 min, and a third litre over 20 min, for a total of 3 l of ice-cold formalin over a total of 2 h. The brain was removed 30 min later, and stored at 4°C in buffered formalin for 48 h. The head was surrounded by ice throughout the perfusion and while the brain remained in situ.

A tissue block encompassing the left hippocampus was dissected free, rinsed with cold 0.1 M phosphate buffered saline (PBS: 9 g sodium chloride, 3.2 g sodium phosphate monobasic, 21.8 g sodium phosphate dibasic/l (pH 7.2)) and immersed in 10% sucrose/PBS for 24 h at 4°C. The block was transferred to cold 20% sucrose/PBS at 4°C, with a fresh change of sucrose buffer every day for 3 days until the tissue block sank. The tissue block was then frozen in isopentane and stored at −80°C.

Serial 10 micron-thick frozen sections were cut through the rostral-caudal extent of the hippocampus, and every 5th section was thaw-mounted onto Superfrost charged slides (VWR). Sections were kept cold and allowed to dry overnight in a 4°C refrigerator. Slides were stored at −80°C until required.

Two animals from the CHX group were removed from the study: both showed poor quality fixation and degenerative artifact to an extent that precluded reliable ISEL/YOYO staining.

### 2.4. ISEL/YOYO staining for detection of apoptotic nuclei

Every tenth section through the main body of the hippocampus was taken for ISEL/YOYO staining, for a total of eight sections per animal. Slides were brought to room temperature, rinsed in PBS buffer, then exposed to methanol (−20°C) for 5 min. Sections were treated with RNase A (100 µg/ml) in 2× SSC (standard saline citrate) (8.82 g sodium citrate, 17.53 g sodium chloride/liter) for 20 min at 37°C, and then rinsed with an excess volume of 2× SSC. Sections were brieﬂy digested with proteinase K (20 µg/ml in Tris–EDTA (TE) buffer (pH 8)) for 2 min at 37°C, and then placed in ice-cold 0.1 M glycine/0.1 M Tris (pH 7.2) for 10 min to halt the digest. Sections were incubated for 10–20 min at room temperature in equilibration buffer (Intergen, S7106) prior to the labeling reaction. Excess buffer was blotted off, terminal transferase enzyme (TdT) reaction mix was applied (Boehringer Mannheim/Roche, 220–582) and sections covered with a strip of parafilm for 1 h at 37°C. The reaction mix contained 2.5 mM cobalt chloride, 35 units TdT (1.4 ul/100 µl) and 0.7 mM (1.4 µl/100 µl) Bodipy-TR-14-dUTP (Molecular Probes, C7618). Slides were given three washes in an excess volume of 2× SSC, at 37°C for 10 min each, followed by a PBS rinse. Sections were then incubated in the dark with YOYO-1 (1:500 in PBS) (Molecular Probes, Y-3601) for 30 min at room temperature, rinsed with PBS, and coverslipped with Aquamount (BDH–Gurr). Sections from HCA animals were run simultaneously with sections from normal age-matched control pigs. The TdT enzyme was omitted from selected sections as a negative control while DNase I-treated sections provided a positive control (data not shown).

Sections were examined first by epifluorescence microscopy (Olympus AX-70, U-MFI/TRITC filter) to determine the number of apoptotic nuclei per section. Slides were then examined by laser confocal microscopy (Leica, TCS 4D) to obtain high resolution digital images of individual nuclei.

### 2.5. Quantitation of apoptosis in the hippocampus

Apoptotic nuclei were identified by bright green YOYO fluorescence in an apoptotic chromatin condensation pattern, combined with bright red ISEL fluorescence. An ocular grid was centered on the CA1-3 neuron lamina, and those apoptotic nuclei found within the grid were counted using a 40× objective. The grid was moved along the entire length of the CA1, 2 and 3 subregions of the hippocampus on each slide. The number of grids per section was totalled and multiplied by a conversion factor to obtain an area value, then multiplied by the section thickness to obtain a volume measure. The number of apoptotic nuclei/mm³ was thus obtained for each slide. Data were averaged from the eight slides counted per animal and plotted for each time point.

### 2.6. Immunocytochemistry for Bax, activated caspase 3 and GAPDH

Sections were brought to room temperature, rinsed in PBS and then exposed to methanol (−20°C) for 5–10 min. Sections to be immunoreacted for GAPDH were exposed to RNAse A (100 µg/ml 2× SSC) for 3 min at 37°C, and then placed immediately in ice-cold 0.1 M glycine/0.1 M Tris buffer (pH 7.2). Slides were then rinsed in PBS at room temperature prior to blocking. All slides were blocked for 20–30 min with 10% normal goat serum (NGS)/0.2% Tween 20/0.1 M PBS. Sections were incubated overnight at 4°C with antisera to Bax (1:200, N-20, Santa Cruz, sc-493) or GAPDH (1:200, Chemicon, MAB374). Sections exposed to antisera for activated caspase 3 (1:100, New England Biolabs, 9661S) were incubated for 48 h at 4°C. Sections were rinsed in PBS and then incubated with Alexa 594-conjugated goat anti-rabbit Alexa 594-conjugated IgG (Bax, caspase 3), or Alexa 594-conjugated goat anti-mouse IgG (GAPDH) (Molecular Probes, A-11012, A-11005) for 40–60 min at 37°C. Slides were rinsed with PBS, then incubated with YOYO-1 (1:500 in PBS) in the dark for 30 min, rinsed with PBS and coverslipped with Aquamount for examination by confocal microscopy.
2.7. GFAP Immunocytochemistry and assessment of reactive gliosis after HCA

A total of 4 slides were taken from the central region of the hippocampus and immunoreacted for glial fibrillary acidic protein (GFAP) to identify astroglia. Sections were allowed to come to room temperature, rinsed in PBS and then exposed to methanol (–20°C) for 10 min. Sections were rinsed briefly in PBS and then blocked for 20–30 min in 10% NGS/0.2% Tween 20/0.1 M PBS. Sections were blotted and then incubated with a monoclonal GFAP antibody (Sigma, G3893) at a dilution of 1:600 in 1% NGS/0.2% Tween 20/0.1 M PBS overnight at 4°C. Slides were rinsed in PBS and incubated for 60 min at 37°C with Alexa 594-conjugated goat anti-mouse IgG. Following a PBS rinse, sections were incubated with YOYO-1 (1:500 in PBS) for 30 min in the dark, rinsed with PBS and cover-slipped with Aquamount prior to confocal imaging.

Three digital images, randomly chosen over the length of the CA1–2 region on each slide, were obtained by confocal microscopy at a fixed magnification, pinhole and voltage using the 40× objective. A total of 12 images from each lamina were obtained per animal. Each image was subjected to thresholding such that GFAP immunoreactivity was highlighted above background so that the total area of GFAP immunoreactivity could be calculated per image using Northern Eclipse image analysis software (Empix Imaging, Toronto, Canada). Area of GFAP immunoreactivity was an average of the 12 images taken from four slides. Three animals were randomly chosen from the CSA, CHX and control groups of the double blind study. Images were also obtained from a normal, age-matched control pig.

2.8. Confocal microscopy

ISEL/YOYO stained sections were examined by confocal microscopy using two independent channels to detect specific ISEL (BODIPY-TR-14-dUTP emission maximum, 625 nm) and YOYO (emission maximum, 509 nm) fluorescence. Alexa 594 (emission maximum, 617 nm) was used to set the detection limits for GAPDH, Bax and the activated caspase 3 fragment immunofluorescence. Consecutive digital images for ISEL/YOYO or immunofluorescence/YOYO were subjected to 32 line averaging to eliminate out of focus fluorescent haze and then stored on a Maxoptix optical data storage system.

2.9. Statistical methods

ISEL/YOYO counts of apoptotic nuclei were carried out by observers fully blinded with regard to the treatment groups. Non-parametric statistics were used to analyze the data, since it could not be demonstrated that the data satisfied the requirements for a normal population distribution. The Kolmogorov–Smirnov two sample test was chosen to compare drug treatment groups with control groups: each group was compared with the control, but the treated animals were not compared with one another. Analyses were performed with Statistica software (Statsoft, Tulsa, OK) for Windows.

3. Results

3.1. Identification of apoptotic nuclei in the hippocampus following HCA

When observed with epifluorescence microscopy, apoptotic nuclei in the pig brain demonstrated an intense bright green YOYO signal in a condensed pattern. This is in contrast to normal nuclei that show only light YOYO staining in the nuclear matrix, with a bright green nucleolus. Nuclei with an apoptotic chromatin condensation pattern also demonstrated a bright red fluorescent signal with BODIPY-Texas Red-dUTP that was distributed in a similar but not identical pattern to the condensed YOYO signal. In contrast, normal non-apoptotic nuclei show only a low background level of ISEL signal.

We observed a range of YOYO-bright, apoptotic condensation patterns in the CA1–3 subregion of the hippocampus. Fig. 1 shows laser confocal images of apoptotic nuclei (ISEL/YOYO positive) that illustrate the range of chromatin condensation patterns and corresponding ISEL signal. It is important to note that the YOYO-stained apoptotic chromatin clumps have smooth edges and are generally round or elliptical, which is consistent with electron microscopic observations of ‘classic’ apoptotic nuclei first described in leukocytes [17]. Fig. 1A1 shows a small, YOYO-bright, highly condensed nucleus and the faint outline of a greatly condensed cell soma. Fig. 1B1 shows the same cell viewed for ISEL fluorescence only, demonstrating an intense nuclear ISEL signal (arrow) but, importantly, no cytoplasmic signal.

Fig. 1A2 depicts another frequently observed YOYO chromatin condensation pattern in the pig hippocampus after HCA. Here a small, condensed nucleus appears with satellite apoptotic bodies containing condensed nuclear chromatin, all bounded by a shrunken cell soma. Fig. 1B2 shows that this condensation pattern is again accompanied by an ISEL signal associated only with the condensed nuclear contents. Figs. 1A3,A4 and 4 show nuclei with multiple, smooth-surfaced YOYO-bright chromatin clumps. Again, these nuclei also demonstrate a positive ISEL signal (Fig. 1B3,4). ISEL/YOYO positive nuclei similar to these examples were always observed as lone degenerating cells, another characteristic feature of apoptotic cell death. Based on these criteria, cells displaying similar ISEL/YOYO staining patterns were considered to be apoptotic, and were counted as positive nuclei.

In contrast, Fig. 2A1,A2 illustrate a second pattern of YOYO-bright chromatin condensation that was also observed in some hippocampal sections. The YOYO-dense bodies in these cells appeared to fill a larger
Fig. 1. ISEL/YOYO positive nuclei in the pig hippocampus 7 days after HCA. Digital confocal images are shown depicting the range of observed apoptotic chromatin condensation patterns with corresponding ISEL signal in the CA1–3 areas of the hippocampus (indicated by white arrows). The left-hand panel (A1–4) shows YOYO-stained sections. The right hand panel (B1–4) shows the same section in the identical plane of focus viewed for ISEL fluorescence. Note that all condensed chromatin bodies are smooth surfaced, and that apoptotic cells are found individually among non-apoptotic neurons. The scale bar is 10 μm.
nuclear/cellular space than observed with the apoptotic nuclei depicted in Fig. 1. Importantly, these condensed chromatin bodies demonstrated an irregular or ‘rough’ surface which is considered to be characteristic of a necrotic chromatin degeneration pattern. It is also noteworthy that such degenerating cells always appeared as a group of cells, which is considered to be characteristic of necrotic cell loss. However, there was no evidence of inflammatory cells in their vicinity. These cells did not demonstrate a detectable ISEL signal (Fig. 2B1,B2), and were therefore not counted as apoptotic cells for the data plots. It is not yet clear whether these cells can be categorized as necrotic or apoptotic.

3.2. Effects of CsA and CHX on apoptosis

The number of apoptotic nuclei/mm³ was plotted as the averaged data from 8 slides per individual pig hippocampus (Fig. 3). Animals were examined at day 7 post-HCA to allow neurobehavioral evaluation prior to sacrifice. As noted in Section 2, two pigs in the CHX-treatment group were removed due to poor quality fixation and degenerative artifacts. An age-matched control pig was subjected to the identical perfusion treatment, but not HCA, and the resulting ISEL/YOYO data plotted to provide a baseline value for apoptosis in the normal pig hippocampus. There was clearly increased apoptotic cell death in all pigs subjected to HCA compared to the normal baseline control pig. There was no significant difference between the number of apoptotic nuclei observed in CSA-treated animals, mean value 4.4 ± 1.63 SEM or CHX-treated animals, mean value 4.0 ± 1.92 SEM, and age-matched control HCA pigs, mean value 4.85 ± 1.69 SEM, (P > 0.10).

3.3. Bax, caspase 3 and GAPDH immunoreactivity

It is not known whether neurons in the pig hippocampus die by a mitochondria-dependent apoptotic pathway, although acute global ischemia in the rodent brain has shown increased Bax protein levels as well as activation of caspase 3. Sections were therefore examined by immunocytochemistry for increased Bax, activated caspase 3 and GAPDH nuclear accumulation. Fig. 4 illustrates Bax and
activated caspase 3 immunoreactivity in normal control sections from the hippocampus (Fig. 4A2,C2) and in HCA-treated pigs (Fig. 4B2,D2). The left-hand panel presents YOYO-staining of the same sections (Fig. 4A1,D1). There did not appear to be any remarkable increase in Bax immunoreactivity either in the HCA pigs or in the drug-treated pigs at 7 days compared to the normal control. However, there were examples of increased immunoreactivity for activated caspase 3 (Fig. 4D2), although these appeared to be associated with those few cells that also demonstrated apoptotic chromatin condensation. No increase in GAPDH immunoreactivity or GAPDH nuclear translocation was observed in any pigs at 7 days post-HCA.

3.4. Reactive gliosis in the hippocampus following HCA

Since ischemic events are known to stimulate reactive gliosis, we decided to examine the hippocampus for evidence of increased GFAP immunoreactivity as a result of HCA with or without CSA and CHX treatment. Astroglia can respond rapidly to changes in the local environment, and, once activated upregulate GFAP and demonstrate exuberant process growth to surround injured neurons. We have measured the relative area of GFAP immunoreactivity randomly throughout the CA1 and CA2 hippocampus. These representative GFAP area values reflect the extent of astroglial hypertrophy.

Fig. 5 shows the averaged pixel area of GFAP immunoreactivity for each treatment group. There was a clear increase in GFAP pixel area in the pigs subjected to HCA compared to the normal, age-matched control pig. However, there was no significant difference detected in averaged GFAP area between CSA-treated animals, mean value 9492.44 ± 1803.09 SEM, or CHX-treated pigs, mean value 13 079.25 ± 2653.85 SEM, and control HCA-only pigs, mean value 8643.17 ± 2682.34 SEM (P > 0.10).

4. Discussion

4.1. Apoptosis/necrosis in the pig hippocampus

This study demonstrates that apoptotic cell death occurs in the pig hippocampus following 90 min of HCA at 20°C. The variety of apoptotic chromatin condensation patterns was unexpected, and appeared to range from the classic, highly condensed apoptotic nucleus to a larger nucleus with multiple, large smooth-surfaced chromatin condensations. Some of these variants may be the same pathway viewed at different stages of the final degenerative phase, although the rough-edged condensation patterns that did not demonstrate ISEL signal appear to be a separate event.

Our findings are reminiscent of those described by other laboratories in which ischemic and excitotoxic events produce morphological degenerative patterns that can share features of apoptotic and necrotic cell death [18]. In vitro and in vivo studies have both shown that increasing the severity of the initiating insult can shift cells from an apoptotic cell death to a necrotic cell death [19–21]. This study points out the need for further molecular/biochemical markers to determine the death pathway invoked, rather than relying solely on endstage degenerative morphologies to decide whether an apoptotic or necrotic pathway has been followed.

4.2. Does HCA induce mitochondria-dependent apoptosis in the pig hippocampus?

Data from rodent global ischemia studies have indicated that Bax and caspase 3 participate in apoptotic cell death pathways [3] but it is not known whether these two apoptotic effector proteins participate in post-HCA apoptosis in the adult pig brain. GAPDH is an important protein in the glycolytic pathway, but it also participates in some mitochondria-dependent apoptotic pathways. A precipitous decline in mitochondrial membrane potential leads to the release of cytochrome c and other pro-apoptotic factors leading to the activation of caspases 9 and 3 [22]. Prior to a decline in mitochondrial membrane potential, GAPDH protein levels increase in the cytosol, and there is an accumulation of GAPDH protein in the nucleus in those cells that will die via apoptosis. In vitro studies have demonstrated that when GAPDH upregulation and nuclear accumulation are prevented using antisense oligonucleotides [23,24], then apoptosis can be blocked.

We found little or no evidence for the participation of Bax or GAPDH protein in apoptosis in the pig hippocampus 7 days after HCA. Similarly, although some cells demon-
strated increases in activated caspase 3 immunoreactivity, these were limited and rare, in contrast to our observations in the Parkinson’s brain [15]. The lack of immunocytochemical evidence may be due to the fact that these proteins are upregulated in the first few days after HCA, as is true in rodent models of global ischemia [3], and may have returned to normal levels in most cells by day 7. Alternatively, apoptotic death after HCA may not be mitochondria-dependent, and thus selective upregulation of these proteins does not occur. Examination of animals from earlier time points using the same antisera, during the first 3 days post-HCA, may help determine whether or not these specific effector proteins participate in ischemia-induced apoptosis in the pig brain. However, alternate antisera must also be tested to determine whether any lack of staining observed is species-dependent.

4.3. Can CsA or CHX decrease HCA-induced neuronal apoptosis?

Our data from the double-blind study indicate that neither treatment with CsA nor CHX significantly decreases the number of apoptotic nuclei in the hippocampus 7 days after HCA. With regard to the CHX-treated animals, this result is not surprising, since behavioral recovery following HCA was no different in CHX-treated animals than in untreated controls. The failure to find a reduction in apoptosis in CsA-treated animals, however, was puzzling in view of improved behavioral and electrophysiological recovery following HCA, as detailed in the accompanying report. Pilot studies (data not shown) as well as the reports of others suggest that the peak of apoptosis occurs between 6 and 72 h after HCA, so our failure to find significant inhibition of apoptosis after CsA treatment 7 days postoperatively may be a result of having looked at a relatively late point in time.

It is possible that CsA could not reach the appropriate concentration in the brain after the first 3 days after HCA because of a restoration in blood–brain barrier (BBB) integrity. CsA may be excluded from the brain by active efflux modulated by P-glycoprotein, a multi-drug transporter [25]. Altered permeability or damage to the BBB has not yet been convincingly demonstrated in this pig model, but data from the rat occlusion models indicate that BBB permeability is altered for a period of time after ischemic insult. In fact, enhancement of neuron survival due to CsA after global ischemia is much greater in rats that have had the BBB compromised by direct injury [14]. Alternatively, as explained in greater detail in the accompanying report, since CsA can also affect inflammatory pathways, some of its effects on neuronal survival may not be via maintenance of the permeability transition pore or, in fact, by any mechanism involving apoptosis.

CHX treatment did not significantly reduce the number of apoptotic nuclei detected 7 days after HCA. This could be because new protein synthesis is not required for apoptosis in the pig model. Although this appears the most likely explanation, it could also be argued that a single bolus injection of CHX after HCA was not sufficient to effectively inhibit new protein synthesis throughout the vulnerable period immediately after HCA and for 7 days thereafter. This seems unlikely since pilot experiments (data not shown) demonstrated that CHX given preoperatively was incompatible with survival of pigs after HCA, and overall protein synthesis was definitely affected by even a single dose of CHX. Taken together, these observations suggest that the role of protein synthesis in apoptosis following HCA in this model is probably better explored using a more specific, and therefore less toxic, inhibitor of protein synthesis.

Since an increase in apoptosis was observed after HCA in untreated control pigs, we do not think that either administration of steroids or exclusive use of female pigs invalidates the basic findings regarding the lack of impact of either of these drugs, although these features of our experimental model may have affected the average numbers of apoptotic nuclei/mm³ seen after HCA. Screening for neuronal apop-
tosis sooner after HCA, however, might allow more definitive determination not only of whether protein synthesis is involved in apoptosis after HCA, but also whether the improved behavioral recovery seen with CsA treatment is a result of an impact on apoptosis or occurs by some other mechanism.

5. Conclusions

This study provides unequivocal evidence that pig hippocampal neurons die via apoptosis following 90 min HCA at 20°C, and that this process extends for at least 7 days. Although immunocytochemical examination 7 days after HCA failed to implicate mitochondrial pathways in this process, or to demonstrate a significant inhibition of apoptosis by CsA or CHX, further studies are required. Earlier histopathological and immunocytochemical scrutiny will be required to better define the pathway of neuronal cell death following HCA, and to determine whether CsA (or CHX) can be effective in improving neuronal survival following HCA.

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