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Immediate-early Gene Expression in Ovine Brain after Cardiopulmonary Bypass and Hypothermic Circulatory Arrest

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Background: **Cardiopulmonary bypass (CPB) and hypothermic circulatory arrest (HCA) are associated with neurological injury. Altered immediate-early gene expression occurs rapidly in the brain in response to ischemia, hypoxia, and severe metabolic stress, which results in long-term changes in the molecular phenotype of neurons. This study determined the effects of CPB and HCA on the expression of the immediate-early gene *c-fos*.**

Methods: **Neonatal lambs were subjected to 2 h of CPB at 38°C (n = 4) or 60 min (n = 6), 90 min (n = 7), and 120 min (n = 6) of HCA at 15°C. One hour after terminating CPB at 38°C, the brains were analyzed for FOS-encoding mRNA and FOS-like immunoreactivity in the hippocampal formation. Other animals (n = 15), subjected to the same CPB and HCA protocol, were allowed to survive 3–5 days before their brains were examined for dead neurons.**

Results: **Minimal *c-fos* mRNA and FOS proteins were observed in neurons of animals subjected to normothermic bypass and of those that served as controls. Non-neuronal FOS proteins were observed in the choroid plexus, ependyma, and blood vessels at all times, including normothermic CPB, but not in the control animals without CPB. The magnitude of *c-fos* mRNA expression in hippocampal neurons increased directly with the duration of HCA. In contrast, expression of FOS proteins peaked after 90 min of HCA and declined significantly thereafter. Dead neurons were seen in surviving animals after 2 h of HCA only.**

Conclusions: **Cardiopulmonary bypass and HCA alter immediate-early gene expression in the brain. Translational processes are impaired after 120 min of HCA and correlate with neuron death in the hippocampus. (Key words: Animals: lambs. Brain: excitatory neurotransmitters; c-fos; immediate-early gene expression; ischemia. Experimental techniques: immunohistochemistry; *in situ* hybridization. Surgery: cardiopulmonary bypass; hypothermic circulatory arrest.)**

THE molecular mechanisms of CNS complications accompanying cardiopulmonary bypass (CPB) and hypothermic circulatory arrest (HCA) are poorly understood. However, recent animal experiments provide evidence that excitotoxic events mediated by glutamate receptors may precipitate the neurological or neuropsychological deficits that can occur after cardiac surgery.[1,2]

Glutamate and aspartate are the major excitatory amino acids in the brain and are responsible for many normal neurological processes, including consciousness, cognition, memory, and synaptogenesis. However under adverse physiologic conditions such as ischemia, hypoxia, or both, excessive activation of glutamate receptors can mediate neuronal injury or death. Olney applied the term *excitotoxicity* to this condition, which describes a common final pathway for neuronal injury and death from various stressful and pathologic conditions.[3]

Excessive extracellular glutamate triggers delayed neuronal death by promoting the influx of calcium into cells by activating N-methyl-D-aspartate (NMDA) or non-NMDA glutamate receptors.[4] Glutamate receptor activation, in turn, stimulates expression of rapidly induced transcriptional activators known as the immediate-early genes. These genes initiate a complex cascade of events that transduct extracellular signals into alterations of

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cellular functions by regulating target gene expression (late-response genes).[5-7] The immediate-early gene *c-fos* is rapidly and transiently induced in neurons within the hippocampal formation of the brain after seizures, hypoxia, and global ischemia through glutamate-mediated NMDA and non-NMDA receptor activation.[8-10] The protein product of *c-fos* mRNA, FOS, modulates transcription of several late-response genes, such as p53, heat-shock protein, bcl-x, tyrosine hydroxylase, and opioid peptides.[10-12] Some of the late-response genes expressed after *c-fos* induction are associated with apoptosis, whereas others enhance cell survival.[7] Although it is not known whether *c-fos* expression is involved with cell survival or cell death, the appearance of nuclear-associated FOS protein is a useful indicator of severely stressed neurons and provides a potential method for assaying pharmacologic interventions. In rodents, for example, FOS expression by CA1 neurons of the hippocampus after global ischemia is associated with the subsequent degeneration of these neurons. Both FOS expression and neuronal death are prevented with competitive and noncompetitive NMDA receptor antagonists such as dextromethorphan and MK-801.[13,14]

The effects of CPB and HCA on immediate-early gene expression in the brain have not been reported. The objectives of this study were (1) to establish a model that defines the relation between CPB and increasing duration of HCA on the expression of FOS-encoding mRNA and FOS protein in the hippocampal formation, and (2) to determine if there is a relation between FOS expression and delayed neuronal death in these neurons. The hippocampus, the brain region associated with memory, learning, and cognition, was selected because it is particularly vulnerable to injury from CPB and HCA.[15]

Materials and Methods

Animal Preparation

Neonatal lambs (weighing 4–6 kg) obtained from Parsons Farms (Springfield, MA) were used in this study. All experimental protocols were approved by the Tufts University School of Medicine Animal Research Committee and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1985). Anesthesia was induced with isoflurane by mask; the animals were tracheally intubated and their lungs were mechanically ventilated with isoflurane, 1–2.5%, in 100% oxygen. Intravenous vecuronium was given for muscle relaxation. Only Ringer's lactate or balanced salt solutions were administered intravenously. Solutions containing glucose were avoided because of reports that hyperglycemia may worsen the deleterious effect of ischemia in neurons.[16]

Animal body temperature was measured with tympanic, nasopharyngeal, and rectal probes. The CPB circuit included a Minimax (Medtronics, Anaheim, CA) pediatric membrane oxygenator, Medtronic pediatric arterial filter, CDI in-line arterial/venous blood gas monitor (3M Healthcare, Ann Arbor, MI), and a Stockert/Shiley roller pump. The pump prime consisted of 100 mg methylprednisolone, 25 mEq sodium bicarbonate, fresh whole sheep blood, 0.5 g/kg mannitol, 0.25 mg/kg furosemide, 2,000 units of heparin, and 300 mg $CaCl_2$. This prime is similar to that used clinically for infants having open-heart surgery with HCA at our institution. A venous cannula (16–18 Fr) was placed in the right atrium through a right thoracotomy incision, and an arterial cannula (10 Fr) was placed in the the right femoral artery and advanced into the descending aorta for CPB.

Animals were divided into four groups. Group 1 (n = 4) had normothermic CPB (38°C) for 2 h; group 2 (n = 6) had CPB with 60 min of HCA; group 3 (n = 7) had CPB with 90 min of HCA; and group 4 (n = 6) had CPB with 120 min of HCA. In all experiments, pump flow rates were maintained at 100–150 $ml \cdot kg^{-1} \cdot min^{-1}$ during CPB. Isoflurane (1–2%) was used during CPB. Mean arterial pressure was maintained between 30 and 50 mmHG during CPB with isoflurane and intravenous fluid administration. A control group (n = 5) received 1–2% isoflurane alone without CPB at normal body temperature for 3 h. Another control group of lambs (n = 3) was anesthetized with isoflurane and then given a 100-mg/kg intravenous bolus of pentylenetetrazol to induce seizures. This seizure model induces expression of *c-fos* and FOS-related antigens in the hippocampus.[17] The experimental animals were cooled to tympanic, rectal, and bladder temperatures of 14°C with core cooling on CPB, cooling blanket, and ice packs around the head. Arterial blood gases were controlled by the alpha-stat strategy. At 14°C, the pump was turned off and venous blood drained by gravity from the animal. Hypothermic circulatory arrest was maintained for 60, 90, or 120 minutes at 14–16°C.

Cardiopulmonary bypass was reinstituted in groups 2

through 4, the animals were rewarmed to 38°C, and CPB was terminated. Additional KCl, Ca^{2+}, and $NaHCO_3$ were given during rewarming as indicated by arterial blood gas sampling. One hour after terminating CPB, a bolus of KCl was administered and the carotid arteries were perfused with 500 ml chilled, heparinized saline for 20 min followed by 1 l of 4% paraformaldehyde for 30 min. The brains were removed rapidly and postfixed in 4% paraformaldehyde for an additional 4 h, stored overnight in 20% sucrose with 0.1 M phosphate-buffered saline, *p*H 7.2, and then stored at −80°C.

Additional lambs (n = 15) subjected to the same protocol just described were allowed to survive 3–6 days after CPB and HCA. The purpose of these experiments was to determine if immediate-early gene expression was associated with subsequent delayed neuronal degeneration. Animals were anesthetized with isoflurane using a mask, tracheally intubated, and their lungs mechanically ventilated with 2% isoflurane. The lambs were killed with intravenous KCl and their brains were perfusion-fixed with paraformaldehyde as previously described. The brains were removed and stored 2–3 days in paraformaldehyde before they were embedded in paraffin. The brains were cut into 5-μm sections and stained with hematoxylin and eosin. The number of dead neurons at ×100 magnification in the same regions of hippocampal formation were counted by a veterinary neuropathologist blinded to the experimental protocol.

In Situ *Hybridization Immunohistochemical Analysis*

Twelve-micron coronal sections were cut and thaw-mounted onto gelatin-coated slides. Plasmids containing either sense or antisense reading frames were transcribed using SP6 polymerase according to previously published procedures.[18] The sense (or control) probe contains the same base sequence as *c-fos* mRNA, whereas the antisense probe is the complementary RNA (cRNA) to *c-fos* mRNA. Thus analysis with the sense probe yields minimal background signal and the antisense probe is used to detect *c-fos* mRNA. Sections were hybridized at 55°C overnight, treated with RNAse to eliminate nonspecifically bound probe, and stringently washed at 55°C in 0.1× standard sodium citrate. Slides were apposed to x-ray film for 1 week and then dipped in NTB2 and exposed for 2–3 weeks. The hybridization signals obtained from analyses using sense *versus* antisense cRNA probes were compared to determine the specificity of the signal, with the cells and tissue exclusively labeled with the antisense probe representing specific hybridization. Sections were stained with thionine to locate the specific regions of hippocampal formation and then examined under dark-field illumination at ×20 magnification for quantification of FOS-encoding mRNA.

Immunohistochemical Analysis

Immunohistochemical analyses were performed as previously described.[13,19] The brains were cut into 12-μm coronal sections and pretreated with 0.1% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase activity (that is, blood cells). Slide-mounted sections were incubated with primary antibody for *c-fos* and fos-related proteins (provided by Dr. M. J. Iadarola, National Institute of Health; final dilution 1:2,000). This peptide sequence is common to three members of the FOS family, *fra-1*, *fra-b*, and *c-fos*. The sections were incubated with biotinylated goat anti-rabbit serum and processed by the avidin-biotin-peroxidase method (ABC; Vector, Burlingame, CA) using diaminobenzidine as the peroxidase substrate. In addition, this procedure was modified to include nickel intensification of the diaminobenzidine reaction. Mounted and coverslipped tissue sections were examined using a Zeiss standard microscope (Zeiss, Heidelberg, Germany) at ×20 magnification, with brown/black reaction product indicating the presence of immobilized antigen. To assess the specificity of immunohistochemical staining, representative tissue sections were processed with primary antiserum preabsorbed with 1 μM of the conserved M-peptide within the FOS protein sequence used to generate antibody and yielded essentially no cellular-specific reaction product. Furthermore, in every immunohistochemical analysis, sections were processed in parallel to avoid interassay differences in labeling intensity. Finally, immunohistochemically detected nuclear-associated reaction product is called "FOS-like immunoreactivity."

Image Analysis

Immunohistochemical data were analyzed by a blinded observer using a MS-DOS 386-based image processing system,[20] which detects and quantitates the number of FOS-like immunoreactive nuclei per anatomic field. Neurons in the hippocampal formation were easily distinguished from glia, ependyma, and choroid cells, which also contained FOS-like immunoreactivity, by their characteristic pyramidal shape, size, and distribution. For *in situ* hybridization immunohisto-

chemical analyses, bright pixels associated with labeled neuronal somata were isolated by removing background from each image using preestablished noise threshold values and an adaptive discriminator. Objects in each filtered image were extracted by locating sets of contiguous active pixels, whereas marginally labeled cells and small artifacts are discarded. For the remaining objects, intensity levels are converted to either grains or levels of radioactivity (fCi) per pixel, and the sum of these values was used to calculate the concentrated hybridized probe contained within each object, expressed as $fCi/\mu m^2$.

Data Analysis

The amount of hybridized probe and the number of FOS-like immunoreactive nuclei within discrete areas of the hippocampal formation are expressed as mean ± SEM. In addition, the number of dead neurons within each region of the hippocampal formation are expressed as mean ± SEM. *In situ* hybridization and immunohistochemical data were evaluated for the treatment groups using the Kruskal-Wallis one-way analysis of variance of the ranks. Durations of HCA among study groups were compared using Dunn's method (InStat program). A probability value less than 0.05 was considered statistically significant.

Results

All animals survived the CPB and HCA periods. The mean cooling times during CPB were similar (24 ± 6 min) for groups 2 through 4. There were no significant differences in tympanic, nasopharyngeal, or rectal temperatures among groups throughout the cooling, arrest, and rewarming phases. There were no differences in mean arterial pressure in all groups during CPB and post-CPB periods. No vasopressors were given after CPB.

In Situ *Hybridization Histochemical Analyses*

Very high concentrations of *c-fos* mRNA were observed in the hippocampal formation (particularly the ventral hippocampus) in the seizure-induced control animals that received pentylenetetrazol. Low concentrations of *c-fos* mRNA were observed in the hippocampal formation of the control animals exposed to general anesthesia only and in those that underwent CPB at 38°C (fig. 1). Cardiopulmonary bypass with HCA resulted in substantial induction of *c-fos* mRNA in the hippocampal formation. The magnitude of *c-fos* gene expression increased linearly with the duration of HCA, with maximum expression after 120 min of HCA (figs. 1 and 2B). Induction of *c-fos* mRNA was not observed in the choroid plexus, ependyma, and blood vessels in any of the experimental or control groups of animals.

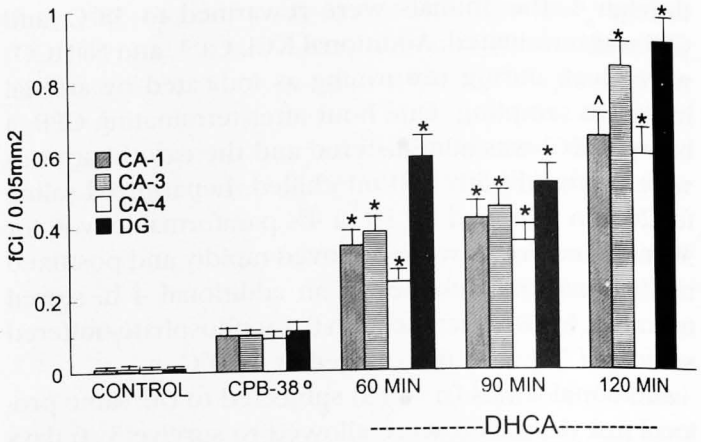

Fig. 1. *c-fos* mRNA expression in CA1, 3, 4, and dentate gyrus regions of hippocampal formation. The transcription of *c-fos* mRNA was significantly different among groups of animals that had HCA and both the controls and the animals that had cardiopulmonary bypass at normothermic (*$P < 0.001$). The transcription of *c-fos* mRNA in the CA-1 region after 120 min of hypothermic circulatory arrest (n = 6) was significantly greater than the amount after 60 (n = 5) or 90 min (n = 7) of hypothermic circulatory arrest (∧$P < 0.01$).

Immunohistochemical Analyses

The pentylenetetrazole-treated lambs in the control group had intense intranuclear FOS-like immunoreactivity in neurons in the hippocampal formation, particularly in the dentate gyrus cells of the ventral hippocampus. The FOS response to pentylenetetrazol observed in lambs is similar to that reported in rodents.[17] Intranuclear FOS-like immunoreactivity was not observed in brain sections from control animals, which had general anesthesia alone. Similarly, negligible FOS-like immunoreactivity was observed in the nuclei of the hippocampal neurons in the animals that had normothermic CPB without circulatory arrest and in those animals that underwent 60 min of HCA (fig. 3). In contrast, intense FOS-like immunoreactivity was detected in neurons and glia of the hippocampal formation in animals subjected to 90 min of HCA (see figs. 2C and 3). Interestingly, the appearance of FOS-like immunoreactivity was equally

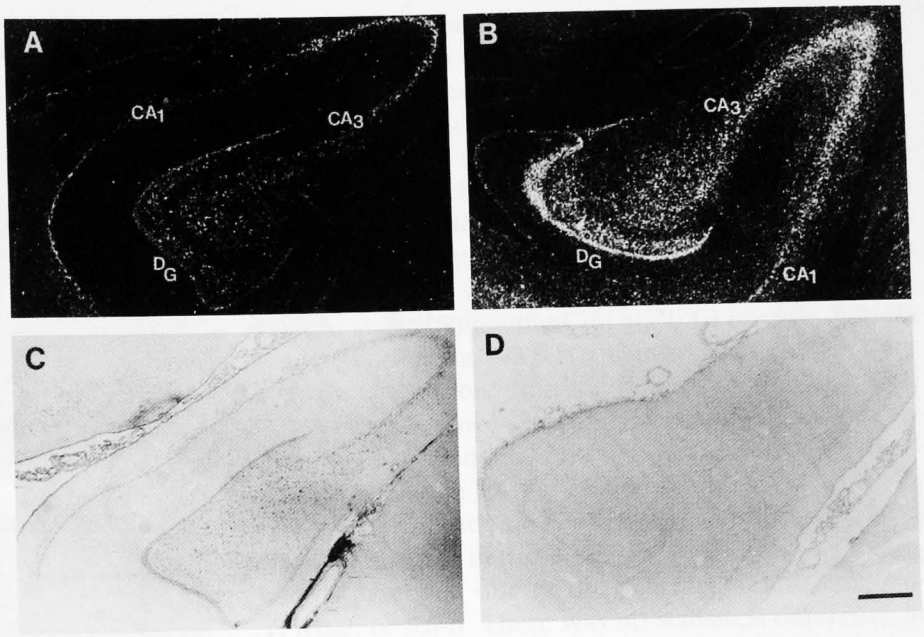

Fig. 2. Photomicrographs of histologic sections of hippocampal formation. Dark-field view of transcribed *c-fos* mRNA after 90 min (*A*) and 120 min (*B*) of hypothermic circulatory arrest. Corresponding sections showing translation of FOS-related proteins after 90 min (*C*) and 120 min (*D*) of hypothermic circulatory arrest. Although *c-fos* mRNA expression was greatest after 120 min of hypothermic circulatory arrest (*B*), there was not a corresponding increase in protein production (*D*). Bar equals 500 μm.

intense in all regions of the hippocampal formation examined: CA1, CA3, CA4, and dentate gyrus.

FOS-like immunoreactivity was observed in non-neuronal cells; that is, glia, ependyma, choroid plexus, and blood vessels in the brain at all points except in the control lambs, which did not have CPB (fig. 4). However, because these structures did not express *c-fos* mRNA, this indicates that one or more of the other genes of the FOS family (*fra-1*, *fra-2*, or *fos-b*) were expressed. There was no statistical difference in the number of non-neuronal cells with intranuclear FOS-like immunoreactivity after normothermic CPB compared with those after 60, 90, or 120 minutes of HCA (fig. 5).

In the surviving animals, neuronal necrosis was only observed in CA1 and CA3 cells of animals subjected to 2 h of HCA (table 1). Dead neurons were identified by their characteristic shrunken nuclei and bright red cytoplasm (fig. 6).

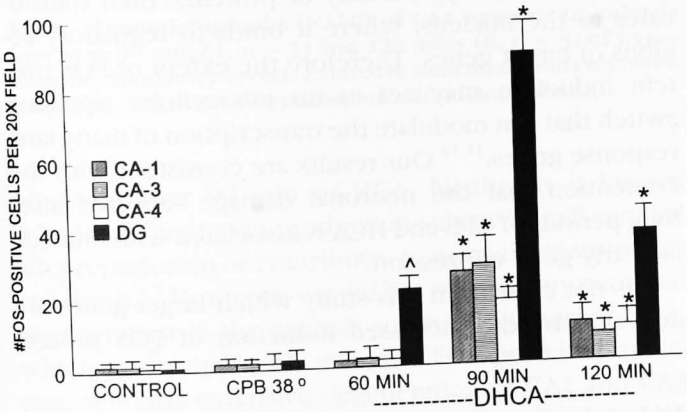

Fig. 3. FOS-related protein synthesis in the hippocampal formation. The translation of FOS-related proteins was significantly greater after 90 min of hypothermic circulatory arrest (HCA) (n = 7; $^*P < 0.001$ for 90 min *vs.* control, normothermic cardiopulmonary bypass, 60 and 120 min of HCA). Translation of FOS-related protein was significantly greater after 60 min of HCA in the dentate gyrus and all regions of the hippocampus after 120 min of HCA compared with controls and animals that had normothermic cardiopulmonary bypass at ($\wedge P < 0.01$).

Discussion

These experiments demonstrated a linear relation between *c-fos* mRNA expression in hippocampal neurons and the duration of HCA. Expression of FOS-related proteins was greatest after 90 min of HCA and decreased significantly thereafter. The intensity and distribution of *c-fos* mRNA and FOS-related proteins observed in these experiments are similar to that reported in other animal models of ischemia and hypoxia, seizures, and head trauma.[8-14] Neuronal death in CA1 and CA3 was observed only after 2 h of HCA, which correlated with a decrease in FOS-related protein production.

Immediate-early genes such as *c-fos* are rapidly expressed throughout the CNS in response to stress, hyp-

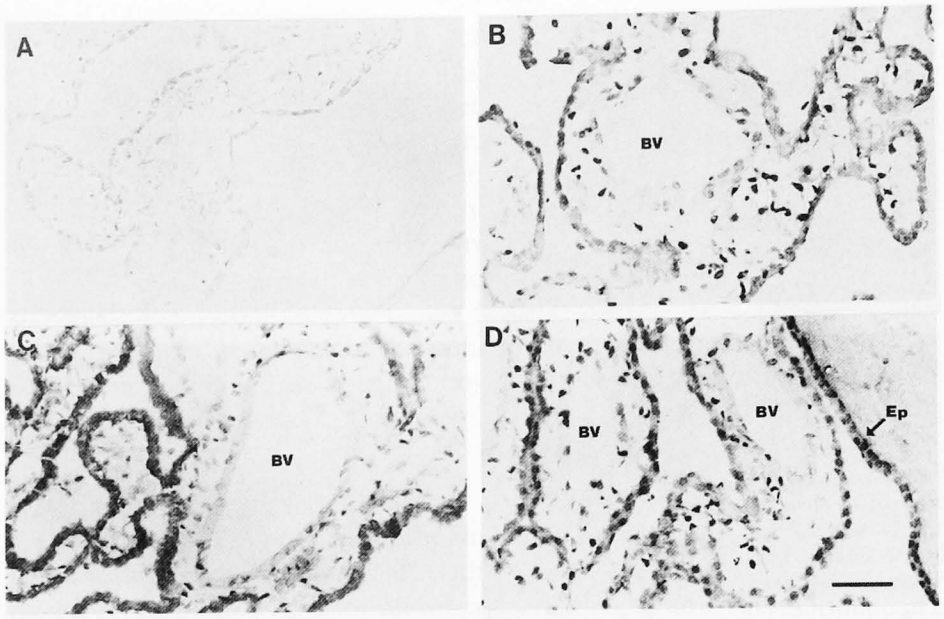

Fig. 4. FOS-related protein synthesis in the choroid plexus. The dark-stained nuclei indicate the presence of the antigens *fra-1*, *fra-2*, or *fos-b*. FOS-like immunoreactivity was not observed in the choroid plexus, ependyma (Ep), or blood vessels (BV) of control animals (*A*), whereas intense signal was seen after normothermic cardiopulmonary bypass (*B*) and after 90 min (*C*) and 120 min (*D*) of hypothermic circulatory arrest. Bar equals 50 μm.

oxia, and ischemia. Although other pathways have been described, the transcription of immediate-early genes is usually initiated by glutamate-mediated membrane depolarizations linked to intracellular second messengers such as calcium, calmodulin, and c-AMP-dependent processes.[3,7,10,21] We assumed in these experiments that the intense *c-fos* expression was due to glutamate-mediated mechanisms, even though extracellular glutamate was not measured. We make this assumption because other animal models of cerebral ischemia, trauma, and seizures have measured increased extracellular glutamate concentrations in association with FOS induction. Furthermore, the distribution of FOS we observed in the hippocampal formation mirrors that of the NMDA form of the glutamate receptor.[8] Once activated, the *c-fos* gene is transcribed in the nucleus to mRNA, which is then translated into protein in the cytoplasm. The protein product, FOS, after forming a heterodimer with a member of the *jun* family of proteins, then translocates to the nucleus, where it binds to regulatory regions of target genes. Therefore the extent of FOS protein induction may act as an intracellular signaling switch that can modulate the transcription of many late-response genes.[11,12] Our results are consistent with the contention that the neuronal damage reported after long periods of CPB and HCA is associated with immediate-early gene expression.

It is not clear from this study which target genes are activated by the increased induction of FOS protein

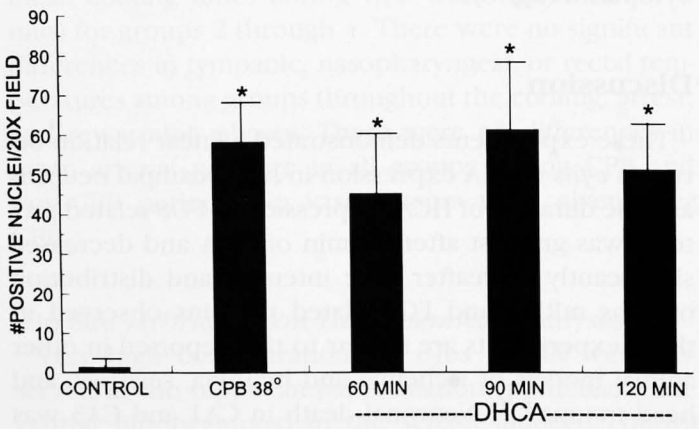

Fig. 5. FOS-related protein synthesis in the choroid plexus. Translation of FOS-related protein (*fra-1*, *fra-2*, *fos-B*) was significantly increased after both normothermic cardiopulmonary bypass and hypothermic circulatory arrest ($P < 0.001$, by analysis of variance).

Table 1. Number of Dead Neurons in the Hippocampal Formation 3–7 Days after 90 and 120 Min of HCA

	CA1	CA3	CA4	DG
90 min HCA	0	0	0	0
120 min HCA	7.8 ± 2.0*	11.3 ± 5.5*	4.5 ± 4.7*	0.33 ± 0.5*

* $P < 0.001$; n = 5; 100 × field.
DG = dentate gyrus.

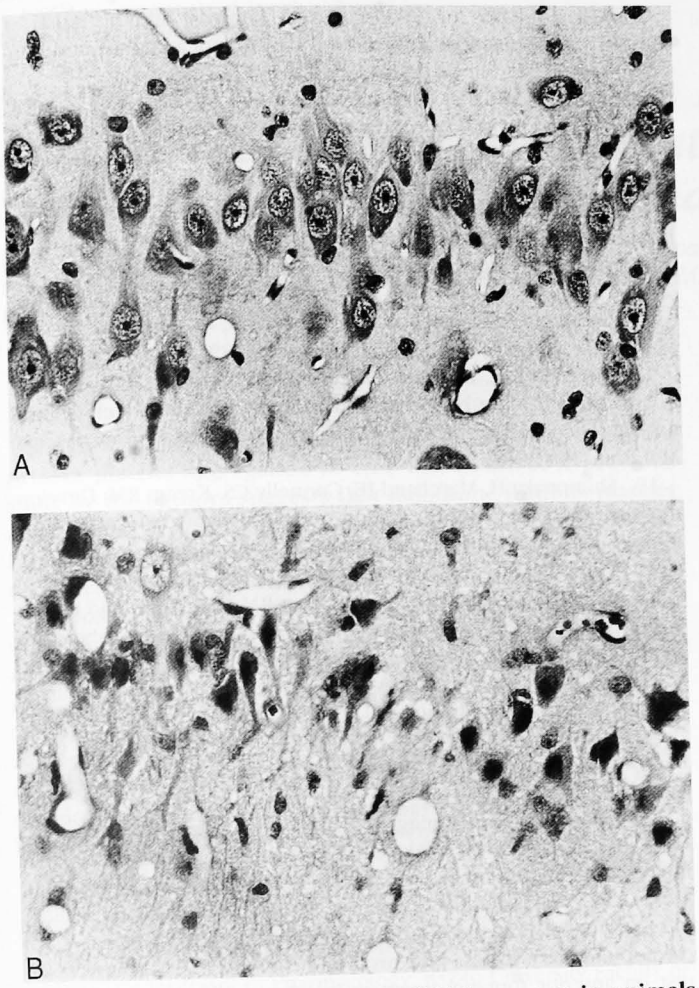

Fig. 6. Photomicrographs (×100) of CA1 neurons in animals surviving 90 min (*A*, n = 5) and 120 mins (*B*, n = 5) of hypothermic circulatory arrest. Extensive neuronal death was seen only after 120 min of hypothermic circulatory arrest.

observed after 90 min of HCA. Neither is it known whether FOS protein production subserves cell survival after reperfusion or contributes to necrotic or apoptotic cell death.[6,7] However, we did not observe any neuronal degeneration in the animals surviving 90 min of HCA, which correlated with maximal FOS protein production, whereas extensive degeneration of CA1 and CA3 neurons was seen after 2 h of HCA when FOS protein production was diminished. This would imply that FOS protein production is necessary for cells to survive. Other animal CPB models have demonstrated that 2 h of HCA is associated with profound neuronal death throughout the hippocampal formation,[1,22] which is consistent with our results.

Measurement of the steady-state level of a specific mRNA and its protein may provide only one index of the alteration of two dynamic processes: synthesis, which includes both transcription (with posttranscriptional modification) and translation, and degradation. Typically, *c-fos* transcription requires about 30 min for expression after ischemia and is visible in the cytoplasm for about 1 h. Intranuclear FOS protein is visible about 45 min after the stimulus and persists for 3 or 4 h. From this perspective, we selected the same time point (45–60 min after terminating CPB at 38°C) in every experiment to consistently detect, quantify, and compare both *c-fos* and FOS protein during reperfusion.

FOS-like immunoreactivity was observed in non-neuronal cells (ependyma, choroid plexus, and blood vessels) in the brain in all CPB and HCA experiments, including normothermic CPB (fig. 4). The absence of *c-fos* mRNA signal in these cells indicates that the antiserum is detecting one of the FOS-related antigens, *fra-1*, *fra-2*, or *fos-B*. There was no statistical difference in the number of cells producing these proteins at normothermic CPB or after HCA (fig. 5). The effect of these proteins in non-neuronal cells, which make CSF in the brain, is not clear, but their presence at all times involving CPB implies that they are responding to the altered cerebral circulation that occurs with CPB. We can speculate that increased CSF production (or decreased reabsorption) accompanies CPB, even though we did not measure CSF volumes in these experiments. Clinically, CPB and HCA cause cerebral edema in infants.[23,24]

Additional studies are needed to define precisely the temporal relations between *c-fos* induction, the occurrence of DNA damage, and the appearance of morphologic changes characteristic of neuronal death. Similarly, whether increased *c-fos* expression plays a direct functional role in excitotoxic-mediated cell death after HCA or mediates attempts at protective mechanisms within neurons remains to be determined. Our laboratory is using this model to study late-response gene expression and the effects of NMDA antagonists.

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