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

Paula M. Bokesch, M.D., Peter A. Seirafi, M.D.,† Kenneth G. Warner, M.D.,‡ James E. Marchand, Ph.D.,§ Richard M. Kream, Ph.D.,‖ Bruce Trapp, Ph.D.#*

Background: **This study determined the induction profiles of immediate-early genes in the ovine brain after cardiopulmonary bypass (CPB) and hypothermic circulatory arrest (HCA), and the effects of the noncompetitive N-methyl-D-aspartate antagonist, aptiganel, on immediate-early gene expression, neuronal necrosis, and functional outcome.**

Methods: **Cannulas were inserted into isoflurane-anesthetized neonatal lambs undergoing CPB. One group received 2.5 mg/kg intravenous aptiganel. Animals underwent 90 or 120 min of HCA at 16°C, were rewarmed to 38°C, and were weaned from CPB. One hour after CPB was discontinued, brain perfusion was fixed and removed for immunohistochemical analysis in one half of the animals. The other half survived 2 or 3 days before their brains were evaluated for neuronal degeneration. Data were analyzed using analysis of variance; $P < 0.05$ was considered significant.**

Results: **Cardiopulmonary bypass and HCA differentially induced c-Jun and Fos proteins in the hippocampal formation, with c-Jun expression increasing with the duration of HCA, whereas Fos protein expressions were greatest after 90 min of HCA. The c-Jun protein was expressed in all neurons except the dentate gyrus. The Fos proteins were expressed in all neurons, including the dentate gyrus. Neuronal necrosis was observed in CA1 (73%) and CA3 (29%) neurons but not in the dentate gyrus after 120 min of HCA. Aptiganel completely inhibited c-Jun expression ($P < 0.001$) but not Fos, improved functional outcome, and attenuated neuronal necrosis ($P < 0.05$).**

Conclusions: **The c-Jun and c-Fos proteins are expressed differentially in hippocampal neurons after CPB and HCA. Expression of c-Jun is associated with neuronal necrosis, whereas Fos protein expression is associated with survival. Aptiganel inhibits c-Jun expression, attenuates neuronal necrosis, and improves outcome. (Key words: Aptiganel; cardiopulmonary bypass; excitotoxicity; immediate-early genes; N-methyl-D-aspartate.)**

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* Staff, Department of Cardiothoracic Anesthesia, The Cleveland Clinic Foundation.

† Fellow.

‡ Assistant Professor, Cardiothoracic Surgery.

§ Assistant Professor.

‖ Professor, Anesthesia Research.

Chairman, Department of Neuroscience, The Cleveland Clinic Foundation.

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Address reprint requests to Dr. Bokesch: Staff, Department of Cardiothoracic Anesthesia, G30, The Cleveland Clinic Foundation, 9500 Avenue, Cleveland, Ohio 44195. Address electronic mail to: bokescp@cesmtp.ccf.org

CARDIOPULMONARY bypass (CPB) and hypothermic circulatory arrest (HCA) are used for brain, vascular, and cardiac surgery. Cardiopulmonary bypass and HCA both can cause significant central nervous system injury that manifests as stroke, seizures, diminished intelligence, developmental abnormalities, choreoathetosis, and neuropsychologic disorders.[1,2] The causes of brain injury after CPB–HCA are multifactorial, resulting from intraoperative microemboli and macroemboli, cerebral hypoperfusion, reperfusion injury, inflammatory and neurohumoral responses, and premorbid disease and postoperative events.[2–4]

Some of the signaling pathways of neuronal injury from CPB and HCA have been identified. The transcription and translation of the immediate-early gene c-*fos* that are rapidly induced in the central nervous system by hypoxia, ischemia, seizures, and trauma[5,6] are also induced by CPB and HCA.[7] The protein products of c-*fos* (c-Fos) and another proto-oncogene, c-*jun* (c-Jun), form heterodimers and homodimers that function as transcrip-

tional regulators at the AP-1 binding site on DNA, inducing the transcription of effector genes for cell survival or death.[8] The relative levels of Fos–Jun homodimers and heterodimers may have positive and negative transcriptional functions.[9]

In the following experiments, animals were studied using a protocol identical to that used previously[10] to determine the relation between the expression of both c-Fos and c-Jun within specific regions of the hippocampal formation after CPB and HCA. The effects of the new noncompetitive N-methyl-D-aspartate antagonist, aptiganel (CERESTAT; Cambridge NeuroScience, Cambridge, MA), on immediate-early gene expression and neuronal survival also were investigated. The N-methyl-D-aspartate receptor antagonists have been shown to inhibit glutamate-mediated excitotoxicity and neuronal injury after ischemia.[10,11] We hypothesized that the differential expression of these immediate-early genes initiates a cascade of events that subsequently leads to neuronal destruction in some cell groups, whereas it may contribute to the survival of other neurons.

Methods

The study protocol was approved by the Animal Research Committee of The Cleveland Clinic Foundation. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 85-23, revised 1985).

Neonatal lambs (average age, 10 days; average weight, 5.9 kg) were anesthetized by mask with isoflurane. After tracheal intubation, the lungs were ventilated mechanically with 100% oxygen and 2% isoflurane. Mean arterial pressure was maintained at 40–50 mmHg throughout the experiment with isoflurane. Vecuronium, at an intravenous dose of 0.1 mg/kg, was administered once for muscle relaxation before incision. Temperature was monitored using temporalis muscle, esophageal, and rectal thermistors and maintained at 38°C at all sites before and after CPB using a heating blanket. Cardiopulmonary bypass was achieved by direct cannulation of the right atrium and the ascending aorta through a right thoracotomy incision. The CPB circuit included a Minimax pediatric membrane oxygenator (Medtronics, Anaheim, CA), a Medtronics pediatric arterial line filter, and a Stockert (Munich, Germany) roller pump. Isoflurane (1 to 2%) was administered while the animals were undergoing CPB. Mean arterial blood pressure was maintained at 40–50 mmHg while undergoing CPB, with perfusion at 100–150 $ml \cdot kg^{-1} \cdot min^{-1}$. The pump prime consisted of 500 ml fresh whole sheep blood, 100 mg hydrocortisone sodium succinate (SoluCortef; Upjohn, Kalamazoo, MI), 25 mEq sodium bicarbonate, 1,500 units heparin, and 300 mg CaCl.

Heparin (300 units/kg) was administered intravenously before the animals were cannulated for CPB. One group of animals (n = 20) received 1.25 mg/kg aptiganel intravenously, 5–10 min before the right atrium was cannulated. A second group of animals (n = 25) received saline vehicle. Twin lambs were used for these experiments; one received aptiganel and the other received the saline vehicle. After CPB was initiated, animal temperatures were cooled to 14–16°C (temporalis muscle, esophageal, and rectal) by surface and core cooling. All animal heads were packed in ice. The CPB pump was turned off, and HCA was maintained for 90 min in 22 animals (14 vehicle treated and 8 aptiganel treated) and for 120 min in 23 animals (11 vehicle treated and 12 aptiganel treated). Animals were rewarmed during CPB to 38°C and weaned from the bypass. Electrolytes (K^+ and Ca^{++}) were corrected as indicated by the arterial blood gases. No inotropes were administered. A subsequent dose of 1.25 mg/kg aptiganel was administered intravenously during rewarming in the aptiganel-treated animals. One hour after discontinuation of CPB, while the animals were still anesthetized with isoflurane, 23 animals were killed with a bolus dose of potassium chloride. The brains were perfusion-fixed with 1,000 ml chilled, heparinized saline, followed by 1,000 ml chilled paraformaldehyde, 4%, and removed for immunohistochemical analyses.

The remaining animals were weaned from mechanical ventilation, extubated, and allowed to recover for 48–72 h. The surviving animals (n = 22) underwent neurologic examination every hour after extubation until they were returned to their mothers and then every 8 h thereafter. Time to achieve major milestones such as standing, bleating, and nursing were recorded. Points for these achievements were assigned as indicated by the ovine behavioral scale (table 1) developed by Crittenden *et al.*[12] After 3 days, the animals were anesthetized again with isoflurane before being killed, and then their brains were removed. The brains were embedded in paraffin, after which 5-μm-thick coronal sections were cut and stained with hematoxylin and eosin. Using the light microscope, a blinded observer counted the number of dead neurons, identified by the presence of bright red

Table 1. Ovine Behavioral Scale: Outcome Scores 24 h after 2 h of HCA

Test	Potential	Aptiganel		Vehicle	
		Median	Range	Median	Range
Eye Opening					
Spontaneous	2				
With stimulus	1	2	0	1	2
Absent	0				
Best motor					
Walks	4				
Stands	3				
Pushes up	2	3	1	2	2
Nonpurposeful	1				
Absent	0				
Speech					
Vocalizes	1	1	0	1	1
Nonvocal	0				
Swallowing					
Nurses	2				
Fed orogastric tube	1	1	1	1	1
Absent swallow	0				
Reaction to pain					
Withdraws	1	1	0	1	1
No response	0				
Total	10	8	2	6	7

Aptiganel-treated animals were significantly better ($P < 0.05$) than vehicle-treated animals.

cytoplasm and condensed nuclei, in the hippocampal formation.

Brains from the acute experiments were cut into 12-μm-thick coronal sections for immunohistochemical analyses, as previously described.[7,10] Briefly, the coronal sections were pretreated with hydrogen peroxide, 0.1%, in methanol for 20 min. Adjacent, slide-mounted sections were incubated with primary antibody for c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) or Fos-related proteins (donated by Dr. Iadarola, National Institutes 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 antirabbit serum and processed by the avidin-biotin-peroxidase method (Vector, Burlingame, CA), using diaminobenzidine as the peroxidase substrate. This procedure was modified to include nickel intensification of the diaminobenzidine reaction. Mounted and coverslipped tissue sections were evaluated using a Zeiss microscope (Oberkochen, Switzerland) at ×20 magnification. The finding of intranuclear brown-black reaction product indicated the presence of immobilized antigen. A blinded observer located and quantified neurons with intranuclear c-Jun-like and Fos-like immunoreactivity within the specific regions of the hippocampal formation using a computer-assisted image processing system.[13] Brain tissue was included from a control group (n = 6) that received general anesthesia with 2% isoflurane for 3 h but that did not undergo CPB.

Neurologic Injury Evaluation

All animals in the survivor groups were assessed neurologically using a modified ovine behavioral scale (table 1).[12] Neurologic deficit scoring consisted of five major components, including level of consciousness, motor and sensory function, feeding behavior, and vocalization. A score of 10 signified normal function and 0 signified brain death. Final neurologic deficits were agreed on by at least two members of the surgical team unaware of the lamb's treatment.

All values are expressed as the mean ± the standard error of the mean. Immunohistochemical data were evaluated for the treatment groups using Kruskal–Wallis one-way analysis of variance on the ranks. Study groups were compared using Dunn's method (SigmaPlot, Jandel Scientific, San Francisco, CA). Behavioral outcomes were analyzed using the median test (JMP Statistics; SAS Institute, Cary, North Carolina). A P value < 0.05 was considered significant.

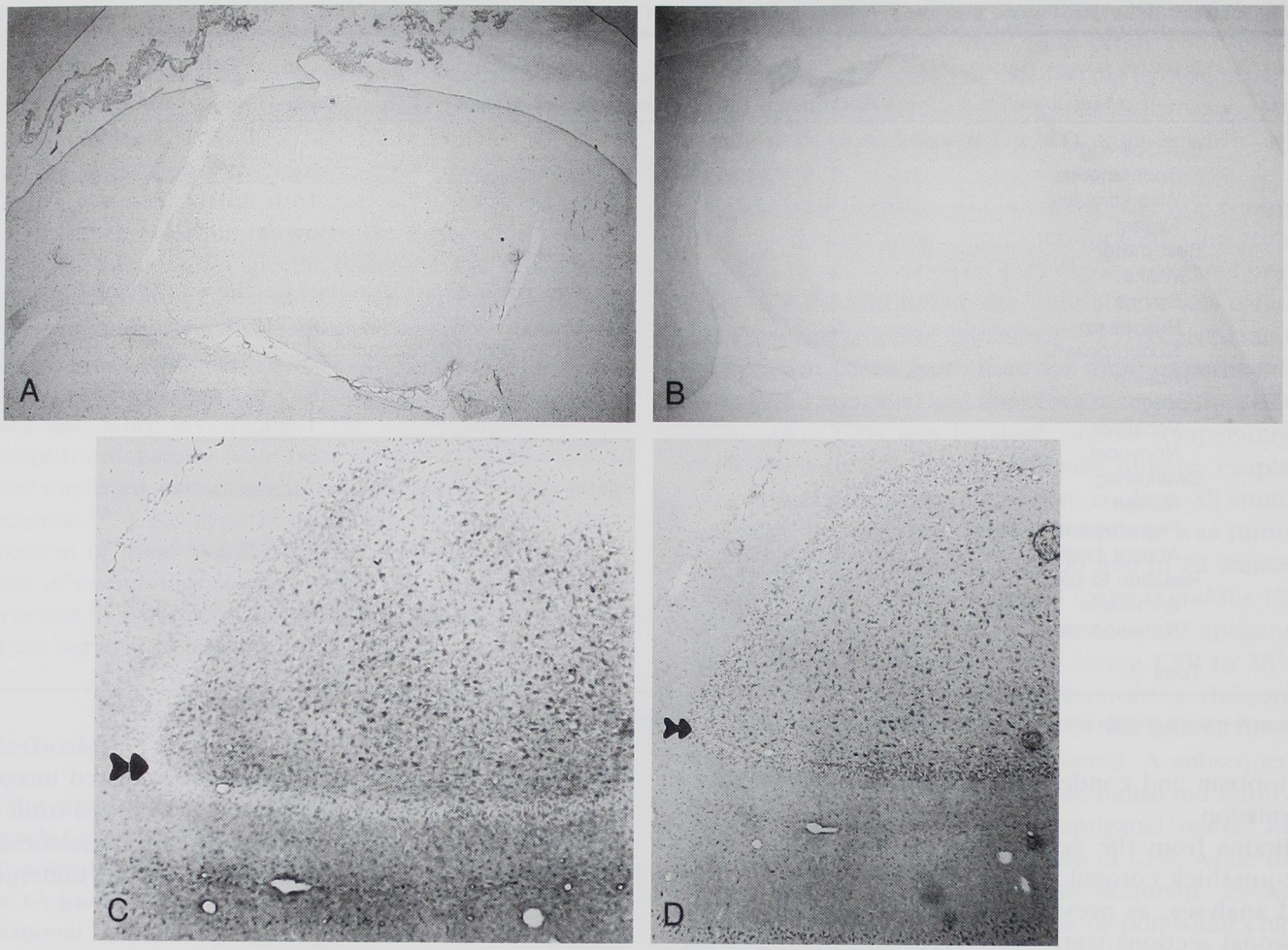

Fig. 1. Photomicrographs (×20) of adjacent 12-μm-thick coronal sections of lamb brain at the level of the dorsal hippocampus. (*A*) Absence of c-Jun protein in control animals after 3 h of general anesthesia. (*B*) Absence of Fos protein expression in control animals after 3 h of general anesthesia. (*C*) Intranuclear c-Jun protein in the hippocampal formation after 120 min of hyperthermic circulatory arrest (HCA). Note the absence of c-Jun protein in the dentate gyrus (DG) granule cells. (*D*) Intranuclear Fos proteins in the hippocampal formation after 120 min of HCA. Note the presence of intranuclear Fos proteins in the DG. Arrows indicate the DG.

Results

The control animals (no CPB) that were administered only isoflurane general anesthesia for 3 h did not have any c-Jun–like or Fos-like immunoreactivity in any neurons of the hippocampal formation or cortex (fig. 1A, B). Cardiopulmonary bypass with increasing duration of HCA in vehicle-treated animals induced a corresponding increase in the expression of c-Jun, with maximal expression after 120 min of HCA (n = 4; fig. 2). All regions within the hippocampal formation, except the dentate gyrus (DG), expressed c-Jun (fig. 1C, fig. 2). Fos-like immunoreactivity was expressed in all regions of the hippocampal formation, including the DG (fig. 1D), and expression was greatest after 90 min of HCA (n = 7; $P <$ 0.001 *vs.* control) in vehicle-treated animals and appeared to decrease in CA1, CA3, and CA4 after 120 min (n = 7; fig. 2).

Aptiganel significantly inhibited Fos immunoreactivity after 90 min of HCA in all regions of the hippocampal formation and the cortex evaluated compared with vehicle-treated animals (n = 5; $P < 0.001$ *vs.* vehicle-treated animals; figs. 2, 3). Aptiganel appeared to increase Fos expression throughout the hippocampal formation after 120 min of HCA compared with vehicle-treated animals after 120 min of HCA (figs. 2, 3). Aptiganel completely inhibited c-Jun expression after both 90 and 120 min of HCA (fig. 3).

In the survival model, the outcomes in these animals were similar to those seen in a separate group of lambs studied using the same protocol and previously report-

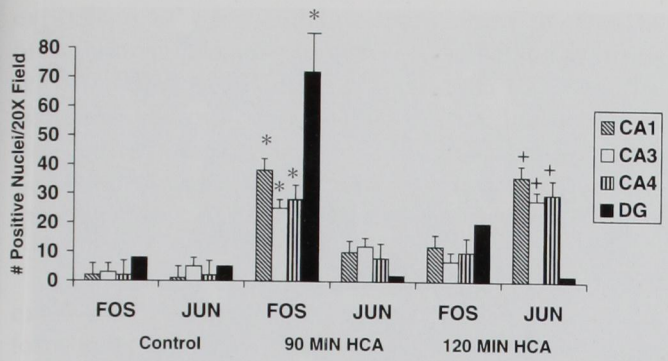

Fig. 2. Immediate-early gene expression after cardiopulmonary bypass and hypothermic circulatory arrest (HCA). Fos protein expression was significantly increased (n = 7; *$P < 0.001$ for Fos expression after 90 min of HCA *vs.* control) throughout the hippocampal formation after 90 min of HCA. In contrast, c-Jun expression was significantly greater (n = 4; †$P < 0.01$ for c-Jun expression after 120 min HCA *vs.* control) in CA1 and CA3 neurons after 120 min of HCA. The c-Jun was never expressed in the dentate gyrus during early reperfusion.

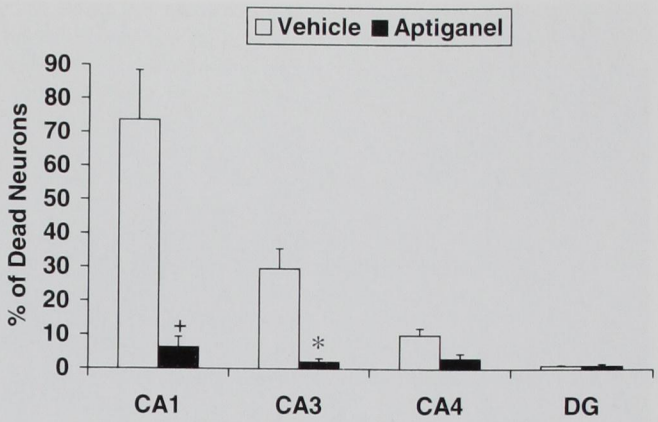

Fig. 4. The percentage of dead neurons in the hippocampal formation 72 h after 120 min of hypothermic circulatory arrest. Pretreatment with aptiganel (n = 5) significantly attenuated neuronal necrosis in CA1 and CA3 (*$P < 0.05$ for aptiganel *vs.* vehicle). Neuronal necrosis was not observed in the dentate gyrus of either vehicle- or aptiganel-treated animals.

ed.[10] None of the animals exposed to 90-min HCA had neurologic impairment. After 120 min of HCA, however, all of the vehicle-treated animals (n = 5) had severe neurologic impairment on the first postoperative day, and two died 24 h after surgery. Aptiganel-treated animals (n = 5) had significantly better functional recovery in the early postoperative period ($P < 0.05$; table 1). All aptiganel-treated animals were ambulating and nursing on the first postoperative day, whereas only one of five vehicle-treated animals could stand after 24 h. Surviving vehicle-treated animals were much slower to recover. Although they had lower scores on the ovine behavioral scale than did the aptiganel-treated animals on the second and third postoperative days, the differences between the treatment groups were not significant (data not shown).

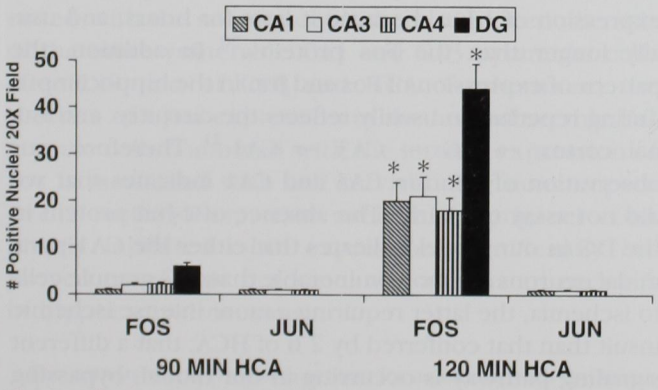

Fig. 3. Effects of aptiganel on immediate-early gene expression after cardiopulmonary bypass and hypothermic circulatory arrest (HCA). Aptiganel decreased c-Jun expression in CA1, CA3, and CA4 neurons after both 90 and 120 min of HCA. Fos protein expression was inhibited by aptiganel after 90 min of HCA (n = 5) but not after 120 min of HCA. *$P < 0.001$ for Fos expression after 90 min of HCA in aptiganel-treated animals compared with vehicle. DG = dentate gyrus.

No neuronal necrosis was seen after 90 min of HCA in either the vehicle-treated or the aptiganel-treated animals. Significant neuronal necrosis was observed in the cortex and the CA1 and CA3 neurons of the vehicle-treated animals (n = 5) after 120 min of HCA, but not in the aptiganel-treated group (n = 5; $P < 0.001$; figs. 4, 5). Necrosis was never observed in the DG neurons in either group.

Discussion

In these experiments we found a previously unreported association between the expression of c-Jun and Fos-related antigens and neuronal necrosis. With increasing duration of HCA, there was a corresponding increase in the expression of c-Jun in CA1 and CA3 neurons, whereas DG neurons did not express c-Jun during early reperfusion (figs. 1C, 2). In contrast, Fos-like immunoreactivity was greatest after 90 min in all neurons of the hippocampal formation, including the DG (fig. 1D), and appeared to decrease after 120 min of HCA, particularly in CA1 and CA3 (fig. 2). Significant neuronal necrosis was observed 3 days after 120 min of HCA in CA1 and CA3 neurons, 73% and 29% respectively, but not in the dentate gyrus (fig. 4). In rodent models of global ischemia, Fos protein immunoreactivity is reported to decrease in the more vulnerable CA1 neurons that subse-

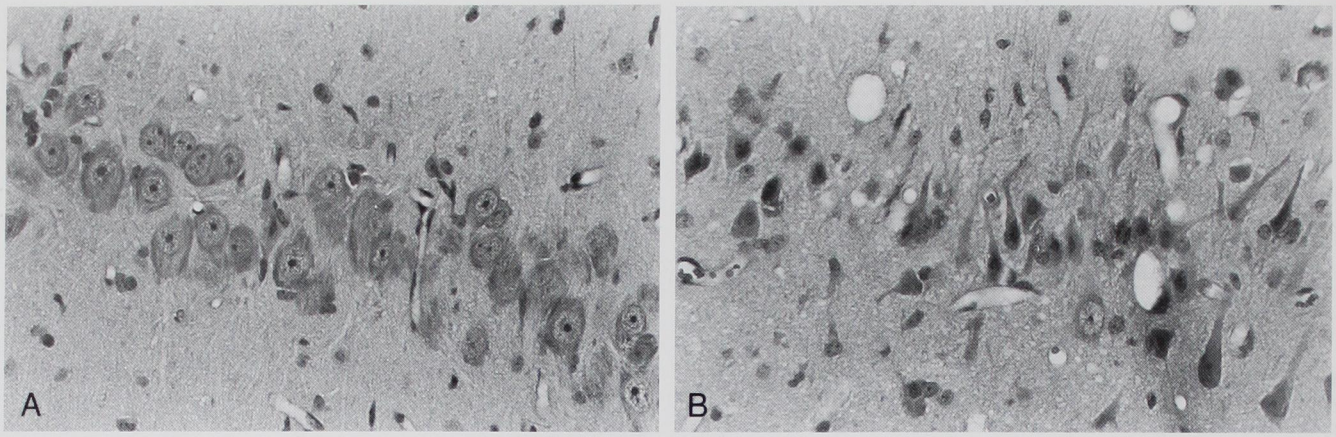

Fig. 5. Photomicrographs (×100) of the CA1 area of the hippocampus 72 h after 120 min of hyperthermic circulatory arrest. (*A*) An aptiganel-treated animal with normal-appearing CA1 neurons. (*B*) A vehicle-treated animal with extensive neuronal degeneration. Hematoxylin and eosin stain, 5-μm-thick coronal sections.

quently die, whereas DG neurons intensely express Fos and survive.[9,14] Neuronal necrosis in the hippocampus (but not the DG) after 2 h of HCA has been reported in other animal models.[11,12]

The results of these experiments support the hypothesis that the expression of c-Jun in excess of Fos is associated with the subsequent necrosis of CA1 (fig. 5) and CA3 neurons (figs. 2, 4). This hypothesis is further supported by the experiments in which animals were treated with aptiganel, a noncompetitive N-methyl-D-aspartate antagonist. Aptiganel, similar to MK-801, acts at a site within the neuronal ion channels coupled to the N-methyl-D-aspartate receptor to inhibit intracellular calcium entry in response to glutamate-mediated depolarization.

Excessive cytosolic calcium initiates a cascade of biochemical events that promote changes in gene expression.[15] Aptiganel completely inhibited c-Jun expression after 90 and 120 min of HCA throughout the hippocampal formation (fig. 3). There was significantly less neuronal necrosis in CA1 and CA3 neurons after 120 min in the aptiganel-treated animals and significantly better functional outcome (figs. 4, 5; table 1).

Other studies using *in situ* hybridization in rodent brains reported c-*jun* mRNA induction in the DG neurons beginning 30 min after transient forebrain ischemia and lasting 3 h.[15,16] However, these investigators did not assay for the translated protein c-Jun. Our results did not show c-Jun protein in the DG after 2 h of HCA, even though c-Jun was expressed in other neurons throughout the hippocampal formation (figs. 1, 2). Although we did not assay for c-*jun* mRNA in these experiments, its transcription is implied because c-Jun protein was translated. However, the reverse is not true; that is, not all transcribed mRNA is translated into the protein. Protein synthesis is affected markedly by ischemia and usually is decreased during early reperfusion.[17,18] Several studies have shown that translational processes may be impaired after ischemia, whereas transcriptional processes remain intact.[7,19] Presumably this is caused by the greater energy requirements to synthesize a protein (translation) than to transcribe a gene. The presence of Fos protein in the DG indicates that cellular energy levels had been restored after HCA to permit efficient *de novo* protein synthesis.

Although the current experiments are limited because we only evaluated a single time point (1 h after weaning from CPB), it is unlikely we would have missed the peak expression of c-Jun, because it lasts for hours, and usually longer than the Fos proteins.[20] In addition, the pattern of expression of Fos and Jun in the hippocampus during reperfusion usually reflects the circuitry: entorhinal cortex → DG → CA3 → CA1.[21] Therefore, our observation of c-Jun in CA3 and CA1 indicates that we did not assay too early. The absence of c-Jun protein in the DG in our model indicates that either the CA1 pyramidal neurons are more vulnerable than DG granule cells to ischemia, the latter requiring a more intense ischemic insult than that conferred by 2 h of HCA; that a different signaling pathway is occurring in our model, bypassing the DG to CA1 neurons; or the mRNA of c-*jun* that has been observed in other ischemia models is not translated.

Other *in vivo* and *in vitro* experiments have related the expression of c-Jun to both neuronal degeneration and survival. Schlingensiepen *et al.*[22] antagonized the

expression of c-Jun and prevented death in neonatal hippocampal neurons. However, conditioning ischemia selectively induces c-Jun in hippocampal CA1 neurons, and these neurons are protected from cell death after a subsequent ischemic insult.[23] These studies indicate that CA1 survival or death may depend on additional signaling events that modulate the transcriptional actions of c-Jun.[9]

The immediate-early gene c-*fos* has been investigated extensively in neural tissue, and its protein product can form heterodimers with c-Jun protein that bind to the AP-1 DNA site and affect the transcription of target genes.[24,25] Although there are other inducible transcription factors in the Jun family, including Jun-B and Jun-D, c-Jun is the predominant transcription factor expressed after neuronal ischemia and it is usually coinduced with c-*fos* in most brain regions.[26] The eventual fate of neurons after c-Jun expression may be a function of the specific homo- or heterodimers formed by c-Jun with itself and Fos proteins (or other transcription factors) and the phosphorylation of these complexes, a potential site for therapeutic interventions.[9,22,27] More studies are needed to determine the combination of phosphorylated dimers within specific regions of the hippocampal formation that result in survival or apoptosis of neurons after HCA. However, the experiments reported here imply that Jun–Jun homodimers predominate over Fos-Jun heterodimers in CA1 after 120 min of HCA, and this homodimer combination is associated with neuronal necrosis in CA1.

In conclusion, this study confirms the contention that the expression of c-Jun in excess of Fos is associated with the subsequent ischemia-induced necrosis of the more vulnerable CA1 and CA3 pyramidal cells after 120 min of CPB and HCA. Furthermore, the absence of c-Jun expression in the less vulnerable DG granule cells combined with the intense expression of Fos is associated with cell survival. The noncompetitive N-methyl-D-aspartate antagonist aptiganel completely inhibited c-Jun expression, significantly improved neurologic outcome, and attenuated necrosis in hippocampal neurons.

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