Blockade of Gap Junctions In Vivo Provides Neuroprotection After Perinatal Global Ischemia

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Background and Purpose—We investigated the contribution of gap junctions to brain damage and delayed neuronal death produced by oxygen-glucose deprivation (OGD).

Methods—Histopathology, molecular biology, and electrophysiological and fluorescence cell death assays in slice cultures after OGD and in developing rats after intrauterine hyoxia-ischemia (HI).

Results—OGD persistently increased gap junction coupling and strongly activated the apoptosis marker caspase-3 in slice cultures. The gap junction blocker carbenoxolone applied to hippocampal slice cultures before, during, or 60 minutes after OGD markedly reduced delayed neuronal death. Administration of carbenoxolone to ischemic pups immediately after intrauterine HI prevented caspase-3 activation and dramatically reduced long-term neuronal damage.

Conclusions—Gap junction blockade may be a useful therapeutic tool to minimize brain damage produced by perinatal and early postnatal HI. (Stroke. 2005;36:2232-2237.)

Key Words: apoptosis ■ carbenoxolone ■ connexin ■ gap junction ■ ischemia

Gap junction channels allow exchange of intracellular ions and molecules as large as 1 kDa between dying and viable brain cells, exchanged that has been suggested to be detrimental or beneficial, depending on the nature of the insult (ie, ischemia or trauma).1 Apoptotic and necrotic signals may spread from stressed to neighboring cells through gap junctions, amplifying the extent of injury.2–5 Agents that close gap junctions, such as insulin,6 insulin-like growth factor-1,7 growth hormone,8 and octanol,9 have been reported to be neuroprotective, but intercellular communication via gap junctions has also been suggested to confer resistance to cellular injury after ischemia,10,11 making the role of gap junctions in response to ischemia unclear.

Materials and Methods

Carbenoxolone Application

In vitro, 75 μmol/L carbenoxolone (CBX; disodium salt; Sigma) was added to slice culture medium.13 In vivo, CBX was administered intraperitoneally twice after intrauterine hypoxia-ischemia (HI), a loading dose (75 mg/kg; ~150 μmol/L) immediately after recovery of respiration, and a second (30 mg/kg) 12 hours later. Similar in vivo dosing regimens desynchronize spinal motor neuron firing13 and directly block dye coupling of anterior subventricular cells14 and retinal neurons.15

Organotypic Hippocampal Slice Cultures

All animal procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals approved by institutional animal ethics committees of Albert Einstein College Medicine and New York Medical College.

Organotypic hippocampal slice cultures16 were prepared from 9- to 12-day-old Sprague-Dawley rats (Taconic) of both sexes and cultured on semipermeable inserts (Millicell CM) over 1 mL medium (50% minimum essential medium; 25% Hanks’ balanced salt solution, 25% heat-inactivated horse serum, 13 mmol/L HEPES, and 2.5 mg/mL glucose). After 6 to 10 days, slice cultures were exposed to oxygen-glucose deprivation (OGD) by transfer to glucose-free medium (in mmol/L: 1.25 CaCl2, 0.9 MgSO4, 5.4 KCl, 0.44 KH2PO4, 137 NaCl, 4.2 NaHCO3, 0.35 NaH2PO4, 16.7 HEPES, and 27.5 mannitol) in a box gassed with 95% N2/5% CO2 for 45 minutes, then returned to normal medium and CO2 until measuring neuronal death.

Propidium Iodide Fluorescence

Media containing 1 μmol/L propidium iodide (PI) was applied for 10 minutes to slices, washed, and PI-labeled dead cells imaged using fluorescein isothiocyanate filters. Fluorescence intensity was measured in equal areas within CA1 stratum pyramidale.

Nucleosomal DNA and Semiquantitative Polymerase Chain Reaction

Nucleosomal DNA fragments were detected using the Cell Death ElisaPlus Kit (Boehringer-Mannheim). RT-PCR assays were per-
formed on total RNA isolated from hippocampal slice cultures with the Thermoscript RT-PCR system (Gibco BRL). Reaction products were analyzed by electrophoresis on 2% agarose gels. Connexin primers were: Mus Cx36 [5'-GAGCAAACGAGAAGATAAGAAG-3'; 3'-CCGCTTCTACATCATCCA-5'] (195 bp) and densitometric assays normalized to 18s mRNA.

Fluorescence Recovery After Photobleaching Slice cultures were loaded by 45-minute application of carboxyfluorescein diacetate (30 μmol/L). CA1 fields of view were photobleached for 2 minutes using the 488-nm laser line of a confocal microscope (Nikon RCM 8000) and images acquired at 1-minute intervals.

In Vivo Intrauterine HI Pregnant rats at term (21 days gestation) were anesthetized, decapitated, the abdomen incised, and uterus quickly isolated from blood supply and surrounding tissue. Acute anoxia was induced by immersing the intact uterus in 37°C saline for 12.5 minutes. Surgical survival was 92±5% after 12.5 minutes of birth anoxia. Controls were delivered by cesarean section without additional HI. Blood gases and pH were measured (RapidLab 855; Bayer) from a mixture from cut cord immediately after delivery.

Dissociated Neuronal Cultures and Exposure to Hypoxia Primary neuronal cultures (<5% glia) were prepared from embryonic day 14.5 C57BL/6 embryos. In 60-mm culture plates, 1.75×10^6 cells were dispersed. To induce hypoxia, cultures were placed in an incubator with 0.5% O₂ (N₂ replacing O₂) for 24 hours (37°C, 5% CO₂).

**Results**

Delayed Neuronal Death in Organotypic Hippocampal Slice Cultures Initial studies characterized the time course of recovery from damage attributable to slice culture preparation. Neuronal death in hippocampal principal layers was assessed by PI staining in principal cell layers daily for the first 10 days in culture (DIC; Figure 1a through 1c). PI staining showed significant neuronal death in slice cultures at 1 DIC that progressively decreased during the first 5 DIC (“recovery window”). By 6 to 10 DIC, cell death was at low steady-state levels. Recordings of Schaffer collateral-evoked field excitatory postsynaptic potentials (fEPSPs) revealed a similar time course of functional recovery (Figure 1d). Based on these data, we studied slice cultures during 6 to 10 DIC (“experimental window”).

Gap Junction Blocker CBX Reduces OGD-Induced Delayed Neuronal Death in Hippocampal Slice Cultures Sustained hypoxia and ischemia produce severe and irreversible functional deficits at Schaffer collateral–CA1 synapses in vitro and widespread delayed neuronal death in all hippocampal principal cell layers. Slice cultures were subjected to 45 minutes of 95% N₂/5% CO₂ plus glucose-free medium (OGD), which produced large numbers of PI-positive pyramidal neurons 24 hours later (Figure 2b). Consistent with our hypothesis that gap junctions contribute to delayed neuronal death, CBX (75 μmol/L) added to culture medium 30 minutes before, during, or 60 minutes after OGD markedly reduced delayed death of CA1 pyramidal neurons assessed by PI staining 24 hours later (Figure 2c and 2d). Treatment of control organotypic slice cultures with CBX alone did not detectably alter viability or morphology of slices 24 hours later (data not shown). The time course of PI staining in the first 24 hours after OGD in CA1 stratum pyramidale (Figure 2e) is suggestive of apoptosis, a conclusion reinforced by the generation of nucleosomal DNA fragmentation (Figure 2e) and activation of the apoptosis-triggering enzyme caspase-3 (Figure 2f). In hippocampal slice cultures after OGD, activated caspase-3 protein increased to 192±9% of control values (n=24 slices from 3 littersates; Student’s t test; P<0.005; control versus OGD).

Consistent with reducing delayed death of CA1 pyramidal neurons, CBX markedly reduced OGD-induced loss of Schaffer collateral synaptic transmission in CA1 stratum radiatum. Figure 2g illustrates these experiments in typical untreated slice cultures, in which OGD caused virtually complete loss of evoked fEPSPs with minimal recovery 24 hours after OGD. In contrast, a second group of hippocampal slices
treated with 75 μmol/L CBX exhibited substantial recovery of evoked fEPSPs (ischemic [ISC]) after identical 45-minute OGD episodes (CA1 EPSP dV/dt 24 hours after OGD; control [n=6] 2.1 ± 0.32 V/s; CBX [n=6] 0.3 ± 0.1 V/s; Student’s t test; P<0.005).

Fluorescence Recovery After Photobleaching Demonstrates Blockade of Junctional Coupling by CBX

To directly measure the extent of cell–cell coupling before and after OGD, cells were loaded by bath application of the gap junction-permeable fluorescent dye carboxyfluorescein diacetate, then an area of the slice was photobleached with high-intensity light. The rate of reappearance of fluorescence is a function of intracellular diffusion of nonbleached dye from neighboring cells through gap junctions. Figure 3a illustrates areas within stratum pyramidale of field CA1 subjected to fluorescence recovery after photobleaching (FRAP) under control conditions and 24 hours after OGD, with and without CBX pretreatment. The top row illustrates carboxyfluorescein-loaded cells before photobleaching; the middle row, 2 minutes, and the bottom, row 9 minutes after photobleaching. Recovery of fluorescence was significantly more rapid in slices subjected to OGD (τISC=56.6 ± 5 s; Student t test; P<0.05) compared with normoxic slices (τCON=117 ± 20 s), whereas number and brightness of cells after initial uptake of dye was not altered, indicating that OGD produced persistent functional upregulation of coupling. Moreover, CBX applied to ischemic cultures 45 minutes before FRAP assay markedly decreased rate of recovery (Figure 3a; ISC+CBX; τISC+CBX=332.6 ± 66 s; Student t test; P<0.05), demonstrating that FRAP is indeed mediated by...
In Vivo Blockade of Gap Junctions With CBX Reduces Mortality and Delayed Developmental Neuropathology After Perinatal HI

The neuroprotection afforded by CBX in vitro suggests that blockade of gap junction channels, during or even after ischemia, might improve recovery and lessen brain damage in vivo, especially early in development, when neuronal gap junction expression is high. To test this hypothesis, transient intrauterine global HI was produced at term. Across all litters, intrauterine HI resulted in changes in pH, PO2, and markedly increased mortality (Table). Consistent with a role for gap junctions in intrauterine HI-induced damage, CBX administered immediately after HI (75 mg/kg) ameliorated the long-term developmental impact of perinatal HI (Table).

Consistent with previous reports, neuropathologies resulting from intrauterine HI varied between litters, ranging from slowed growth to gross encephalopathy, including marked developmental abnormalities in hippocampus, neocortex, and cerebellum. By postnatal day (P21) 21, 16% of HI rat pups weighed <40% less than normoxic and <20% less than HI CBX-treated littermates (ANOVA; P<0.005; control 38.5±1 g; HI 23±4 g; CBX-treated HI rats 29.2±3 g). Histopathological analyses of hematoxylin-eosin–stained sections from brains of 5 P9 to P21 rats subjected to perinatal HI compared with controls and CBX-treated littermates revealed hypercellularity in the hilar region of the hippocampal dentate gyrus (DG), a site of late neurogenesis (DG cells/0.015 mm2 for normoxic P21 animals=11±2; HI=37±7; HI+CBX=13±2; n=16 fields in 5 animals; P<0.001; Figure 4, hippocampus, white arrow). Three of 5 exhibited abnormal cerebellar development with atrophic folia and superficial sulci. Hypercellularity and thickness abnormalities of the external granular layer were also evident (cells/0.05 mm2 for normoxic animals=44±2.5; HI=58±3; HI+CBX=46±2.5; n=26 fields in 5 animals; P<0.05; EGL thickness in normoxic animals=24.5±3 µm; HI=85±12 µm; HI+CBX=34±4 µm; n=25 fields in 5 animals; P<0.01; Figure 4, cerebellum). Two animals showed cortical laminae disorganization not restricted to primary sensory areas, but also present in associative regions of HI animals. Quantification of cells in layer 5 of somatomotor cortex revealed a mean of 3.4±0.5 dying cells/100 µm2 in HI versus 0.5±0.1 dying cells/100 µm2 in normoxic and 1.5±0.5 dying cells/100 µm2 in HI+CBX (n=23 fields in 5 animals; P<0.001). Analysis at higher magnification revealed shrinkage of cytoplasm and condensation of the nucleus of pyramidal cells, suggestive of cell damage (Figure 4; neocortex, ISC, white arrows). Three animals showed ventricular dilation (data not shown). Consistent with the hypothesis that gap junctional coupling is a necessary contributor to these long-term consequences, CBX treatment dramatically reduced the extent of histopathological damage in hippocampus (Figure 4; ISC+CBX), neocortex, and cerebellum.

**Gap Junction Blockade Prevents Activation of Caspase-3 by Intrauterine HI**

To assess effects of HI, and gap junctional blockade after HI, activation of the apoptosis-triggering enzyme caspase-3,
CBX Does Not Directly Inhibit Caspase-3 Cleavage in Dissociated Neurons

To exclude the possibility that CBX may inhibit cleavage of caspase-3 independent of its blockade on gap junctions, we examined hypoxia and camptothecin-induced accumulation of activated caspase-3 in freshly dissociated neurons in culture (n=2). Application of CBX (0.3 to 300 μmol/L) did not alter hypoxia or camptothecin-induced activation of caspase-3 (Figure 5b). In contrast, the general caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone effectively prevented caspase-3 cleavage (Figure 5b).

**Discussion**

In OGD-treated hippocampal organotypic in vitro slice cultures and intrauterine HI in vivo, the relatively selective gap junction blocker CBX was potently neuroprotective, even when administered 60 minutes after ischemia.

The more rapid recovery of fluorescence we observed after photobleaching in organotypic slice cultures 24 hours after ischemia (Figure 3a) demonstrates that OGD persistently increases coupling in vitro. Although FRAP was measured in the CA1 pyramidal cell layer, neuronal and glial coupling are both likely to contribute to the response to ischemia. These results are consistent with findings in an in vitro trauma model that OGD enhances gap junction coupling and promotes bystander cell killing. Introducing gap junction proteins (connexins) into cell lines has been shown to enhance bystander cell death. Pharmacological and antisense blockade of gap junctions in hippocampal slice cultures decreases their vulnerability to traumatic injury and HI, and gap junction blockade has been shown to reduce infarct size after arterial occlusion. Although deletion of some connexins has been reported to augment, not decrease, infarct size in rodent stroke models, one cannot easily dissociate effects of reduced coupling from secondary effects of altered blood flow or effects downstream of altered connexin expression. Our study helps clarify these results by showing that a net blockade of gap junctions confers neuroprotection immediately after a perinatal HI insult.

Although studies suggest that the majority of neuronal loss 24 hours after perinatal HI is apoptotic, necrosis can also contribute. DNA fragmentation and caspase activation are hallmarks of apoptosis. Nucleosomal DNA fragmentation began to rise by 3 hours after OGD in slice cultures, with a 20-fold increase by 12 hours; PI fluorescence showed a similar time course. The decrease in nucleosomal signal after 12 hours appears to be attributable to nucleosomal degradation that overtakes the rate of production. The good temporal correlation between these measures suggests an apoptosis-like process is induced by OGD in slice cultures.

Although it remains to be shown directly that activation of caspase-3 by OGD caused eventual neuronal death, the pronounced appearance of p17 subunits in P1 brains preceded delayed neuronal death and neuropathologic changes at 9 and 21 days of age. CBX treatment produced marked, parallel reductions in caspase-3 activation and delayed neuronal death and eventual long-term brain damage but did not directly inhibit caspase-3 cleavage in virtually uncoupled primary cultured neurons, consistent with a causal connection between cell coupling, activation of apoptotic pathways, and neuronal death. Because gap junctions do not pass molecules larger than ~1 kDa, ions such as Na⁺ and Ca²⁺, and reactive free radicals, are likely candidates to be involved in the spread of damage. Because CBX has been shown to inhibit voltage-gated Ca²⁺ channels and synaptic transmission in the retina, it remains to be determined to which extent a combination of calcium channel blockers with gap junction blockade might be an advantageous therapeutic approach. However, substantial neuroprotection after HI or trauma has been shown to be conferred by selectively reducing connexin expression with antisense.

The high rate of survival of newborn rats given CBX intraperitoneally demonstrates that there are centrally effective concentrations of CBX that avoid the well-known cardiac effects and intrauterine HI in vivo, the relatively selective gap junction blocker CBX was potently neuroprotective, even when administered 60 minutes after ischemia.

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The high rate of survival of newborn rats given CBX intraperitoneally demonstrates that there are centrally effective concentrations of CBX that avoid the well-known cardiac
toxicity of gap junction blockers. Because intrauterine HI makes the entire fetus ischemic, the therapeutic effect of CBX could be on the central nervous system, peripheral organs, or vasculature.

Although in clinical practice this may not matter, our in vitro experiments in isolated neural tissue show that central nervous system gap junction blockade confers direct neuroprotection independent of vasculature or any peripheral structure, indicating significant therapeutic potential of gap junction blockers for the treatment of perinatal HI, which currently has no effective therapy.

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