Excitotoxic Neurodegeneration Induced by Deprivation of Oxygen and Glucose in Isolated Retina

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PURPOSE. Ischemic neurodegeneration contributes to many retinal diseases. An isolated retina model has been used to examine the neuronal cell death induced by deprivation of oxygen and glucose (simulated ischemia) as a model for ischemic disease.

METHODS. Neurodegeneration in the isolated chick embryo retina was induced by simulated ischemia and assessed using biochemical (lactate dehydrogenase release) and morphologic (light microscopy) techniques.

RESULTS. Simulated ischemia led to lactate dehydrogenase release gradually in a period of 6 to 24 hours. Light microscopic observations demonstrated morphologic cell degeneration well before lactate dehydrogenase release occurred. N-Methyl-D-aspartate (NMDA) and non-NMDA receptor blockers individually provided partial protection, and the combination was fully protective. No protection was provided if the antagonists were added after simulated ischemia. When NMDA receptors were blocked by MK-801, cyclothiazide, an inhibitor of desensitization at non-NMDA receptors, enhanced lactate dehydrogenase released after 1 or 2 hours of simulated ischemia. Low concentrations of glucose effectively prevented lactate dehydrogenase release, despite anoxic conditions.

CONCLUSIONS. The isolated retina provided a convenient system to characterize quantitatively ischemic cell death. Retinal ischemic neurodegeneration is an excitotoxic process that involves overactivation of NMDA and non-NMDA glutamate receptors. Blockade of both of these receptor subtypes was necessary for complete neuroprotection. Receptor desensitization played a protective role. If even low concentrations of glucose were delivered to an ischemic retina in vitro, substantial neuroprotection could be achieved. This may have implications for the management of acute retinal ischemic episodes. (Invest Ophthalmol Vis Sci. 1998;39:416-423)
protected by antagonists of NMDA receptors. Activation of non-NMDA receptors leads to acute morphologic changes in these cultured neurons (for example, swelling induced by Na+ and water influx), but a fatal pathologic cascade is usually not initiated unless the non-NMDA receptor activation is extreme and prolonged.13,26,29 Conversely, in the chick retina preparation, overactivation of non-NMDA receptors swiftly leads to the pathomorphologic changes characteristic of cell death.20,22,30 Cerebellar granule cells in culture are also rapidly killed on overactivation of non-NMDA receptors.16 Treatment with NMDA or non-NMDA receptor agonists leads to necrosis of distinct populations of neurons in acutely prepared cerebellar slices.31-33 In several whole animal models of neural ischemia, antagonists of non-NMDA receptors are more effective neuroprotectants than NMDA antagonists.34-38

Most studies using isolated retina have examined the acute changes that occur on excitotoxic insult of the tissue, whereas in most of the culture models cell death is assessed by biochemical measures or cell counting hours afterward. It is possible that some of the retinal changes are transient and may not be predictive of eventual cell death. This may explain some of the differences between the retina and the culture models. However, recently we adapted the chick embryo retina model to permit maintenance in isolation for 24 hours and examination of the delayed aspects of excitotoxic neurodegeneration.39 Studies in this preparation demonstrate that activation of either non-NMDA or NMDA receptor leads to delayed excitotoxic neurodegeneration, assessed histologically or biochemically.

We extend these studies here by performing a detailed characterization of the neurodegeneration induced by simulated ischemia in the isolated chick embryo retina. A quantitative biochemical measure of cell degeneration, lactate dehydrogenase (LDH) release, is used and compared with light microscopic evaluations.

**METHODS**

**Chick Embryo Retina Preparation**

All experiments adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Chick embryos, 14 to 16 days old, were decapitated, and their retinas were rapidly removed and cut into thirds. During dissection the eyes were kept at 0°C to 4°C, but the retinas, once removed, were maintained at 25°C. Each retinal segment was incubated in 1 ml balanced salt solution in a sealed 7-ml glass vial. The composition of the control medium was 124 mM NaCl; 5 mM KCl; 2 mM CaCl2; 2 mM MgCl2; 1.25 mM K2HPO4; 22 mM NaHCO3; 20 mM glucose; 30 μM phenol red; pH 7.4. The medium was oxygenated, and the pH was adjusted by equilibrating it with 95% O2/5% CO2, which also provided the atmosphere in the sealed vial. For simulated ischemia, the medium contained no glucose and was bubbled with 95% N2/5% CO2. The retinas were incubated in the simulated ischemia medium for the specified time, then washed with several changes of control medium and incubated, usually for 22 to 24 hours, at 25°C. Pharmacologic agents were present during or after the period of simulated ischemia, as described. When present, MK-801 was at 10 μM, GYKI 52466 at 100 μM, and cyclothiazide at 100 μM. These were maximally effective concentrations, based on concentration–response experiments (not shown).

**Lactate Dehydrogenase Assay**

An aliquot of the medium was removed at the end of the simulated ischemia period, and another aliquot was removed at the end of the experimental period (usually 22-24 hours later). The LDH content of each aliquot was determined and used as an index of acute and delayed neurodegeneration, respectively. To control for the differences in sizes of the individual retinal segments, at the end of the experiment, each retina was frozen and thawed in the remaining buffer to which 0.1% Triton X-100 was added, thereby breaking all cell membranes and liberating cellular LDH. The lysate was centrifuged, and LDH was measured in an aliquot of the supernatant. The LDH released into the buffer before and after incubation were normalized to a percentage of the total retinal LDH content. To pool data from some experiments, they instead were normalized to maximal LDH released by the experimental condition (for example, when the retina was processed for histology and was not lysed to obtain the total retinal LDH value).

LDH was measured spectrophotometrically, by the rate of disappearance of NADH (measured at 340 nm). The assay was performed in a total volume of 300 μl in individual wells of a 96-well plate, using a kinetic plate reader (Molecular Devices, Menlo Park, CA) online to a desktop computer. The LDH sample and NADH (0.1 mg/ml in 0.1 M potassium phosphate, pH 7.4) were added to each well. The reaction was started by the addition (to eight wells at a time with a multitip pipettor) of sodium pyruvate at 2 mM. The rate of disappearance of NADH is linear for several minutes, and readings are taken from each well by the plate reader every 5 seconds for 2 minutes. A typical experiment that contained 60 retinal segments required analysis of 180 LDH samples (preincubation, postincubation, total LDH). Each sample was analyzed in quadruplicate, so eight 96-well plates were serially set up and run. The assay had a greater than 20-fold linear range. In each experiment, each condition was tested on three retinal segments. All data shown represent the means and standard errors of mean results from at least three separate experiments.

A potential problem with the use of the LDH assay to quantitate cellular injury is that if the experimental procedure alters the total amount of LDH in the tissue under study, as well as the released LDH, the extent of injury will not be accurately reflected by the measurements of the released LDH. To determine whether simulated ischemia altered the total LDH content of the retina, we reanalyzed data obtained in 20 independent experiments to compare total LDH (released and residual) in retinas subjected to 2-hour simulated ischemia (followed by overnight incubation in normal oxygen- and glucose-containing buffer) with that in retinas treated similarly but not subjected to the 2-hour simulated ischemia. Normalizing to the LDH in control retinas as 100% (±6.1 [SE]), the LDH content of retinas subjected to simulated ischemia was 95.4% (±6.1 [SE]). These data indicate that simulated ischemia per se does not alter the total LDH.

**Histology**

Retinal segments were fixed by immersion in phosphate-buffered solution containing 1.5% glutaraldehyde and 1% paraformaldehyde, then fixed in 1% osmium tetroxide and embedded in araldite. One-micrometer sections were prepared, mounted, and stained with methylene blue-azure II for evaluation using a light microscope. To provide for biochemical and histologic observations on the same retinal specimens, an aliquot of the
Simulated Ischemia Leads to Biochemical and Morphologic Signs of Neuronal Degeneration

Previous investigations have established that, under control conditions, the isolated retinas were considered healthy by morphologic and biochemical criteria immediately on dissection and for at least 24 hours of incubation in isolation.\textsuperscript{39} To assess retinal damage and death caused by the deprivation of oxygen and glucose (simulated ischemia), the isolated retinas were incubated in the absence of oxygen and glucose for different periods of time, then returned to the control medium for the remainder of a 24-hour period. LDH released from the retina at the end of the simulated ischemia period (acute) and the total released during the experiment were measured (Fig. 1). Little LDH was released acutely, even with 2 hours of incubation in simulated ischemia medium. Approximately 5% of retinal LDH was released at the end of a 6-hour simulated ischemia period. However, a delayed and gradual release of substantial amounts of LDH was triggered by relatively short incubations in the absence of oxygen and glucose: approximately 10% after a 1-hour incubation and approximately 20% after a 2-hour incubation (Fig. 1). The fraction of retinal LDH released after a 6-hour simulated ischemia incubation was approximately 35%. Because a substantial, but submaximal, delayed LDH release occurred after 2 hours of simulated ischemia, this exposure time was used as the standard in most subsequent experiments.

How delayed was the LDH release that followed simulated ischemia? Aliquots of medium were taken at varying periods after the 2-hour simulated ischemia and were assayed for LDH (Fig. 2). The release was gradual, with some release apparent by 4 hours after incubation, but only 50% to 60% was complete even at 16 hours after simulated ischemia. This demonstrates the slowly evolving nature of this manifestation of toxicity.

Light microscopic pathomorphology of retinas at various times after a 2-hour period of simulated ischemia is presented in Figure 3. At the end of the 2-hour incubation (Fig. 3; acute), there was marked edema in all retinal layers (except perhaps in the still immature photoreceptor layer): cell bodies were swollen and watery in the inner nuclear layer (INL) and ganglion cell layer (GCL), and the plexiform layers had the cheesecloth-like appearance suggestive of many fluid-filled, distended processes. Even at this time, many cells appeared to have condensed, pyknotic nuclei surrounded by protein-poor (nonstaining) cytoplasm (bulls-eye profiles), suggesting severe chromatin disease. These changes are hallmarks of cell degeneration and death. Over time, more cells in the INL and GCL showed the changes characteristic of dying cells, and many degenerated to mere fragments of nuclear and cellular debris. By 16 hours and especially 24 hours, many INK and GCL cells had already died, leaving only remnants. Not all retinal cells died by 24 hours, however: Many healthy cell bodies were apparent in the photoreceptor layer and the outer half of the inner nuclear layer, and some were apparent in the ganglion cell layer. Fewer normal-appearing cell bodies were observed in the inner half of the inner nuclear layer.

\begin{figure}
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\includegraphics[width=\textwidth]{fig1}
\caption{Lactate dehydrogenase (LDH) release after exposure of chick embryo retina to simulated ischemia for increasing times. Chick embryo retinas were incubated in the absence of oxygen and glucose for different lengths of time, then placed in oxygenated, glucose-containing medium for the remainder of the 24-hour experimental period. LDH released into the medium, at the end of the simulated ischemia period and at the end of the 24 hours, was measured. The LDH released at the end of the ischemic period (acute) and the sum of the LDH released acutely and after the postischemic period (Total) are shown. In this and all other figures, values are mean ± SEM for three experiments.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Time course of lactate dehydrogenase (LDH) release after 2 hours of simulated ischemia. Chick embryo retinas were incubated in the absence of oxygen and glucose for 2 hours, then placed in oxygenated, glucose-containing medium for the remainder of the 24-hour experimental period. LDH released into the medium was measured at the end of the simulated ischemia and at 4, 16, or 24 hours later. Values are mean ± SEM for three experiments, normalized to mean maximal LDH released in each experiment.}
\end{figure}
Ischemic Cell Death Is Caused by Overactivation of NMDA and Non-NMDA Receptors

In most systems studied, ischemic neuronal degeneration is excitotoxic—that is, excessive activation of glutamate receptors plays a causal role in the disease process. To test this here, the glutamate receptor antagonists MK-801 (selective for NMDA receptors) and GYKI 52466 (selective for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [AMPA]-preferring receptors) were added singly or in combination to retinas subjected to 2 hours of simulated ischemia (Figs. 4, 5). Neither antagonist induced LDH release in control retinas (not shown). Each antagonist individually decreased simulated ischemia-induced LDH release: MK-801 provided approximately 84% blockage, whereas GYKI 52466 provided approximately 56% blockage, and, when added together, the protection was near complete. These results indicate that both NMDA and non-NMDA receptors contribute to the LDH release induced by simulated ischemia. The morphology of these retinas is shown in Figure 5. Without antagonists, cell death was evident in the INL (particularly in the inner half) and the GCL, and there were many spaces in the neuropil of the inner plexiform layer, representing residual swelling or tissue loss (Fig. 5B). MK-801 provided full protection of cells in the GCL, and in many fields there was complete protection of cells in the INL as well. However, there were always areas with pathomorphologic changes in some cells of the INL and in the appearance of the inner plexiform layer, as illustrated in Figure 5C. GYKI 52466 also provided partial protection (Fig. 5D), but a particular population of cells protected by GYKI 52466 was not clearly evident. In the presence of both antagonists, none of the pathomorphologic changes was evident (Fig. 5E).

When the antagonists were added only after 2 hours of simulated ischemia, no significant protection was afforded by either individually, and the combination was only partially protective. This partial protection was highly variable from experiment to experiment and was not statistically significant (Fig. 6).

Blockade of Desensitization-Enhanced Non-NMDA Receptor-Mediated Ischemic Cell Death If NMDA Receptors Are Blocked

The results from the experiments with antagonists suggested that non-NMDA receptors contribute to cell death in retina-simulated ischemia. Cyclothiazide is an agent that potentiates electrophysiological responses at AMPA-preferring, non-NMDA
FIGURE 5. Light micrographs of isolated chick retinas incubated under control (A. Control) or simulated ischemia (B. SI) conditions in the absence or presence of antagonists (MK, MK-801, 10 μM; GYKI, GYKI 52446, 100 μM) individually or combined. The N-methyl-D-aspartate (NMDA) and the non-NMDA receptor antagonists must be present for the protection of all neurons.

receptors by blocking desensitization. Would cyclothiazide potentiate simulated ischemia-induced LDH release? LDH release induced by either 1 hour or 2 hours of simulated ischemia was unchanged when cyclothiazide (100 μM) was included (Fig. 7A). However, if the NMDA receptor-mediated component of LDH release was eliminated by including MK-801, cyclothiazide provided a significant potentiation (Fig. 7B), suggesting that AMPA-preferring receptors contribute to the neurotoxicity.

Low Concentrations of Glucose Are Effective At Inhibiting Ischemic Cell Death

Because glycolytic (anaerobic) metabolism may play an important role in the retina, the effects of glucose addition to oxygen-depleted media were examined. There was a concentration-dependent decrease in LDH release with added glucose (Fig. 8). Half the LDH release was blocked with 100 μM glucose, and there was no LDH release above background with concentrations of 10 mM or more.

FIGURE 6. Glutamate antagonists provided after 2-hour simulated ischemia are ineffective blockers of lactate dehydrogenase (LDH) release. The conditions are as in Figure 4, except the antagonists were added at the end of the simulated ischemia period. When administered after the simulated ischemia, the antagonists singly did not block LDH release and in combination did provide partial, but not statistically significant, protection. Values are mean ± SEM for four experiments.

DISCUSSION

In previous studies of isolated chick retina, short periods of simulated ischemia were shown to cause pathomorphologic changes characteristic of excitotoxic neurodegeneration. In the present study, by maintaining retinas in isolation for 24 hours, delayed features of this process could be examined.

In this and our previous study, the isolated retinas were maintained at 25°C. Hypothermia provides protection against ischemic neurodegeneration in in vivo and in vitro systems. Lowering the temperature of the incubation from 37°C to 25°C eliminated a variable and high background presumably by preventing the small tears and injuries that may occur during the dissection from evolving into excitotoxic lesions in a portion of the tissue. Presumably, by lowering the temperature, we slowed the neuropathologic processes. We have not eliminated many important and salient features of ischemic neurodegeneration, because reasonable times of incubation under simulated ischemia conditions lead to biochemical and morphologic hallmarks of damage, and the excitotoxic nature of this damage is apparent from our results.

Combined oxygen-glucose deprivation of isolated chick embryo retinas led to retinal degeneration that was quantitatively graded by the extent of delayed LDH release (Fig. 1). It should be noted that the maximum LDH released never equaled 100% in our results. Even after 24 hours of simulated ischemia (not shown), only approximately 50% of retinal LDH was found in the medium. This most likely reflects binding of LDH to tissue components. Therefore, the data reflecting percentage of release should not be interpreted absolutely (such as the percentage of neurons or retinal volume injured).

Even relatively long periods of simulated ischemia, up to 6 hours, did not result in large amounts of LDH release by the end of the simulated ischemia period (Fig. 1), although profound morphologic changes took place at early time points (Fig. 3). The simulated ischemia appeared to trigger a neurodegenerative process that required several hours to manifest as LDH release (Fig. 2) even though morphologically dead cells accumulate faster than LDH is released. LDH release is usually...
Simulated Ischemia in Isolated Retina

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When used alone, the NMDA receptor antagonist MK-801 provided substantial, but incomplete, protection. These results are consistent with studies of cultured cortical neurons, in which MK-801 provided incomplete protection against prolonged periods (>45 minutes) of simulated ischemia.\(^{28}\) Significant partial protection was also provided in our system by the AMPA receptor antagonist GYKI 52466. In studies of cultured cortical neurons, the AMPA receptor blocker CNQX alone did not provide any protection against LDH release by simulated ischemia,\(^{15,28}\) although it did enhance the partial protection provided by MK-801 against prolonged periods of simulated ischemia.\(^{28}\) The present results indicate that both classes of ionotropic glutamate receptor contribute to simulated ischemia-induced neurodegeneration in isolated retina. Similar results were reported using an in vivo rat model of retinal ischemia: non-NMDA and NMDA receptor blockade were necessary for optimal neuroprotection as assessed histologically 1 hour after occlusion of the retinal circulation.\(^{49}\) In a study of acute excitotoxic damage resulting from chemically induced hypoglycemia and anoxia in isolated chick retina, NMDA receptors appeared to be primarily responsible for the damage in moderate lesions, with non-NMDA receptors contributing to severe lesions.\(^{55}\) Although it is difficult to compare the paradigms, perhaps the 2-hour damage we used here as a moderate lesion is more severe than the moderate lesion induced in the studies of Zeekvalk and Nicklas.\(^{24,25}\) In other isolated neuronal systems, simulated ischemia-induced neurodegeneration is wholly and exclusively prevented by NMDA receptor antagonists,\(^{15,28,29}\) except when the simulated ischemia is extended for prolonged periods. Apparently, different neuronal populations degenerate by different mechanisms: This may reflect distinct receptor subtypes, postreceptor mechanisms, or both.

Another possibility is that network and tissue properties of the isolated, but largely intact, retina account for differences between neurodegeneration here and in dissociated neuronal preparations. Regardless of the mechanism, this model may more closely reflect certain features of in vivo ischemic damage. For example, there is considerable evidence that non-NMDA receptors contribute to ischemic neurodegeneration in vivo.\(^{34,36,37,50}\)

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**FIGURE 7.** Cyclothiazide enhances lactate dehydrogenase (LDH) release only after N-methyl-D-aspartate (NMDA) receptor blockade. Cyclothiazide (CTZ) blocks desensitization at α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-preferring glutamate receptor. This agent did not increase the LDH release induced by either 1 hour or 2 hours of simulated ischemia (A). However, after NMDA receptor blockade with MK-801 (B), CTZ caused a significant increase in LDH release (*P-test, MK versus MK + CTZ, P < 0.03). There was no effect when AMPA receptors were blocked instead with GYKI 52466. Values are mean ± SEM for three or four experiments.

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**FIGURE 8.** Glucose addition prevents simulated ischemia (SI)-induced lactate dehydrogenase (LDH) release. Relatively low concentrations of glucose block a significant fraction of SI-induced LDH release (*P < 0.03 relative to SI). Values are mean ± SEM for three experiments.
To further examine the role of non-NMDA receptors in simulated ischemia-induced ischemic neurodegeneration, we studied the effects of cyclorrhizide, which potentiates current flow through AMPA (but not NMDA or kainic acid) receptors by blocking desensitization. Only when NMDA receptor-mediated LDH release was blocked with MK-801 did cyclorrhizide potentiate LDH release (Fig. 7). One interpretation of these results is that the population of neurons susceptible to AMPA-receptor-induced cell death also contains NMDA receptors that are sufficient to cause neurodegeneration when they are overstimulated.

Administration of glutamate antagonists after the initiation of ischemia has been shown to have neuroprotective efficacy in some in vitro and in vivo model systems. The potential clinical usefulness of these antagonists would be enhanced if neuroprotection did not depend on administration before ischemia began. No protection was observed in isolated retina when the individual subtype-selective antagonists were administered after the 2-hour simulated ischemia (Fig. 6). However, partial blockade of LDH release was observed when the combination of NMDA and non-NMDA antagonists was provided. Such combination pharmacotherapy may therefore have better efficacy than individual subtype-selective agents in other systems.

Current dogma holds that extracellular Ca\(^{2+}\) is causally involved in delayed excitotoxic neurodegeneration, and this conclusion is based on observations of attenuated toxicity in several model systems in vitro in the absence of extracellular Ca\(^{2+}\). Our work attempted to examine the effects of manipulations of extracellular Ca\(^{2+}\) on neurodegeneration induced by simulated ischemia in this system, but lowering the extracellular Ca\(^{2+}\) concentration below the standard 2 mM level rapidly led to substantial LDH release and cell death (not shown). Many other investigators have reported toxicity, or exacerbation of toxicity, on extracellular Ca\(^{2+}\) removal. Our studies of excitotoxic cell death induced by non-NMDA receptor agonists in this system indicate that this process is independent of extracellular Ca\(^{2+}\) despite the presence of Ca\(^{2+}\)-permeable AMPA receptors in some vertebrate retinas. Definitive answers concerning the mechanisms of ischemic neurodegeneration in this system are not yet available.

Aerobic and anaerobic metabolism is used by the retina. Therefore, we predict that, under hypoxic conditions, available glucose should maintain energy stores and tissue health. This is precisely what was found (Fig. 8). Even concentrations of glucose as low as 100 μM provided significant blockade of LDH release. These results are consistent with studies demonstrating acute excitotoxic damage results from poisoning either glycolysis or electron transport. We have shown previously that intravitreal administration of glucose forestalls neurodegenerative changes in an in vivo model of irreversible retinal circulatory occlusion. Despite the well-known finding that endogenous or induced systemic hyperglycemia is contraindicated in stroke, our results suggest that direct delivery of glucose to ischemic neural tissue may be beneficial.

References


