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A610

ISCHEMIC-HYPOXIC INJURY IN CULTURED NEURONS AND ASTROCYTES MEASURED BY A DYE TECHNIQUE.

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Cell culture models are increasingly popular to study brain cell hypoxia and ischemia. Release of such markers as the macromolecule lactate dehydrogenase (LDH) has been used to determine average survival of an ischemic/hypoxic insult in a cell population. Using this technique we have observed an extremely slow LDH release from cultured neurons and astrocytes during and after ischemic/hypoxic cell damage. This obviously contrasts with the very rapid neuronal death during cerebral ischemia/hypoxia in vivo. We have therefore initiated studies using the fluorescein diacetate (FDA)/propidium-iodide (PI) dye technique, which permits assessment by fluorescence microscopy of death in individual cells in the culture. FDA is a non-fluorescent compound that crosses membranes readily and is converted to fluorescein by esterases present in living cells. Fluorescein does not readily cross membranes and thus is trapped within living cells. PI, on the other hand, does not cross intact cell membranes and only enters dead or severely injured cells, where it binds to DNA; (e.g., Jones and Senft, J. Histochem. Cytochem. 33, 877, 1985; Manev et al., Molec. Pharm. 36, 106, 1989; Didier et al., J. Neurosci. Res. 27, 251, 1990).

Methods: Primary cell cultures of cerebral neurons were obtained from 15-day old mouse fetuses and cerebellar granule cell neurons from one week old mice. Astrocyte cell proliferation was inhibited with 40 µM of cytosine arabinoside. Primary cultures of astrocytes were obtained from neonatal mice. Neuronal cultures were studied after at least 8 days and astrocytic cultures after at least three weeks. Cells were exposed to i) control conditions, ii) severe hypoxia (1-3% O_2) and normal medium, iii) normoxic conditions and culture medium depleted of glucose and glutamine, and iv) hypoxia and substrate depleted medium. Following exposure to an insult at 37°C the culture medium was removed and the cultures washed in a balanced salt solution before adding 15 µg/ml FDA and 4.6 µg/ml PI for a ten minute period. After, washing, the cultures were examined under a fluorescence microscope.

Results: Cerebellar granule cells (a glutamatergic preparation) showed very considerable cell death after ischemia or hypoxia for as little as 30 min. After 2 hrs, the vast majority of cells were dead under these conditions. The NMDA antagonist MK 801 completely protected the cells after 2 hrs but not after prolonged (6 hrs) insults. Cortical interneurons (a mainly GABAergic preparation) showed some cell death (about 50%) after 30 min of incubation, even in control cultures (a result of medium change). This was not observed when glutamine was omitted from the medium and MK 801 showed some protective effect. Exposure to hypoxia or ischemia caused no additional cell death. Even after 2 hrs, a considerable fraction of the cells survived. Astrocytes showed no cell death under any of the conditions described above but extensive damage (80% cell death) after 12 hrs of ischemia. No damage occurred after 12 hrs exposure to hypoxia or substrate deprivation. 24 hrs of hypoxia also caused no cell death but 24 hrs of substrate deprivation killed many of the astrocytes.

Discussion: The present results suggest that neurons suffer an early, cytotoxic death in ischemia/hypoxia, whereas astrocytes suffer a late death due to exhaustion of energy substrates. The same happens for neurons protected against cytotoxicity. Rational in vivo interventions therefore should probably comprise both protection against cytotoxicity and interventions geared toward improvement of metabolic status. Moreover, the present method is in agreement with LDH release in astrocytes but more sensitive than LDH release in neurons.

A611

TITLE: EXTRACELLULAR ALKALOSIS EXACERBATES INJURY OF CORTICAL NEURONS DUE TO OXYGEN-GLUCOSE DEPRIVATION *IN VITRO*

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Introduction: While the effects of acidosis on neuronal injury especially during ischemic stress have been the subject of study in many laboratories, the effects of alkalosis on the vulnerability of neurons and glia to injury have not been well studied. We report here the direct cellular effect of alkalosis on cultured cortical neurons and glia and the effect of alkalosis on the vulnerability of these cells to oxygen glucose deprivation injury. These primary cultures from fetal and newborn mouse neocortex are well characterized and permit assessment of direct effects on brain parenchymal cells without effects on the cerebral circulation. As glutamate toxicity is widely agreed to be an important component of cerebral injury in such circumstances as hypoxic/ischemic injury we chose to quantitate the effect of extracellular alkalosis on injury induced by direct glutamate toxicity and by combined oxygen and glucose deprivation.

Methods: Cortical cell cultures from fetal mice for mixed cultures (neurons and glia) or newborn mice (glia without neurons) were made as previously described (1). Injury due to glutamate toxicity was assessed 24 h after a 5 min exposure to 500 µM glutamate. For oxygen-glucose deprivation cultures were placed in an anoxic chamber (O_2 < 0.2%) and washed into balanced salt solution previously equilibrated with anoxic gas and lacking glucose. Deprivation was terminated by either washing into buffered salt solution with glucose and oxygen or by adding glucose back in a small volume and removal from the chamber. Injury was assessed at 24 h by measuring lactate dehydrogenase (LDH) released into the medium. Intracellular accumulation of calcium was measured by adding a trace amount of ^{45}Ca to the extracellular medium only for the period of oxygen-glucose deprivation, thoroughly washing the cells, then solubilizing them with hot 0.2% SDS. Aliquots were counted in a scintillation counter.

Results: In mixed cultures alkalosis was found to progressively worsen glutamate toxicity increasing injury at pH 7.4, 60 ± 3% of maximal LDH release, to 78 ± 1% at pH 8.0 (for n=4, mean ± SEM). Oxygen-glucose deprivation injury increased from 22 ± 3% to 48 ± 3% at pH 8.0 for the same duration of insult (for n=4, mean ± SEM). For 9 h oxygen-glucose deprivation no pH dependence of glial injury was detected over the range 7.4 to 8.0. To produce glial injury 9-12 h of deprivation is required, whereas neuronal injury is essentially complete after 1 h. Furthermore, astrocytes tolerated prolonged incubation at alkaline pH demonstrating little injury after 24 h. The extent of calcium accumulation by mixed cultures during oxygen glucose deprivation at pH 8.0 was nearly double that at pH 7.4, whereas glial cultures showed considerably less total accumulation of calcium and calcium accumulation was independent of pH.

Discussion: These results suggest that glial injury is not strongly influenced by extracellular alkalosis, whereas oxygen glucose deprivation neuronal injury is sensitive to alkalosis, consistent with an increase in NMDA receptor activation and increased influx of calcium into the cells. Recently, it has been suggested that the extent of rebound alkalosis after ischemia may better predict injury than the degree of acidosis (2). Patients alkalotic for any reason may be more vulnerable to ischemic neuronal injury.

References

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