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Hypoxic Injury to Developing Glial Cells: Protective Effect of High Glucose

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ABSTRACT. Hypoxic injury to differentiating glial cells is a critical event in the development of periventricular leukomalacia, the major hypoxic-ischemic lesion of the premature infant. This study has addressed the effects of hypoxia on differentiating glial cells, primarily astrocytes. Primary cultures of dissociated newborn rat brain, which are composed predominantly of differentiating astroglia, were used. Efflux of lactate dehydrogenase, an enzyme enriched in astroglia, was used to quantitate cellular injury. Three major findings are reported. First, differentiating astrocytes were resistant to hypoxic injury for many hours, although by 24 h of hypoxia severe cellular injury (lactate dehydrogenase efflux of 86% of total and morphologic changes) was obvious. Second, increase of glucose in the culture medium from the approximately physiological concentration of 5.6 to 15 mM had a marked protective effect versus hypoxia, *i.e.* lactate dehydrogenase efflux was totally prevented during 24 h of hypoxia in 15 mM glucose. Third, the protective effect of high glucose appeared to be related to increased utilization by glycolysis, because there was a direct correlation between the resistance to hypoxic cellular injury and the amount of lactate generated and of glucose consumed by the cells. Thus, the cells with the lowest lactate dehydrogenase efflux (and highest glucose supplementations) had medium lactate concentrations as high as 32–36 mM. These concentrations of lactate are approximately double the reported threshold concentration of lactate considered to produce cellular necrosis in *in vivo* models of hypoxic injury, primarily in mature animals. The data raise the possibility that hypoxic injury to differentiating glia can be prevented or ameliorated by increase in glucose availability. (*Pediatr Res* 27: 186–190, 1990)

Abbreviations

DMEM, Dulbecco's modified Eagle's medium
LDH, lactate dehydrogenase

Hypoxic-ischemic brain injury incurred in the perinatal period is an extremely important clinical problem. This form of brain injury accounts for the largest proportion of the major nonprogressive neurologic deficits manifested in infancy and childhood under the rubrics, cerebral palsy and mental retardation (1). In recent years a large amount of research has been directed toward elucidation of the mechanisms of neuronal death with hypoxia. Indeed studies of neurons in cell cultures (2, 3), as well as in other experimental models (4), have emphasized the importance of excitatory amino acid neurotransmitters in the mediation of hypoxic neuronal death. However, relatively little work has been directed toward elucidation of mechanisms of hypoxic death of glia, especially developing glia. The particular importance of this research for the neonatal period is related to the fact that the dominant neuropathology in the human premature infant subjected to hypoxic-ischemic insult is not neuronal injury but rather injury to periventricular white matter, rich in developing glia but devoid of neurons (1). Neuropathologic data indicate that the glial cell is the particular target of such hypoxic injury (5, 6). The particular glial cell type(s) most affected are not yet known conclusively.

In this study we use a model of developing glial cells, *i.e.* primary glial cell cultures derived from newborn rat brain. Although containing both developing astrocytes and oligodendroglia, these cultures are composed of approximately 90–95% astrocytes, which undergo rapid differentiation in the 3rd wk in culture (see "Materials and Methods"). We undertook this study with three major objectives. First, because of a recent report that fully mature astrocytes exhibit no major signs of cell injury for as long as 12 h of hypoxia (7), we wished to determine the relative vulnerability of differentiating astrocytes to duration of hypoxia. Second, because in a variety of *in vivo* models of hypoxic-ischemic injury there is controversy regarding whether glucose supplementation is beneficial or deleterious (8–17), we studied the effect of exogenous glucose levels on vulnerability to hypoxia. Third, because in a variety of *in vivo* models of hypoxic-ischemic injury there is controversy regarding the role of high lactate levels in the mediation of cell death (8, 10, 13, 15, 18–21), we determined the relation of extracellular lactate levels to the degree of hypoxic astrocytic injury.

MATERIALS AND METHODS

Materials. Newborn Sprague-Dawley rats were purchased from Sasco, Inc. (Omaha, NE). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture flasks and other disposable culture materials were from Becton-Dickinson (Oxnard, CA) and from Corning (Corning, NY), except for Plastek culture dishes which were obtained from Tekmat Corp., (Ashland, MA). Nylon meshes were supplied by Small Parts, Inc. (Miami, FL). Tissue culture media were obtained from the Tissue Culture Support Center (Washington University Medical School (St. Louis, MO). FCS was purchased from HyClone (Logan, UT).

Cell culture. Primary glial cultures of the cerebral hemispheres of newborn rats were prepared by a modification of earlier methods (22–24), essentially as previously described (25). After decapitation, the brains of the newborn rats were removed to sterile Petri dishes containing a 1:1 mixture of PBS (pH 7.4) and

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DMEM at 37°C. With the assistance of a dissecting microscope, the cerebellar hemispheres, brainstem, and meninges were removed and discarded. The cerebral hemispheres were mechanically dissociated by sequential passage through sterile nylon monofilament mesh with pore sizes of 125, 37, and 20 μm, respectively. The dissociated cells were suspended in sufficient volume of DMEM with 10% FCS to yield a ratio of one brain to 9 mL of cell suspension. The cell suspension was transferred in 3-mL aliquots to 25-cm² Plastek tissue dishes. The cultures were incubated at 37°C in 95% air/5% CO_2 and 95% humidity. The culture medium was first changed after 3–4 d in culture and subsequently three times per week with DMEM containing 10% FCS. The cultures were used for experiments on the 18th d in culture.

The composition of these glial primary cultures has been shown to be approximately 90–95% astroglial, by immunocytochemical and ultrastructural techniques (24, 26–29). The remaining cells are oligodendroglia. Neurons are absent. Study of such enzymatic markers as glutamine synthetase for astrocytes and 2′,3′-cyclic nucleotide 3′-phosphohydrolase for oligodendroglia by ourselves (30, 31) and by others (24, 32, 33) has shown that these expressions of glial differentiation increase most rapidly during the 3rd wk in culture. For that reason, the experiments described herein were carried out on cells grown for 18 d in culture.

In vitro model of hypoxia. The *in vitro* model of hypoxia was similar to that described by Yu *et al.* (7). Thus, culture dishes were placed in a humidified modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) that was purged with 95% N_2/5% CO_2 for 10 min at 12 L/min and sealed. Yu *et al.* (7) showed that purging with this gaseous mixture at 10 L/min for 10 min results in an atmosphere with undetectable oxygen. The incubation medium was fresh DMEM with varying concentrations of glucose, as indicated, which also had been bubbled with 95% N_2/5% CO_2 at 1.0 L/min for 5 min. The chamber was placed inside a standard incubator at 37°C for the times indicated in "Results." Control cultures were incubated in identical media under aerobic conditions.

In the experiments described in "Results," the media and surviving cells were collected immediately after the prescribed period of hypoxia or normoxia for the assays. In either case, after the media were collected, the cells were washed three times with Tris-NaCl (pH 7.40). One mL of 50 mM Tris-HCl (pH 7.50) then was added to the flasks and the cells were collected by scraping with a rubber policeman. (The medium was centrifuged to sediment detached cells, although in several pilot experiments LDH activity of the medium before and after centrifugation was virtually identical.) Cells and media, were then disrupted in a polytron homogenizer for 20 s before performing assays for LDH activity in the cells and media; for lactate concentration in the media, and for total protein content in the cells.

LDH. LDH activity of the cell homogenates and incubation media were determined immediately after collection. LDH activity was measured spectrophotometrically with pyruvate and NADH in a phosphate buffer according to the method of Bergmeyer and Bernt (34). One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH/min.

Percent LDH efflux into the media (% LDH efflux) was calculated for each of the hypoxic experimental and the normoxic control conditions by the following formula:

$$\frac{\text{LDH activity of media}}{\text{total LDH activity (cells and media)}} \times 100\% = \%\ \text{LDH efflux}$$

For each experimental condition, the percent LDH efflux reported is the difference between the percent LDH efflux of the hypoxic cultures and the percent LDH efflux of the normoxic control cultures. The percent LDH efflux of the normoxic control cultures at all concentrations of glucose studied was consistently very low, *i.e.* generally 1–3%. In pilot experiments, percent LDH efflux was shown to be an excellent correlate of cell death, determined either morphologically or by measurement of total protein derived from cells remaining on the surface of the flasks. Koh and Choi (35) similarly have demonstrated the value of LDH efflux in the quantitation of cortical neuronal injury in cell culture.

Lactate and glucose determinations. The lactate concentration (μmol/mL) of the medium was determined spectrophotometrically by the method of Gutman and Wahlefeld (36). The glucose concentration (μmol/mL) of the medium was determined spectrophotometrically by the method of Bergmeyer *et al.* (37).

Total protein content. The total protein content of the cultures was determined by the method of Bradford (38).

RESULTS

Effect of hypoxia on LDH efflux. Initially we evaluated the effect of hypoxia on the glial cells as a function of duration of hypoxia at two concentrations of glucose in the culture medium (Fig. 1). Hypoxia had no apparent effect on the cells during the first 6 h, but by 24 h of hypoxia cells in 5.6 mM glucose, an approximately physiologic concentration, were strikingly affected, as manifested by LDH efflux of 80%. Notably, however, increase of medium glucose to 25 mM totally protected the cells during 24 h of hypoxia.

The phase microscopic appearance of the cultures reflected the LDH data (Fig. 2). Thus, control cultures in 5.6 mM glucose and aerobic conditions exhibited the typical appearance of a predominant bed layer of phase-light, epitheloid cells characteristic of astrocytes, upon which are a much smaller number of phase-dark cells characteristic of oligodendroglia (24) (Fig. 2*A*). After 24 h of hypoxia and 5.6 mM glucose, there is widespread disintegration of cells (Fig. 2*B*). [After 2 h of hypoxia and 5.6 mM glucose, cellular morphology was clearly distorted, with swelling of processes and vacuolization of cytoplasm (not shown).] In contrast, after 24 h of hypoxia and 25 mM glucose, no appreciable change in morphology can be identified (Fig. 2*C*). Moreover, total cellular protein content in the latter cultures was not different from that in the normoxic cultures (data not shown).

Effect of glucose on hypoxia-induced LDH efflux. We next evaluated the concentration dependence of the protective effect of glucose on the LDH efflux caused by 24 h of hypoxia (Fig. 3). A striking concentration dependence of the protective effect of

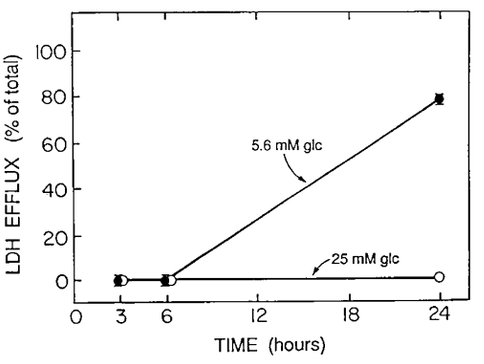

Fig. 1. Effect of hypoxia on LDH efflux. Primary cultures of developing glia were obtained from dissociated cerebral hemispheres of newborn rats and grown in culture for 18 d (see "Materials and Methods"). At that time the cultures were placed in fresh DMEM with the indicated concentrations of glucose (*glc*), incubated in 95% nitrogen/5% CO_2 for the indicated times, and LDH efflux determined as described in "Materials and Methods." Values are means ± SD of separate determinations performed on three cultures. Essentially identical results were obtained in four separate experiments.

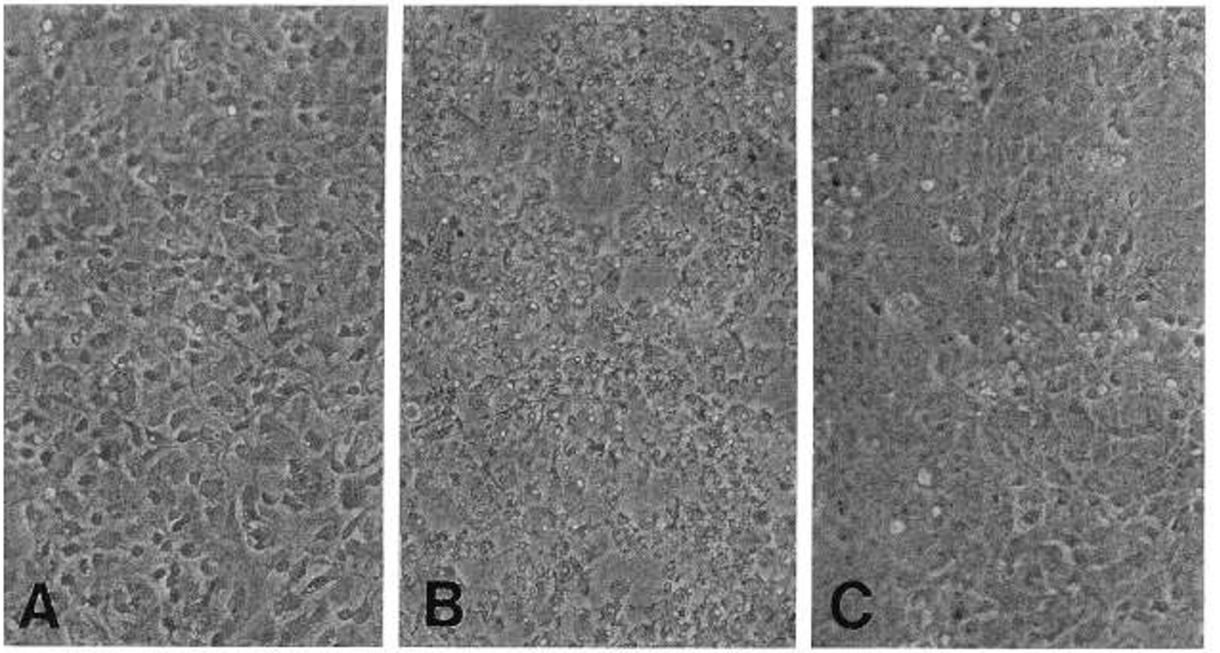

Fig. 2. Phase-microscopic appearances of 18-d glial cultures that have been grown for 24 h in fresh DMEM and under the following conditions: *A*, 5.6 mM glucose, normoxia (95% air/5% CO_2) (control); *B*, 5.6 mM glucose, hypoxia (95% nitrogen/5% CO_2); and *C*, 25 mM glucose, hypoxia (95% nitrogen/5% CO_2). The cultures were examined by phase-contrast microscopy (×100).

glucose was apparent, such that concentrations of 5.6 and 10 mM were associated with 80–90% LDH efflux, whereas a concentration of 15 mM glucose totally prevented hypoxia-induced LDH efflux.

To ensure that the striking beneficial effect of 15 mM glucose did not reflect simply an osmotic effect and to evaluate the possibility that other glycolytic intermediates might also protect the glial cells, we added to hypoxic cultures (and normoxic controls) either 10 mM pyruvate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, sucrose, or sorbitol in addition to 5.6 mM glucose. No protective effect was observed with any of the additions (data not shown). The lack of protective effect of fructose-1,6-diphosphate is unlike observations made with fully differentiated astrocytes (39).

Relation of lactate concentration to LDH efflux of hypoxic cells. Because studies of ischemic animal models (derived principally from mature animals) suggest that high lactate concentrations may mediate hypoxic cell death and that there is a critical threshold of approximately 16–20 mM lactate above which cell death occurs (8, 18–20), we evaluated the relation of medium lactate concentrations to LDH efflux of cells made hypoxic. Thus, cells were exposed to 24 h of hypoxia in various concentrations of glucose (Fig. 4). Whereas lactate concentrations in the culture medium of control cells did not exceed a concentration of approximately 12 mM, lactate concentrations in cells made hypoxic increased markedly as a function of the glucose concentration in the medium. Indeed, peak concentrations of lactate (32–36 mM) were achieved at medium glucose concentrations (15–25 mM) that totally protected the cells from hypoxia (compare Fig. 3). Thus, in this model system cells least affected by hypoxia were exposed to the highest levels of lactate.

As a measure of glucose utilization, we quantitated the glucose concentration remaining in the medium after the 24 h of hypoxia and subtracted this quantity from the starting concentration to determine the amount of glucose consumed. The values for glucose utilization were, for flasks in 3, 5.6, 10, 15 and 25 mM glucose; 9, 17, 30, 44, 55 μmol, respectively, for the 24-h period. Thus, increasing glucose utilization occurred in concert with increasing lactate production.

The pH of the medium after 24 h of hypoxia did not differ from the pH of the medium of control flasks at medium glucose concentrations below 15 mM (data not shown). The lowest pH in hypoxic flasks, 6.70, was observed at the highest medium glucose concentration (25 mM).

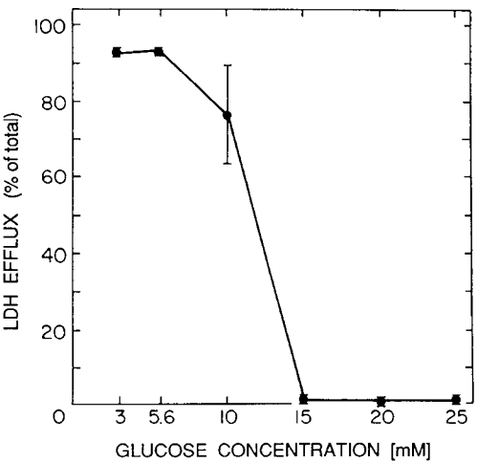

Fig. 3. Effect of glucose on hypoxia-induced LDH efflux. Primary cultures were grown for 18 d and subjected to 24 h of hypoxia, as described in the legend to Figure 1, with the indicated concentrations of glucose in the culture medium. Values are means ± SD, as described in the legend to Figure 1. Essentially identical results were obtained in three separate experiments.

DISCUSSION

We addressed the effects of hypoxia on differentiating glial cells, primarily astrocytes. That our data reflect effects of hypoxia on differentiating astrocytes rather than oligodendroglia relates not only to the marked predominance of astrocytes in these glial primary cultures but also to our use of LDH efflux as a marker for hypoxic injury. Thus, LDH has been shown to be a major constituent of astrocytes, but not oligodendrocytes in glial primary cultures (40). The major findings of the study, therefore,

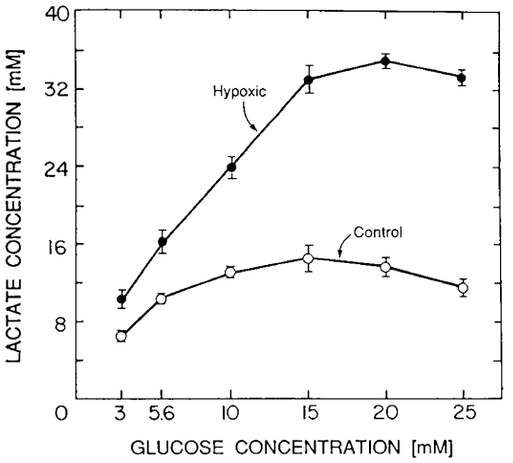

Fig. 4. Relation of lactate concentration in the culture medium after 24 h of hypoxia to the concentration of glucose in the culture medium (at the onset of hypoxia). The conditions of the experiment were identical to those described in the legend to Figure 2. Values are means ± SD as described in the legend to Figure 1. Essentially identical results were obtained in three separate experiments.

relate to the relative vulnerability of differentiating astrocytes to hypoxia, the effect of exogenous glucose levels on this vulnerability, and the relation of medium lactate concentrations to hypoxic astrocytic injury.

Concerning the relative vulnerability of the differentiating astrocytes contained in these primary cultures to hypoxia, it is clear that these cells can tolerate hours of hypoxia with no effect on cellular integrity, as assessed by LDH efflux or morphologic appearance. This relative resistance of the differentiating astrocytes to hypoxia contrasts with the sensitivity of cultured neurons, which exhibit severe injury after several hours (41, 42). Nevertheless, after 24 h of hypoxia in medium containing 5.6 mM glucose, the astrocytes do exhibit severe cellular injury, manifested by LDH efflux of 80–90% and by morphologic evidence for widespread cellular disintegration. The finding of relative resistance of differentiating astrocytes to hypoxic injury for many hours in an approximately physiologic concentration of 5.6 mM glucose is similar to that reported in 7.5 mM glucose for fully mature cultured astrocytes (grown in culture for more than 4 wk and treated continuously with dibutyryl cyclic AMP after 2 wk in culture to promote and maintain differentiation) (7).

The beneficial effect of elevation of medium levels to 15–25 mM regarding hypoxic astrocytic injury is dramatic and not previously described. Thus, the differentiating astrocytes tolerated 24 h of hypoxia with no increase in LDH efflux above the minimal control values when the medium glucose level was 15 mM or more. This protective effect of elevated glucose levels is reminiscent of that shown in developing animal models of hypoxia and/or ischemia (15, 16, 43, 44). Moreover, the beneficial effect of increased glucose levels as shown in the current experiments and in the experiments with developing animals is unlike the deleterious effect of increased glucose observed in mature animal models of hypoxia and/or ischemia (8–13). The mechanisms underlying the beneficial effect of glucose in our experiments with differentiating glia and in experiments with developing animals are unclear, as are the mechanisms for the differences in the effects of glucose supplementation between immature and mature animals. Factors of potential importance include differences in glycogen stores, glucose transport and utilization, rates of utilization of high energy phosphate levels, and lactate accumulation and/or utilization, but more data are needed to define the relative importance of each of these in each system.

Concerning the relation of lactate concentrations to hypoxic astrocytic injury, our data indicate that lactate levels far in excess of the 16–20 mM threshold considered important for cellular death in *in vivo* models of hypoxia (8, 18–20) are compatible with complete protection of differentiating astrocytes. Indeed, the demonstration that high lactate levels are associated with the high medium glucose levels that lead to protection from hypoxia suggests that glucose utilization via glycolysis is the principal means by which the protective effect of glucose occurs. Our demonstration that the quantity of glucose consumed during the 24 h of hypoxia increased nearly linearly with quantity of glucose in the starting medium, and similarly with the quantity of lactate measured in the medium after the 24 h of hypoxia, strongly supports this conclusion. Moreover, our findings are not consistent with the concept of a threshold concentration of lactate of approximately 16–20 mM that is considered to mediate hypoxic cell death in *in vivo* models of ischemic injury to brain. Although most such models involve mature brain, one involves developing brain [fetal sheep (20)] and glial-enriched regions thereof [cerebral white matter (20)]. One explanation for the difference between our findings with differentiating astrocytes in culture and those derived from the *in vivo* models is the possibility of an artificially greater buffering capacity of the culture medium compared to the potentially more limited buffering capacity of the extracellular space in brain *in vivo*. In this regard, there is evidence to suggest that it is not lactate *per se* that is injurious but rather the lowered pH associated with its accumulation (45, 46). In our experiments, the lowest pH, which was observed in the flasks supplemented with 25 mM glucose, is only slightly above that necessary to produce tissue injury in most animal models (42), but was compatible with complete protection of the developing glia in our system.

Clearly further data are needed on these issues, because the possibility that glucose supplementation could be beneficial in prevention of hypoxic injury in developing brain, suggested initially by studies of animal models, and now by our studies at the cellular level, has important clinical implications. Thus, available evidence indicates that unlike the typically intrauterine hypoxic injury of the full-term infant, hypoxic brain injury in the premature infant occurs postnatally, during the neonatal period (1), when such preventative intervention as glucose supplementation could be attempted more readily. Nevertheless, in the absence of data concerning the effect of such supplementation on brain pH and lactate levels, potentially obtainable in the human infant by nuclear magnetic resonance spectroscopy (47), recommendation for such supplementation cannot be made with certainty about its safety.

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