Journal of Cerebral Blood Flow and Metabolism
9:20–28 © 1989 Raven Press, Ltd., New York

Hypoxia-Induced Dysfunctions and Injury of Astrocytes in Primary Cell Cultures

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Summary: The effects of severe hypoxia were studied in a primary culture of astrocytes prepared from newborn rat cerebral cortex. Hypoxia was created by placing cultures in an airtight chamber that was flushed with 95% N_2/5% CO_2 for 15 min before being sealed. The hypoxic environment was maintained constant for up to 24 h. During the first 12 h of hypoxia, astrocytes showed no morphological changes by phase-contrast microscopy. After 18 h of hypoxia, some astrocytes in culture became swollen and started to detach from the culture dish. All cells in the culture were destroyed after 24 h of hypoxia. The lactate dehydrogenase level in the culture medium increased more than tenfold between 12 and 24 h of hypoxia. Glutamate uptake was inhibited 80% by similar hypoxic conditions. The cell volume of astrocytes, as measured by 3-O-methyl-[^{14}C]-D-glucose uptake, was increased. These observations suggested cell membrane dysfunction. The malondialdehyde level of hypoxic cultures increased twofold after 24 h of hypoxia. Verapamil (0.5 m*M*), furosemide (1 m*M*), indomethacin (1 m*M*), $MgCl_2$ (10 m*M*), and mannitol (10 m*M*) reduced but never completely abolished the release of lactate dehydrogenase from hypoxic astrocytes. These data suggest multifactorial causes for severe injury in hypoxic astrocytes. **Key Words:** Hypoxia—Astrocytes—Oxygen tension—Cell injury—Primary culture.

The brain needs a continuous supply of oxygen and glucose for its functional integrity. Interruption of the supply of these substances during various pathological conditions, such as ischemia and stroke, initiates a sequence of biochemical events that can lead to swelling, leakage of intracellular material, and structural as well as functional damage of brain cells (Baethmann, 1978; Nemoto, 1985; Siesjö, 1985; Rothman and Olney, 1986). Numerous in vivo studies have been performed to elucidate the mechanisms involved in this type of brain injury. However, the results are difficult to interpret because multiple parameters are liable to change simultaneously in brain under experimental conditions. This makes it difficult to determine the relevant mechanisms involved. Moreover, cerebral tissue is composed of many different cell types that can be expected to respond independently and differently to a given experimental or clinical condition. These problems can be circumvented partly by using brain cell cultures (Kimelberg, 1983; Hertz et al., 1985).

The injury caused by ischemia, which includes severe hypoxia, substrate deprivation, and failure to remove toxic metabolic products, is multifactorial (Ljunggren et al., 1974). There is ultrastructural evidence that astrocytic swelling is an early and primary event following cerebral ischemia (Chiang et al., 1968; Kimelberg and Ransom, 1986). Astrocytes are important in the control of extracellular water content and ion concentrations in mammalian brain (Hertz and Schousboe, 1975; Hertz, 1982; Kimelberg and Ransom, 1986). Astrocytes also contain receptors and high affinity uptake systems for various neurotransmitters. These receptors and high affinity systems are important to provide a normal physiological microenvironment for neurons to function properly. Dysfunction of astrocytes would lead to a sequence of pathological events such as loss of cellular volume control, an increase of brain tissue volume, both cellular and vasogenic edema, a rise in intracranial pressure, cerebral herniation,

Received December 10, 1987; accepted July 26, 1988.

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Abbreviations used: dBc AMP, dibutyryl cyclic AMP; FCS, fetal calf serum; LDH, lactate dehydrogenase; MDA, malondialdehyde; 3-MG, 3-0-methyl-D-glucose; SOD, superoxide dismutase; MEM, modified Eagles medium.

and, finally, arrest of cerebral circulation (Hossmann, 1985). We used intact primary cultures of rat cerebral cortical astrocytes as a model to examine the dysfunction of astrocytes under severe hypoxia. This culture system has been well established and routinely used by investigators for various biochemical, physiological, and pharmacological studies of brain cells (Hertz and Schousboe, 1975; Schousboe et al., 1980; Sensenbrenner et al., 1980; Hertz, 1981, 1982; Kimelberg, 1983; Kimelberg and Ransom, 1986). Studies of this nature are of fundamental importance for understanding the molecular and biochemical mechanisms involved in the hypoxia-induced dysfunction of astrocytes.

MATERIALS AND METHODS

Cell culture

Primary cultures of rat cerebral cortical astrocytes were prepared from cortices of newborn Sprague–Dawley rats (Simonsen, Gilory, CA, U.S.A.) as described previously (Yu et al., 1986). The neopallium, i.e., the portion of cortex dorsal and lateral to the lateral ventricles, was obtained aseptically from the brain. The neopallia, which were freed of meninges, were cut into small cubes (<1 mm^3) in a modified Eagles minimum essential tissue culture medium (MEM) (Hertz et al., 1985) with fetal calf serum (FCS) (from Sterile System, Logan, UT, U.S.A.). The tissue was disrupted by vortex mixing for 1 min and the suspension was passed through two sterile nylon Nitex sieves (from L. and S. H. Thompson, Montreal, Canada) with pore sizes of 80 μm (first sieving) and 10 μm (second sieving). A volume of cell suspension equivalent to approximately one-30th of brain was placed in a 60 mm Falcon tissue dish (Beckman Dickinson, Oxnard, CA, U.S.A.). Fresh MEM supplemented with 20% FCS was added to a final volume of 3 ml. All cultures were incubated at 37°C in a 95%:5% (vol/vol) mixture of atmospheric air and CO_2 with 95% humidity. The culture medium was changed after 3 days of seeding and subsequently two times per week with MEM containing 10% FCS. After 2 weeks, the cultures reached confluence and were grown in MEM with 0.25 m*M* dibutyryl cyclic AMP (dBcAMP) (Sigma, St. Louis, MO, U.S.A.). The cultures were used for experiments after they were 3 weeks old.

In vitro hypoxia model

To simulate in vivo hypoxia, cultures were put in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, U.S.A.) that was purged with 5% CO_2/95% N_2 for 15 min (flow rate = 10 L/min). The resultant atmosphere contained 0% oxygen (Fig. 1A). Then the chamber was placed inside an incubator and maintained at 37°C. The concentration of oxygen in the culture incubation medium was determined by a blood-gas analyzer (Corning, Medfield, MA, U.S.A.). Although the atmospheric oxygen content was zero, there was a small amount of O_2 present in the culture medium even after 24 h of hypoxia (Fig. 1B). Astrocytic respiration was then allowed to consume some of the remaining oxygen. We defined severe hypoxia as that condition when the PO_2 in the medium fell below 25 mm Hg, a time that usually occurred 12 h after the onset of hypoxia (Fig. 1B). Cultures were incubated in the airtight chamber for the same length of time under aerobic conditions to serve as control.

Effects of verapamil (0.5 m*M*), furosemide (1 m*M*), indomethacin (1 m*M*), $MgCl_2$ (10 m*M*), mannitol (10 m*M*), human recombinant superoxide dismutase (SOD) (300 units/culture), catalase (300 unit/culture), α-tocopherol (1 m*M*), and ascorbate (1 m*M*) were studied on the hypoxic astrocytes. Stock solutions of these compounds were

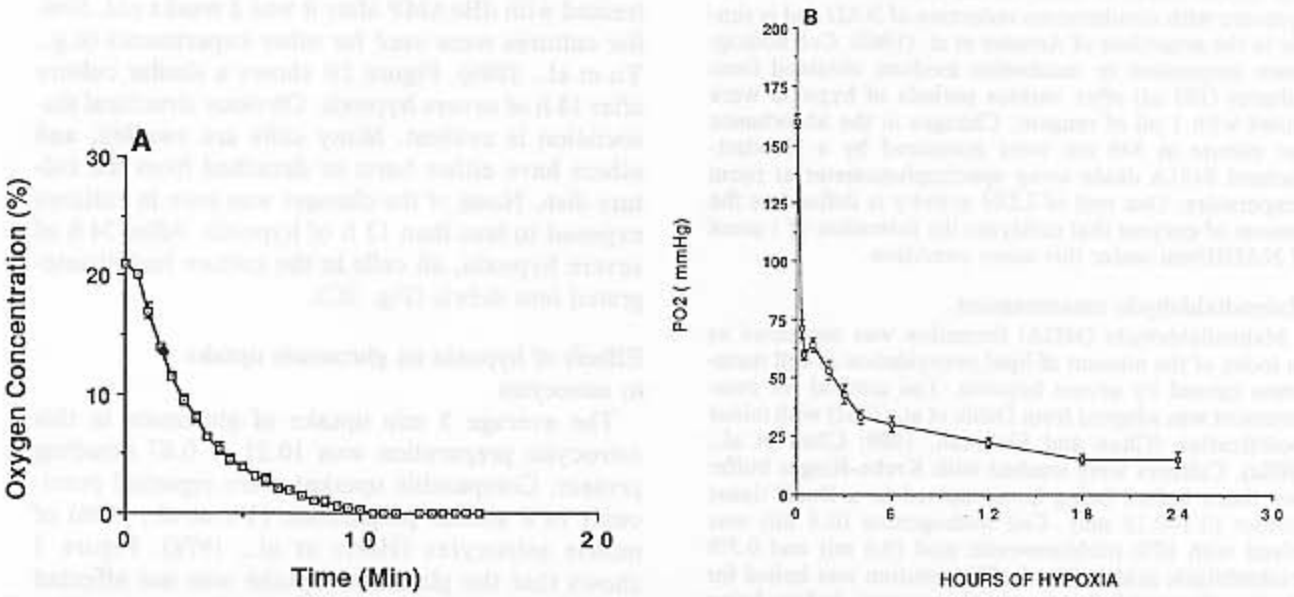

FIG. 1. (A) Changes in the percentage of oxygen as a function of time in the modular incubator chamber flushed with 95% N_2/5% CO_2. The oxygen tension was measured by a low oxygen alarm monitor IL 402 (Instrumentation Laboratory Inc., MA, U.S.A.). The flow rate was 10 L/min (2 psi). **(B)** The change of PO_2 in culture medium as a function of time of hypoxia in the modular incubation chamber with 0% environmental oxygen.

added directly to the culture medium before placing the culture into the modular incubator chamber.

Glutamate uptake studies

Uptake of glutamate was determined as previously described by Yu et al. (1984, 1986). To determine the effects of hypoxia on glutamate uptake, cultures were incubated under severe hypoxia, as described above, for 6, 12, 18, and 24 h in the modular incubator chamber. Control cultures were incubated for the same period without exposure to hypoxia. At the end of the hypoxia incubation period, [U-^{14}C]glutamate (Amersham, Arlington Heights, IL, U.S.A.) was added directly to the culture. The final concentration of the amino acid was 50 μM with radioactivity at 0.1 μCi/ml. Uptake incubation lasted for 5 min, a time short enough to insure initial uptake (Hertz et al., 1978) and to minimize the loss of accumulated amino acid as carbon dioxide, a metabolic process that may be quite pronounced with glutamate (Yu et al., 1982; Yu and Hertz, 1983). After the incubation period, the cultures were rapidly washed twice with ice-cold MEM. One milliliter of 1N NaOH was added, and the radioactivity and protein content in the dissolved cultures were determined, the former using a Beckman LS7000 scintillation counter and the latter using the technique of Lowry et al. (1951). The uptake in 5 min was calculated from the radioactivity per milligram of protein and the specific activity in the incubation media.

Lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) (DD, EC 1.1.1.27) activities in culture cell homogenate and incubation medium were measured utilizing the Sigma diagnostic lactate dehydrogenase reagent (Sigma Diagnostics, St. Louis, MO, U.S.A.). Following the incubation period, the culture medium was pipetted and centrifuged and detached cells and debris were removed prior to the assay of LDH activity. Culture homogenate was obtained by scraping the cells followed by homogenizing with 3 ml of ice-cold MEM. The measurement is based on the oxidation of lactate to pyruvate with simultaneous reduction of NAD and is similar to the procedure of Amador et al. (1963). Cell homogenate suspension or incubation medium obtained from cultures (100 μl) after various periods of hypoxia were mixed with 1 ml of reagent. Changes in the absorbance per minute at 340 nm were measured by a Hewlett-Packard 8451A diode array spectrophotometer at room temperature. One unit of LDH activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH/min under this assay condition.

Malondialdehyde measurement

Malondialdehyde (MDA) formation was measured as an index of the amount of lipid peroxidation in cell membrane caused by severe hypoxia. The method for measurement was adapted from Dahle et al. (1962) with minor modification (Chan and Fishman, 1980; Chan et al., 1988*a*). Cultures were washed with Krebs–Ringer buffer two times before being homogenized in a Duall tissue grinder (0.1–0.15 nm). Cell homogenate (0.4 ml) was mixed with 10% trichloroacetic acid (0.6 ml) and 0.5% thiobarbituric acid (0.25 ml). The solution was boiled for 15 min, then cooled to room temperature before being centrifuged at 2500 rpm for 5 min to precipitate the protein. The absorbance of the supernatant was read against a Krebs–Ringer blank at 535 nm using a Hewlett-Packard 8451A diode array spectrophotometer at room temperature. The amount of MDA in the sample was quantified from a standard curve, using 1,1,3,3-tetraethoxylpropane (Mallinckrodt, Paris, KY, U.S.A.) as a standard.

Measurement of 3-O-methyl-D-glucose uptake

Intracellular water space of intact astrocytes in culture was measured by the method of Kletzien et al. (1975) using 3-O-[^{14}C]-methyl-D-glucose (3-MG) with minor modifications. The technique took advantage of the fact that nonmetabolizable hexose, 3-MG, is transported into cells and reaches intracellular concentrations equal to the extracellular concentration. Phloretin is used to inhibit back-diffusion of 3-MG during washing (Kletzien et al., 1975). The method requires the determination of the amount of hexose taken up at equilibrium vs. the protein content of the culture. The cultures were made hypoxic for 20 h; then the culture medium was removed and the cells were rinsed three times with MEM (37°C). Following the rinsing, 2.5 ml of MEM containing 1 mM of 3-MG with radioactivity of 1 μCi/ml (304.7 μCi/mmol; NEN) was added to the culture and the incubation was carried out in a CO_2 incubator at 37°C for 30 min. After the incubation, the medium was decanted and the cells were washed three times with ice-cold MEM that contained 1 mM phloretin. The cells were then digested in 1N NaOH and samples were taken for protein determination (Lowry et al., 1951) and scintillation counting (Beckman LS7000 scintillation counter). The radioactivities of 3-MG were converted to intracellular water space according to the calculation of Kletzien et al. (1975). The results were expressed as microliters of H_2O per mg protein.

RESULTS

Effects of hypoxia on cellular morphology

Figure 2A shows a normal culture of astrocytes before it was exposed to hypoxia. The culture was treated with dBcAMP after it was 2 weeks old. Similar cultures were used for other experiments (e.g., Yu et al., 1986). Figure 2B shows a similar culture after 18 h of severe hypoxia. Obvious structural dissociation is evident. Many cells are swollen, and others have either burst or detached from the culture dish. None of the changes was seen in cultures exposed to less than 12 h of hypoxia. After 24 h of severe hypoxia, all cells in the culture had disintegrated into debris (Fig. 2C).

Effects of hypoxia on glutamate uptake in astrocytes

The average 5 min uptake of glutamate in this astrocytic preparation was 10.21 ± 0.67 nmol/mg protein. Comparable uptakes were reported previously in a similar preparation (Yu et al., 1986) of mouse astrocytes (Hertz et al., 1978). Figure 3 shows that the glutamate uptake was not affected by severe hypoxia during the first 12 h of exposure whereas it was reduced by almost 80% after 18 h of hypoxia. The uptake remained low thereafter.

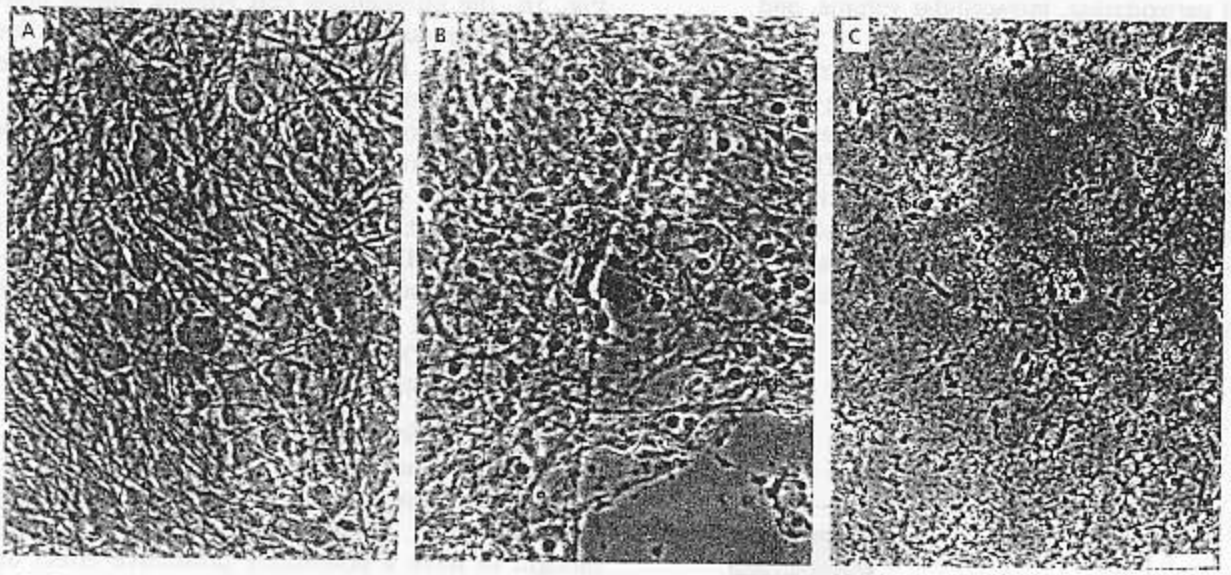

FIG. 2. Phase-contrast micrographs of primary cultures of cerebral cortical astrocytes. **(A)** Normal control culture without exposure to hypoxia; **(B)** culture after 18 h of hypoxia; **(C)** culture after 24 h of hypoxia. These cultures were over 4 weeks old and treated with dBcAMP after they were 2 weeks old. The bar represents 40 μm.

Lactate dehydrogenase measurement

A control astrocytic culture contained about 1.2 units of LDH (i.e., equivalent to approximately 400 units/L of culture medium if all the intracellular LDH was released into the medium). The LDH level in the medium of a normoxic culture was very low. Medium with 5–10% FCS had 30–40 units of LDH/L, whereas serum-free medium had less than 10 units of LDH/L. All of the experiments were done with medium containing 5% FCS because the amount of serum in the medium seems to affect the time course of hypoxia-induced damage (Chan et al., unpublished results). Figure 4 shows the changes of LDH content in the culture medium as a function of time of exposure to severe hypoxia. The level of LDH did not change during the first 12 h of hypoxia; then it began to rise. This indicated leakage of this intracellular enzyme into the extracellular space. The LDH level in the medium reached a maximum of 350–450 units/L and remained at a high level after 24 to 48 h of hypoxia. This suggests complete loss of membrane integrity between 24 to 48 h because this level of LDH is equivalent to the total intracellular LDH content of a normoxic culture.

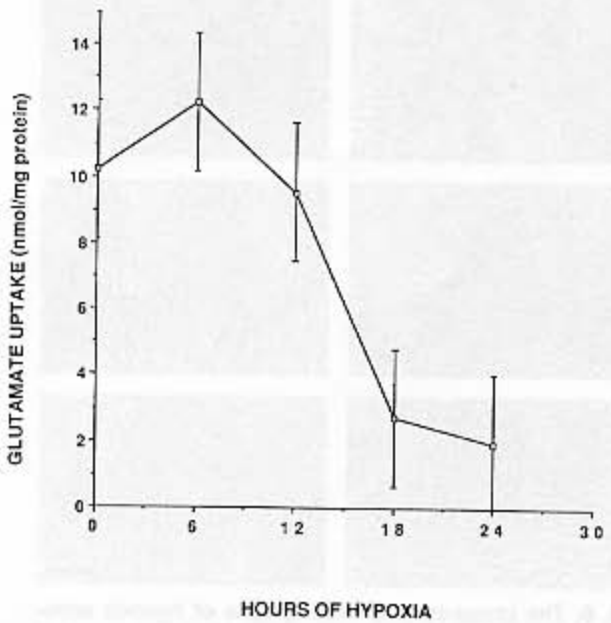

FIG. 3. Uptake of [U-^{14}C]-glutamate in primary cultures of astrocytes as a function of exposure time to hypoxia. Uptakes were measured at 5 min. The concentration of glutamate was 50 μ*M*. Results are means of at least six experiments and SEM values are shown by vertical bars.

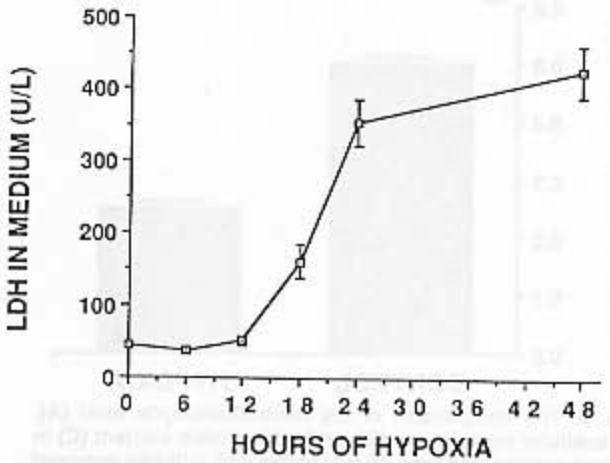

FIG. 4. The release of lactate dehydrogenase from primary cultures of astrocytes as a function of time of exposure to severe hypoxia. Results are means of 18 individual experiments and SEM values are shown by vertical bars.

Lipid peroxidation, intracellular volume, and protein content

The average level of MDA in a control astrocytic culture was about 1.16 ± 0.07 nmol/mg protein. Following 18 h of severe hypoxia, the level of MDA increased about twofold (Fig. 5A). This indicates peroxidation of lipids during hypoxic incubation.

The intracellular water space of astrocytes was measured under normoxia and hypoxia. Under normal conditions, the average intracellular water space was 4.48 ± 0.16 μl/mg protein. As shown in Fig. 5B, the intracellular cell volume almost doubled after the culture was exposed to hypoxia for more than 18 h, i.e., when the LDH content in the medium was above 160 units/L.

The protein contents of cultures under normoxia and hypoxia were also compared. The average protein content of cultures of astrocytes under normoxia was 500 ± 14 μg/dish and remained constant during the first 12 h of hypoxia. After 18 h of hypoxia, it decreased to about 251 ± 16 μg/culture while the LDH level in the culture medium increased (Fig. 5C). There was no measurable protein on the culture plates after 24 h of hypoxia.

Possible mechanisms underlying hypoxia-induced astrocytic dysfunction

We studied the possible mechanisms underlying hypoxic injury of astrocytes by using agents thought to have a potentially protective effect on hypoxic cells. Cell morphology and LDH content of hypoxic astrocytes were determined with and without these agents present.

Figure 6A shows the morphology of cultured astrocytes after 20 h of hypoxia. The dramatic changes seen in hypoxic cultures (Fig. 6A) were partly prevented by the addition of 0.5 m*M* verapamil (Fig. 6B), 1 m*M* furosemide (Fig. 6C), 1 m*M* indomethacin (Fig. 6D), 10 m*M* $MgCl_2$ (Fig. 6E), or 10 m*M* mannitol (Fig. 6F) to the culture medium

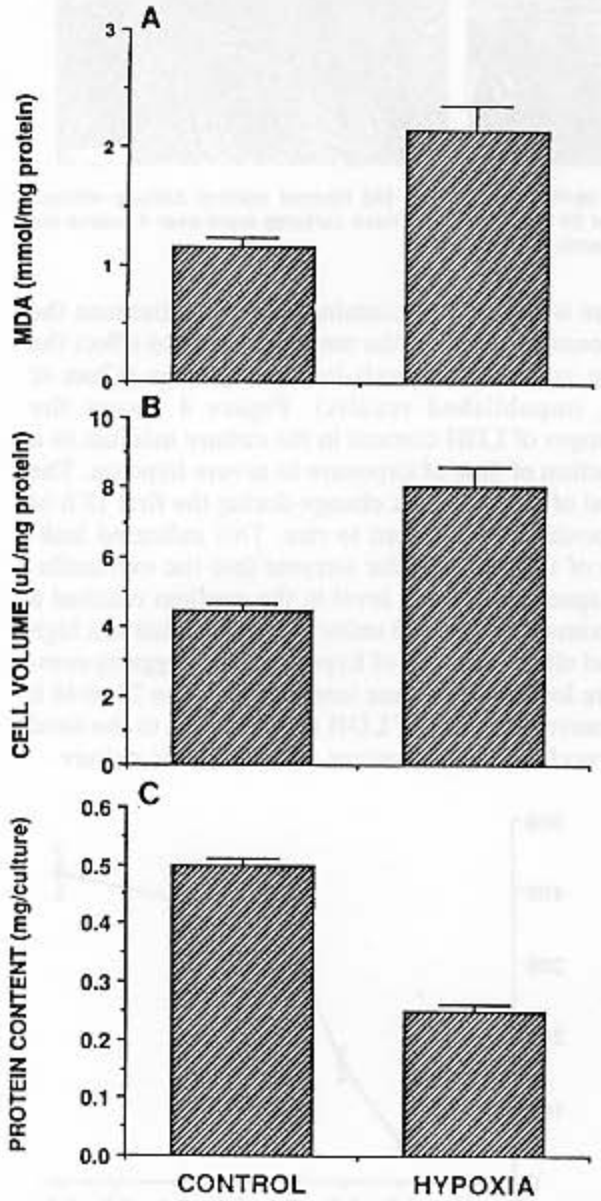

FIG. 5. The comparison of the malondialdehyde level (A), intracellular water space (B), and total protein content (C) in normal primary cultures of astrocytes and cultures exposed to over 18 h of severe hypoxia. Results are means of 6, 4, and 16 individual experiments for A, B, and C, respectively. The SEM are represented by vertical bars.

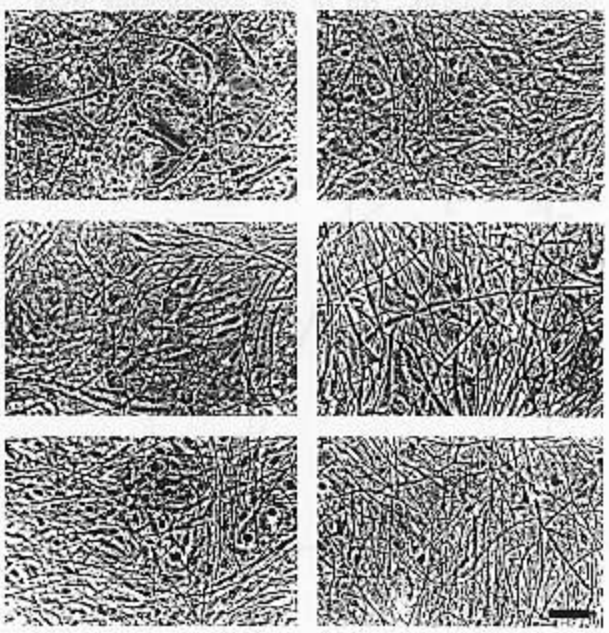

FIG. 6. The phase-contrast micrographs of hypoxic astrocytes (A) compared to similar astrocytes in the presence of verapamil (B), furosemide (C), indomethacin (D), $MgCl_2$ (E), and mannitol (F). Concentrations of these agents were the same as in Table 1. These photographs were taken 20 h after hypoxia began. The bar represents 50 μm.

prior to the cells being subjected to hypoxic conditions. Human recombinant SOD (300 units/culture, kindly supplied by Chiron Corporation, Emeryville, CA, U.S.A.), catalase (300 units per culture), and α-tocopherol (1 m*M*) did not prevent the morphological change in hypoxic astrocytes.

Table 1 demonstrates the effects of these drugs on hypoxia-induced LDH release from astrocytes. Verapamil (0.5 m*M*), furosemide (1 m*M*), and indomethacin (1 m*M*) significantly ($p < 0.001$) reduced the release of LDH from the hypoxic cells by over 60%, indicating partial cell protection by these agents. $MgCl_2$ (10 m*M*) and mannitol (10 m*M*) also significantly reduced the cellular LDH efflux into the culture medium ($p < 0.05$). SOD, catalase, and α-tocopherol did not reduce the LDH efflux from the cells into the incubation medium.

DISCUSSION

The observed morphology and biochemistry of the astrocytes from rat cerebral cortex in primary culture were very similar to those described previously for mouse (Hertz et al., 1978, 1985) and rat (Kimelberg, 1983). The staining of glial fibrillary acidic protein (GFAP) with anti-GFAP serum demonstrated that over 95% of the cultured cells were GFAP positive. The cultures had a stable protein content of 600–700 μg/60 mm culture dish at 3–4 weeks after seeding and were viable in vitro for at least 6 months. The amount of ATP in this culture was 30 nmol/mg protein (Gregory, Welsh, Yu, and Chan, unpublished data), a level similar to the value reported in adult rat brain (Lewis et al., 1974). The degree of differentiation of astrocytes in culture compared to astrocytes that have developed in the intact adult nervous system has been a subject of extensive study by many investigators. A substantial amount of information is currently available regarding the normal metabolism and function of astrocytes in culture, indicating that the preparations reached a reasonable degree of functional maturation (for reviews: Schousboe et al., 1980; Hertz, 1981, 1982; Kimelberg, 1983; Hertz et al., 1985). It has been shown that astrocytes in culture contain significant electrically silent ion transport pathways to maintain ion homeostasis, a function known to be important in situ (Hertz, 1982; Kimelberg, 1983). Receptors for a number of transmitters such as norepinephrine, adenosine, and prostaglandins have been located in cultured astrocytes (reviewed by Van Calker and Hamprecht, 1980). Astrocytes in cultures can take up both GABA and glutamate by Na^+-dependent, high affinity uptake systems (Hertz, 1982). Adenosine, aspartate, and taurine are also taken up by cultured astrocytes (Hertz, 1982). Uptake of some of these transmitters has been observed in astrocytes in situ by autoradiography (Hokfelt and Ljungdahl, 1971; Hertz, 1982). Together with other well-established immunological (Hansson et al., 1980, 1985), electrophysiological (Kimelberg et al., 1979; Moonen et al., 1980), and morphological properties, the primary culture prepared for the current studies are considered mature astrocytes.

TABLE 1. *The release of lactate dehydrogenase from astrocytes into media in the presence of various agents after 20 h of hypoxia*

Drugs	Concentrations	LDH release (units/L)[a]
Control		156.27 ± 8.22 (n = 14)
SOD	300 units/culture	184.24 ± 15.11 (n = 3)
Catalase	300 units/culture	176.33 ± 16.23 (n = 4)
Verapamil	0.5 m*M*	79.16 ± 7.50 (n = 10)[b]
Furosemide	1.0 m*M*	54.62 ± 10.98 (n = 10)[b]
Indomethacin	1.0 m*M*	66.23 ± 6.35 (n = 10)[b]
$MgCl_2$	10.0 m*M*	100.30 ± 24.24 (n = 9)[c]
Mannitol	10.0 m*M*	108.78 ± 17.71 (n = 9)[c]
α-Tocopherol	1.0 m*M*	139.89 ± 15.11 (n = 3)

[a] Means ± SEM (number of experiments).
[b] $p < 0.001$. [c] $p < 0.05$. Unpaired Student's t test compared to control group.

The present studies have clearly demonstrated that cerebral cortical astrocytes in primary culture were injured by severe hypoxia. The change in cell morphology was dramatic (Fig. 1). Destruction of intact cells, e.g., swelling and disintegration of the cell to cell contact in culture, was time dependent. Biochemical measurements corroborated the morphological observation. Between 12 to 18 h of severe hypoxia, the LDH content of the medium increased and the uptake of glutamate decreased. Inhibition of glutamate uptake indicates derangement of one of the important biochemical functions of astrocytes in situ (Hertz, 1982). The inhibition may be caused by an energy failure of hypoxic astrocytes (Rothman and Olney, 1986), or by disruption of the integrity of the cell membrane, which is indicated by the leakage of the intracellular LDH into the extracellular medium. The dysfunction was further evidenced by the cell swelling and the loss of protein in the hypoxic culture. These changes were observable and measurable only when the PO_2 in the medium was below 25 mm Hg but above 0. This implies that severe hypoxia but not anoxia is required to damage cultured astrocytes.

The observed inhibition of glutamate uptake agrees with the findings of others, i.e., during hypoxia and other pathological conditions, uptake of this neurotransmitter amino acid was inhibited, but its release was enhanced (Arnfred and Hertz, 1971;

Benveniste et al., 1984; Hirsch and Gibson, 1984; Chan et al., 1985). We have shown previously in a similar astrocytic preparation that glutamate uptake is inhibited by the presence of arachidonic acid and other polyunsaturated fatty acids (Yu et al., 1986). These fatty acids are known to be released under various insults to the brain. The findings further support the intimacy between glutamate and free fatty acids in the process of cell damage under various pathological insults. The defect of glutamate uptake in astrocytes would lead to an accumulation of this excitotoxic amino acid in the extracellular space and subsequent receptor-mediated neuronal cell death (Olney, 1983; Meldrum, 1985; Rothman and Olney, 1986). This may explain the observation of Benveniste et al. (1984) that anoxia induced an increase in the concentration of extracellular glutamate in hippocampus in vivo. In addition, high concentrations of extracellular glutamate can induce depolarization of astrocytes (Bowman and Kimelberg, 1984) that would open up ion channels (Kimelberg and Ransom, 1986) and allow the entry of sodium and water into the cells. This would result in astrocytic swelling, which may lead to cell lysis.

The mechanisms for hypoxia-induced astrocytic injuries are not clear at present. There are a number of factors known to play a role. They are extracellular K^+ (Moller et al., 1974), lactic acid and/or pH (Siesjö, 1985; Norenberg et al., 1987), ammonia (Norenberg, 1981), excitatory neurotransmitter amino acids (Rothman and Olney, 1986; Chan et al., 1979, 1988*b*), free fatty acids and oxygen radicals (Chan and Fishman, 1985), and factors affecting sodium pump activity that would lead to shifts of electrolytes between the intracellular and extracellular compartments (Siesjö, 1985). It has been shown that the increase in intracellular calcium and chloride following the increase of sodium influx with various insults is important for cell swelling and cell death (Kimelberg and Ransom, 1986; Rothman and Olney, 1986). This is especially true in the case of calcium, which damages cells by several mechanisms (Baudry et al., 1981; Farber et al., 1981; Wolfe, 1982; Rothman and Olney, 1986). The beneficial effects of verapamil and furosemide on hypoxic cells demonstrated that hypoxic astrocytic cell damage may involve both calcium and chloride. The protective mechanism of Mg^{2+} may be related to its ability to bind to the Ca^{2+} channels. High concentrations of magnesium can interfere with transmitter release and selectively block the N-methyl-D-aspartic acid (NMDA) receptor-associated Ca^{2+} channels (Kass and Lipton, 1982; Rothman, 1983; Nowak et al., 1984; Choi, 1987). However, the existence of the NMDA receptor in cultured astrocytes is unknown (Chan et al., 1987). The possible action of Mg^{2+} on NMDA receptor-mediated Ca^{2+} channels requires further elucidation.

The finding of increased levels of MDA content in hypoxic cells indicates lipid peroxidation, which is a process closely related to free radical formation and release of polyunsaturated fatty acids, especially arachidonic acid (Chan and Fishman, 1985). We have previously reported that arachidonic acid and its radical metabolites are key determinants of membrane injury in astrocytes (Chan et al., 1988*b*). Furthermore, arachidonic acid is a precursor to prostaglandins, thromboxane, and leukotrienes (Moncada, 1983; Chan and Fishman, 1985), which are known to play a role in various insults. The protective effect of indomethacin, a cyclooxygenase inhibitor, may indicate the involvement of these eicosanoids in hypoxic astrocytic injury. The lack of protective effect of free-radical scavengers, e.g., SOD, catalase, and α-tocopherol, on hypoxic astrocytes does not contradict the hypothesis of oxygen radical involvement in the injury because the site of action of radical formation during hypoxia appears to be intracellular. Exogenously added SOD and catalase may not easily penetrate the intact cell and reach the targets for their scavenging action. One possible way to clear this uncertainty is to use a carrier such as liposome to transport exogenous specific radical scavengers into the cells. Chan et al. (1988*b*) have shown that liposome-entrapped SOD reduces intracellular formation of superoxide radicals in astrocytes. Mannitol, a hydroxyl radical scavenger, slightly but significantly delayed the structural perturbation of hypoxic astrocytes. These data suggest that hydroxyl radicals may be involved in the development of hypoxic injury in astrocytes.

The studies of the protective effects of the several agents on hypoxic astrocytes demonstrated that some of them are beneficial. Each of these compounds has its own specific effect on a particular mechanism. Although they slowed down the hypoxia-induced astrocytic damage, none of them completely prevented the release of LDH. However, in the accompanying study, we have demonstrated that fructose-1,6-diphosphate (FDP) in the presence of hypoxia completely prevented the release of LDH from hypoxic astrocytes within the experimental period (Gregory et al., 1989). Therefore, FDP may prove to be a useful tool in the study of the cellular mechanisms of hypoxic injury in cultured astrocytes.

In conclusion, for years it has been known that hypoxia causes cellular damage in vivo. We have

used a model that allows the effects of severe hypoxia to be studied in astrocytes without the complication of other physiological factors such as hypertension, hypo- or hyperglycemia, the blood–brain barrier, etc. These studies have clearly shown that severe hypoxia perturbed both structure and function of astrocytes in culture, causing problems such as swelling, loss of cell membrane integrity, and decreased uptake of extracellular glutamate. The mechanisms involved in the cellular damage are multifactorial.

Acknowledgment: We thank Dr. R. A. Fishman for his criticism, Dr. Sylvia F. Chen for her technical help, and Ms. Dianne Esson and Dr. R. F. Jansen for help in the word processing of this manuscript. This work was supported by NIH Grants NS-14543, NS-25372, and NS-26092.

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