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Pyruvate Reduces the Necrotic but not Apoptotic Component of OGD/Reoxygenation Injury in Primary Murine Cortical Neuronal Cultures

Hokyou Lee[1], Jong Youl Kim[1,2], Jong Eun Lee[1,2], Won Taek Lee[1] and Kyung Ah Park[1]*

[1]Department of Anatomy and [2]BK21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Korea

ABSTRACT

Pure neuronal cell cultures from fetal mice (15-day gestation) were cultured in order to investigate the effects of pyruvate in hypoxia. Primary neurons were injured in proportion to the oxygen concentration and duration of hypoxia in the culture. When concentrated pyruvate was supplied in the oxygen-glucose deprived condition (OGD), cell injury was prevented. In contrast, pyruvate did not attenuate apoptosis when supplied during reoxygenation after OGD. Hoechst-PI nuclear staining and Annexin-PI FACS analysis data were consistent with these observations. Although lactate, after being converted to pyruvate, goes through the same metabolic pathway as pyruvate, it did not increase cellular ATP levels as much as pyruvate, and did not induce apoptosis. We also investigated the expression of LDH isotypes, which catalyze the final step of anaerobic metabolism, glycolysis, in OGD primary cultured neurons with and without pyruvate or lactate. The expression of LDH-1 and LDH-5 was distributed differentially under OGD and reperfusion. During oxygen-glucose deprivation, LDH-1 was predominant and LDH-5 was often not detected at all. However, LDH-5 was the dominant isotype during reoxygenation.

Key words: pyruvate, neuroprotection, OGD, reperfusion injury, cortical neuron

INTRODUCTION

Because brain cells use only glucose as a nutrient for energy, it is necessary to understand how glucose metabolism and neuronal activity are tightly coupled to blood flow and energy metabolism. Interruption of the blood supply to the brain results in energy failure. Such death may occur through either necrosis or apoptosis. Ischemia and/or metabolic impairment accompany traumatic central nervous system injury (Fujita and Ueda, 2003). The ischemic area induced by middle cerebral artery occlusion can be divided into an ischemic core and a penumbra. In the core, there is a rapid necrotic death, whereas in the penumbra apoptosis occurs more slowly (Ferrer and Planas, 2003). Since necrotic cells induce secondary damage, due to damage of surrounding cells, while apoptotic cells do not (MacManus and Linnik, 1997), a switch in the mode of cell death from necrosis to apoptosis could play a role in ameliorating the spread of cell death in the brain. The evolution from reversible to ir-

*To whom correspondence should be addressed.
TEL: 82-2-2228-1643, FAX: 82-2-365-0700
e-mail: kapark@yumc.yonsei.ac.kr

reversible damage in necrosis involves progressive derangements of energy and substrate metabolism. During ischemia, a deficiency of metabolites to the brain is the primary factor which initiates a cascade of cellular events, leading to neurodegeneration through both apoptotic and necrotic mechanisms (Zipfel et al., 2000). Under anaerobic conditions, glycogen is rapidly depleted (Swanson, 1992), and the TCA cycle cannot operate in the absence of oxygen, so an alternative source of NAD^+ is provided through the conversion of pyruvate to lactate. This production of lactate has long been considered a by-product of maintaining glycolysis and, furthermore, lactate has been implicated in acidosis, which may contribute to neuronal degeneration (Siesjo et al., 1996).

Although the molecular mechanisms underlying apoptosis are now well characterized, little is known about the mechanisms underlying necrosis. Most recently, it has been demonstrated that some neuronal conditioned medium factors switch the cell death mode from necrosis to apoptosis (Ueda and Fujita, 2004). This switch in the mode of cell death is correlated with cellular ATP levels, as it is altered by various reagents (Eguchi et al., 1997; Leist et al., 1997; Ueda and Fujita, 2004). Since high energy levels are required for the execution of apoptosis but not for necrosis, ATP may have a key role in regulation the mode of cell death. However, it remains to be seen whether an increase in cellular ATP levels might switch necrotic cell death to apoptotic cell death.

Lactate dehydrogenase (LDH) is the enzyme that catalyzes the final step of glycolysis. LDH is a tetramer composed of four polypetide chains. There are five component isoenzymes as a result of the five different combinations produced by two polypeptide chains encoded by separate genes (M and H; Bittar et al., 1996). LDH-1 is composed of four H subunits, and LDH-5 of four M subunits. The prevailing type of LDH varies according to tissue type. In the heart, for example, the H gene is more active than the M gene, the latter being strongly expressed in the skeletal muscle. As the number of M over H chains increases, the LDH isoenzyme becomes more efficient in catalyzing the conversion of pyruvate to lactate (LDH-5), while an increase in H over M chains (LDH-1) favors the conversion of pyruvate to acetyl-coenzyme A that enters the citric acid (Krebs) cycle (Bittar et al., 1996; Koukourakis et al., 2003). In the adult brain, LDH-1 is predominant, thus supporting the notion that the brain relies mostly on aerobic metabolism (Poitry-Yamate et al., 1995; Laughton et al., 2000). It is of interest, however, that differential distribution of LDH-1 and LDH-5 mRNA suggests that the brain might contain two metabolically distinct populations of cells. One population, expressing the LDH-1 form, would act as a lactate sink by using lactate as an energy fuel to support oxidative metabolism. Another population, expressing the LDH-5 form could represent, in part, astrocytes and would act as a lactate source and rely on glycolytic metabolism (Poitry-Yamate & Tsacopoulos, 1992; Bittar et al., 1996).

In this study, we investigated whether a switch in the mode of cell death is affected by an increase in cellular ATP levels, or associated with the distribution of LDH isotypes, by adding pyruvate under oxygen deprivation. By better understanding the changes in neurons based on the duration of exposure to hypoxia or anoxia, and the mechanisms controlling pyruvate metabolism, it may be possible to design and test alternatives that can protect neural cells from injury induced by hypoxia or anoxia.

MATERIALS AND METHODS

Primary neuronal cell culture

Cell cultures were prepared from fetal mice (15 day gestation). Neocortices were freed of meninges and blood vessels. Tissue was suspended in medium, trypsinized, triturated and plated in 24-well plates coated with poly-D-lysine and laminin at a density of 3.5 hemispheres per plate. Twenty four to 48 hours after plating, 60% of the culture medium was replaced with glial conditioned medium (GCM) and cytosine arabinoside (Ara-C, 3 mM). Cultures were used on day 10.

Anoxic and hypoxic injury

Cultures were transferred to an anaerobic chamber (Forma Scientific Co) (O_2, tension $<0.2\%$ (0 ppm), 5% (1.5 ppm), 10% (3.0 ppm) and 20% (6.1 ppm)), washed in deoxygenated, glucose-free balanced salt solution (BSS_0) and glucose-balanced

salt solution ($BSS_{5.5}$), and incubated at 37°C for 0.5, 1, 2, 5, and 10 hrs. OGD was stopped by adding glucose to a final concentration of 5.5 mM and returning cultures to the normoxic incubator for 24 h.

Treatment of glucose metabolites

Glucose (0 mM, 5.5 mM), pyruvate 6 mM, and lactate 6 mM were added to the culture medium 30 minutes before and after anoxic or hypoxic injury. Injury was assessed at the end of reperfusion.

LDH analysis

Cell lysis was quantified by assay of lactate dehydrogenase (LDH) activity released into the culture medium (Kho and Choi, 1987). Total LDH release corresponding to complete astrocyte death was determined at the end of each experiment following freezing at −70°C and rapid thawing.

Hoechst-propidium iodide nuclear staining

Injury was evaluated morphologically by phase-contrast light microscopy and staining of non-viable cells with propidium iodide and live cells with Hoechst 33258 dye. Nuclear morphology and membrane integrity were assessed simultaneously. Cells were observed using an Olympus diaphot microscope equipped for epifluorescence with a UV filter block.

Annexin V-FITC/propidium iodine flow cytometric analysis

Flow cytometric determination of apoptosis was performed using a commercially available Annexin V-FITC/propidium iodine apoptosis detection kit (R&D Systems, Minneapolis, MN). Untreated and treated cells were collected after 24 hours of incubation by trypsinization and centrifugation at 500×g for 5～10 minutes at room temperature. Cells were washed and resuspended in ice cold PBS and pelleted by centrifugation. Cells were then resuspended in Annexin V incubation reagent at a concentration of 1×10^6 cells/ 100 ul, and incubated in the dark for 15 minutes at room temperature. Binding buffer was then added to each sample. Samples were analyzed within one hour by flow cytometry, and evaluated based on the percentage of cells staining low or high for Annexin V (apoptotic cells) and propidium iodide (necrotic cells). Measurements of fluorescence were made with a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry systems, USA) using a 488 nm argon ion laser.

Reverse transcription-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed to estimate mRNA levels of LDH-1 and -5 isotypes. Primers used were LDH-1 (5'- CGTCGTCTGCGACTGGCGCAATGAG-CTTTTCCTTAAGGGTTGC-3' and 5'- GTCCTTCAG-CTTCTGGTTGATGACGCTGGTCAGTCCCCGAGCA TT -3'), LDH-5 (5'- CTTAAGAAGGATTCACAATCA-GCTGGTCCTTGAGGGCTGCC -3' and 5'- CTGCT-CCTTGTCTGCATCCGTGCCCAGCTGCGGG -3'). G-APDH cDNA was amplified with sense 5'-ATGTCG-TGGAGTCTACTGGT-3' and antisense 5'-TGGCAT-GGACTGTGGTG-3' primers.

Western blot

Expression of GAPDH proteins was estimated by immunoblotting of cultured neurons after OGD injury. Western blot analysis was performed using anti-GAPDH (ab8245; abcam, Cambridgeshire, UK), and anti-actin antibodies (Santa Cruz Biotechnology, CA). Equal amounts of protein, 50 μg per lane, were separated on an 10% polyacrylamide gel and electrotransferred onto Immobilon-NC membrane (Milipore, MA). Immunoreactive bands were visualized with the ECL detection system using Kodak X-AR film.

Statistical analysis

Data were expressed as the mean±SD. Statistical tests to determine differences between groups were performed using SigmaStat (Jandel Corp., CA). ANOVA followed by a multiple comparisons procedure, the Student-Newman-Keul's test was used.

RESULTS

Pyruvate treatment during reoxygenation induced apoptosis after oxygen-glucose deprivation

The cortical neurons were injured during oxygen-glucose deprivation (OGD). Addition of pyruvate during oxygen-glucose deprivation (OGD) increased

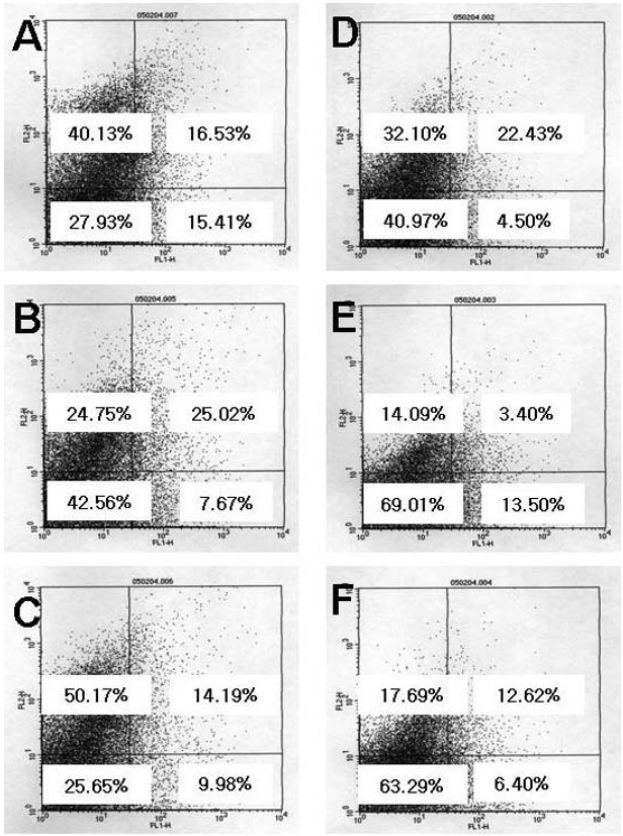

Fig. 1. Representative fluorescence-activated cell sorter (FACS) analysis of apoptosis in primary cultured neurons treated with glucose metabolites under oxygen-glucose deprivation. Lower right quadrants of the box (Annexin V positive and PI negative) represent percentages of apoptotic cells with preserved plasma membrane integrity, and upper right quadrants (Annexin V positive and PI positive) represent necrotic or late-stage apoptotic cells. Cells that are only PI positive (upper left quadrant) are necrotic. Untreated cells did not stain with Annexin V or PI, suggesting that most were intact, live cells (X axis: Annexin V, Y axis: PI). (A) Untreated neurons. (B, C) Neurons treated with glucose metabolites during oxygen glucose deprivation (OGD). (D, E, F) Neurons treated with glucose metabolites during reoxygenation after OGD injury. (D) Neurons treated with glucose (11 mM). (B, E) Neurons treated with pyruvate (6 mM). (C, F) Neurons treated with lactate (6 mM).

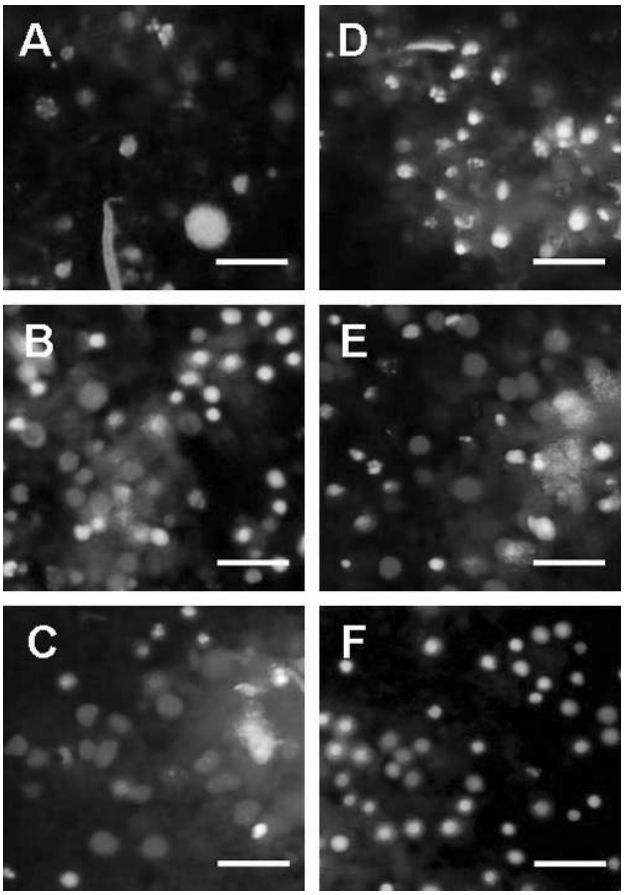

Fig. 2. Photomicrograph of primary cultured cortical neurons treated with pyruvate (6 mM) and lactate (6 mM) during reoxygenation after oxygen-glucose deprivation. Cells were stained with propidium-iodide PI and Hoechst dye 24 h (A, B, C) and 48 h (D, E, F) after insults. (A, D) Untreated neurons. (B, E) Neurons treated with pyruvate (6 mM) during reoxygenation after OGD. (C, F) Neurons treated with lactate (6 mM) during reoxygenation after oxygen glucose deprivation (OGD). Bar=25 mm.

the survival of cortical neurons, but lactate didn't (Fig. 1A～C). In contrast, pyruvate induced apoptosis in the cells when supplied during reoxygenation after OGD (Fig. 1D～F, 2).

To evaluate apoptosis, cultured neurons treated with pyruvate (6 mM) and lactate (6 mM) during reoxygenation of OGD were stained with Annexin V and propidium iodide (PI), an important marker for distinguishing early apoptosis and necrosis. Untreated cells were not stained by Annexin V and PI, suggesting that most of them were intact live cells. The percentage of apoptosis in neurons treated with pyruvate during reoxygenation was higher than that of the untreated or lactate treated groups (Fig. 1E).

Pyruvate increased cellular ATP levels after oxygen-glucose deprivation

We hypothesized that the fundamental cause of ATP exhaustion might be closely associated with glucose metabolism. Addition of pyruvate increased the cellular ATP levels in cortical neurons during oxygen-glucose deprivation (OGD) by addition of pyruvate (Fig. 3), whereas addition of lactate decreased ATP levels. Pyruvate enhanced the survival of cortical neurons under OGD, but induced apop-

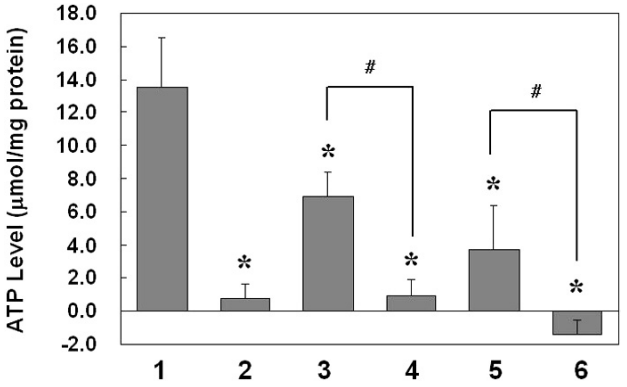

Fig. 3. Cellular ATP levels in cultures treated with different glucose metabolites after oxygen-glucose deprivation injury. Adenosine triphosphate (ATP) levels were calculated from standard ATP solution. All data are mean±S.D from three independent experiments. *: $p<0.01$ compared to untreated group; #: $p<0.01$ compared to lactate treated group. 1: Normal control neurons. 2: Untreated neurons after oxygen glucose deprivation (OGD) injury. 3, 4: Neurons treated with glucose metabolites during OGD. 5, 6: Neurons treated with glucose metabolites during reoxygenation after OGD injury. 3, 5: Neurons treated with pyruvate (6 mM). 4, 6: Neurons treated with lactate (6 mM).

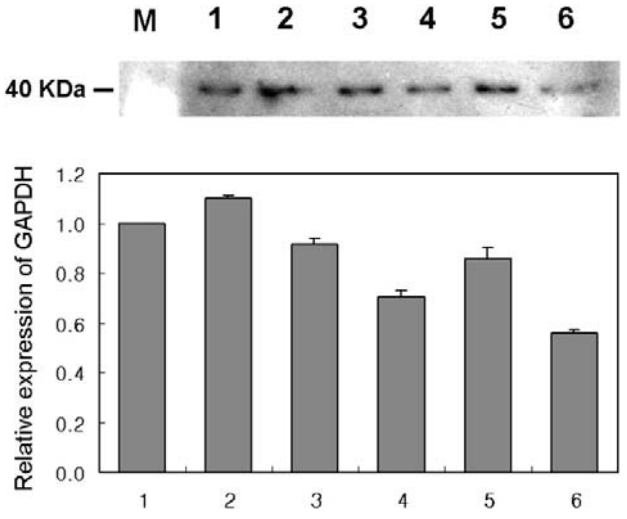

Fig. 4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in primary cultured neurons treated with glucose metabolites during oxygen-glucose deprivation injury. 1: Normal control. 2: Untreated neurons after oxygen glucose deprivation (OGD) injury. 3, 4: Neurons treated with glucose metabolites during OGD. 5, 6: Neurons treated with glucose metabolites during reoxygenation after OGD injury. 3, 5: Neurons treated with pyruvate (6 mM). 4, 6: Neurons treated with lactate (6 mM).

tosis when added during reoxygenation. This switch in the mode of cell death by addition of pyruvate during reoxygenation occurred simultaneously with a decrease in cellular ATP levels (Fig. 3). Since high energy levels are required for the execution of

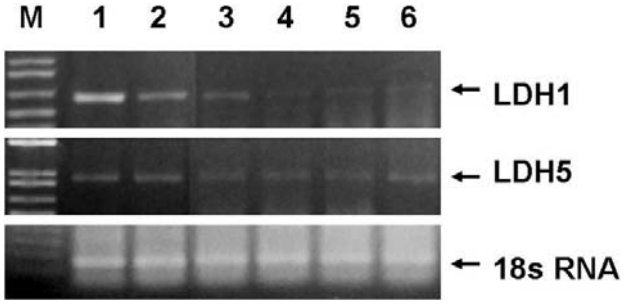

Fig. 5. RT-PCR analysis of lactate dehydrogenase (LDH) isotypes. Products were loaded onto 2% TAE-agarose gels containing ethidium bromide. The left lane (M) represents DNA markers. 1: Normal control. 2: Untreated neurons after oxygen glucose deprivation (OGD) injury. 3, 4: Neurons treated with glucose metabolites during OGD. 5, 6: Neurons treated with glucose metabolites during reoxygenation after OGD injury. 3, 5: Neurons treated with pyruvate (6 mM). 4, 6: Neurons treated with lactate (6 mM). Values were normalized using the 18s ribosomal RNA signal observed under identical RT-PCR conditions. All data were obtained from three independent experiments.

apoptosis but not for necrosis, ATP may play a key role in regulating the mode of cell death.

Inhibition of hypoxia-induced GAPDH expression by pyruvate

Under the assumption that the fundamental cause of ATP exhaustion might be closely associated with glucose metabolism, we also investigated the effect of glucose, pyruvate and lactate on the expression of GAPDH in the neurons. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) regulates ATP synthesis during glycolysis under hypoxia or anoxia. We measured GAPDH levels in cultured primary neurons treated with glucose metabolites during OGD or reoxygenation after OGD. GAPDH expression was reduced in neurons treated with lactate during OGD or reoxygenation. However, pyruvate attenuated this suppression of GAPDH expression and partially prevented ATP depletion (Fig. 3, 4).

Expression of lactate dehydrogenase LDH-1 and LDH-5 isoforms in primary cultured neurons and astrocytes during oxygen-glucose deprivation and reoxygenation-reperfusion

We assessed LDH isoform expression in primary cultured neurons adapted to altitude and low oxygen availability (O_2, tension $<0.2\%$, 0 ppm), with or without pyruvate and lactate. LDH-1 and LDH-5 were differentially distributed under OGD

and reperfusion (Fig. 5). During oxygen-glucose deprivation, LDH-1 was the predominant enzyme for glycolysis, while LDH-5 was predominant during reoxygenation.

DISCUSSION

In this study, we observed that cultured primary neurons are protected from oxygen-glucose deprivation (OGD) by the addition of pyruvate. Pyruvate, in particular, showed favorable effects that made it a favorable alternative to glucose. Although the potential of pyruvate as a drug has not yet been well studied, many researchers are conducting studies, as ATP is now considered to be a major cause of cell death (Ying et al., 2002; Leong et al., 2003; Wang wt al., 2003). Glycogen in the brain is metabolized into lactate, acidifying the intracellular environment and producing free radicals that damage cells and serve as the main cause brain cell death (Ying et al., 2002). In this study, supplying pyruvate exogenously during oxygen-glucose deprivation (OGD) could prevent neuronal death when cells were exposed to anoxia. However, when supplied during reoxygenation after OGD, pyruvate induced apoptosis. We demonstrated that this switch of cell death mode by addition of pyruvate during reoxygenation coincided with a decrease in cellular ATP levels, suggesting that the fundamental cause of ATP exhaustion might be closely associated with glucose metabolism.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) regulates ATP synthesis during glycolysis under hypoxia or anoxia (Sheline and Choi, 1998). In this study, we measured GAPDH levels in primary cultured neurons treated with glucose metabolites during OGD or reoxygenation after OGD. GAPDH expression was reduced in neurons treated with lactate during OGD or reoxygenastion. Addition of pyruvate, however, attenuated this suppression of GAPDH expression and partially prevented ATP depletion. ATP depletion occurs through the blockade of the NAD+-dependent, glyceraldehyde-3-phosphate dehydrogenase step of glycolysis (Szabo and Dawson, 1998; Sheline et al., 2000). Blockade at this step would limit both production of glycolytic ATP and the flux of glucose carbon into the mitochondrial tricarboxylic acid (TCA) cycle. If blockade of glycolytic flux by NAD+ depletion is a major factor in neuronal cell death, it should be possible to rescue cells by supplying substrates that enter the TCA cycle by routes other than glycolysis (Leong et al., 2003). Here we show that pyruvate prevented neuronal cell death when administered during OGD or reoxygenation after OGD. Our findings suggest that delivery of pyruvate may be a useful strategy for rescuing neurons from OGD insult.

Under acute hypoxic conditions, some researchers have demonstrated overexpression of genes encoding proteins involved in the homeostatic response to hypoxia, i.e., anaerobic metabolism (Semenza, 2000). Overexpression of these genes is essentially due to the stabilization of hypoxia inducible factor 1 (HIF-1; Wang and Semenza, 1993). Among these genes, some encoding glycolytic isozymes such as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase A are up-regulated at the transcriptional level (Semenza et al., 1996). It seems logical that in low oxygen conditions, the glycolysis rate would increase in order to compensate for oxidative energy metabolism decay. Since glycolysis produces only two molecules of ATP per glucose oxidized, glucose transport has to be improved to maintain a constant energy supply (Loike et al., 1992), and pyruvate to lactate transformation must be efficient in order to keep a high NAD+/ NADH ratio and therefore to allow continued glycolytic flux (Sheline and Choi, 1998). In cells, pyruvate reduction is catalyzed by lactate dehydrogenase (LDH; E.C. 1.1.1.27), so we explored regulation of LDH isotype expression in neurons treated with pyruvate and lactate. LDH (EC 1.1.1.27) catalyzes the interconversion of pyruvate and lactate, and is thus essential for both production and utilization of lactate. It is a tetrameric enzyme which can consist of three different subunits arising from three distinct genes: LDH-A (or M for muscle, or LDH-5), LDH-B (or H for heart, or LDH-1) and LDH-C (or X; Bittar et al., 1996; Koukourakis et al., 2003). The LDH-A and LDH-B gene products are more widely expressed, and both are found in the CNS (Gerhardt-Hansen W., 1968). They combine together to form five distinct isoenzymes: LDH-1 (or B4), LDH-2 (or A1B3), LDH-3 (or A2B2), LDH-4 (or A3B1) and LDH-5 (or A4). We

showed that the expression of LDH isotypes was distributed differentially during OGD or reperfusion injury. The LDH-1 form was predominant during oxygen-glucose deprivation for glycolysis and LDH-5 was predominant during reoxygenation. Expression of the various isoenzymes is often taken as a reflection of its metabolism. Tissues such as skeletal muscle which are enriched in LDH-5 subunits are indeed more glycolytic, while tissues such as heart which highly express LDH-1 have predominantly oxidative metabolism (Markert et al., 1975). This conclusion partly stems from differences in the catalytic properties of the isoenzymes. The LDH-1 isoenzyme has a high affinity for its substrate and is allosterically inhibited by high levels of pyruvate, while LDH-5 has a low affinity but a high capacity for its substrate and is not allosterically inhibited (Bishop et al., 1972; Nitisewojo and Hultin, 1976). Other isoenzymes display intermediate properties between these two extremes in relation to the ratio of LDH-1 and LDH-5 subunits. Thus, it is considered that LDH-1-enriched isoenzymes are better suited to convert lactate into pyruvate (hence their enrichment in oxidative tissues), while LDH-5-enriched isoenzymes are better adapted for environments where large amounts of lactate are produced (such as in glycolytic tissues). The potential role of lactate as an important energy substrate in the CNS has recently attracted significant interest (Magistretti et al., 1999).

It has been proposed that an intercellular exchange of lactate could take place between brain cells (Bittar et al., 1996; McKenna et al., 1993; Pellerin and Magistretti, 1994). One population of cells would be more glycolytic and act as a lactate source, while another would be predominantly oxidative and consume lactate. Results obtained thus far are consistent with the notion that astrocytes are the predominant lactate-producing cells, while neurons are predominantly those using lactate as an energy source (Magistretti et al., 1999). A few other studies which separated the various isoenzymes by electrophoresis have indicated, however, that LDH-1-containing isoenzymes are the most abundant in the adult brain (Gerhardt-Hansen, 1968; Maker et al., 1972; Laughton et al., 2000). Our results are consistent with previous studies. We have demonstrated that LDH-1 and LDH-5 were distributed differentially under OGD and reperfusion. During OGD, the LDH-1 form was predominant, and LDH-5 form was predominant during reoxygenation.

As mentioned previously, the LDH-1 isoform is usually enriched in oxidative tissues, and thus expected to be expressed in areas where markers of oxidative metabolism, such as cytochrome oxidase, should also be abundantly present (Friede and Fleming, 1963). Our results with LDH-1 in this study are compatible with this model. Cellular localization of LDH isoform mRNAs in the brain has been performed (Laughton et al., 2000), but cannot provide a definitive answer, since the overall distribution of LDH-1 mRNA would be a major neuronal localization. In our study, however, primary cultured neurons expressed both LDH-1 and LDH-5 mRNA, and their expression was different depending on the oxygen environment. Developmental studies have supported our result that, although the LDH-5 form is predominant in the early postnatal period, it is gradually overcome by the LDH-1 form, a change which is apparently related to neuropil growth, synapse formation, myelination and increase in aerobic metabolism (Bonavita et al., 1964; Maker et al., 1972). Interestingly, recent reports have demonstrated that glial cells appear to contain a substantial amount of the LDH-5 form (Tholey et al., 1981; Venkov et al., 1976) and are generally considered to be metabolically more glycolytic, since they produce large amounts of lactate (Tsacopoulos and Magistretti, 1996). This is supported by more recent observations indicating that LDH-5 immunoreactivity is abundant in astrocytes from both the hippocampus and the visual cortex of human brain (Bittar et al., 1996). We didn't observe the expression of LDH-1 and LDH-5 in cultured astrocyte in this study. It is clear, however, that such an interpretation does not exclude the possibility that neurons express LDH-5 mRNA, as there is indeed evidence that neurons do contain LDH-5-containing isoforms with a subcellular distribution distinct from the LDH-1-containing isoforms (Brumberg and Pevzner, 1975).

In this study, we showed that neurons are injured in proportion to the duration of exposure to hypoxia and oxygen concentration in the culture. Oxygen deprivation and glucose administration were closed associated. When exposed to anoxia/hypoxia, cell

injury was prevented by the addition of high concentrations of glucose. High concentrations of pyruvate could also prevent injury. Meanwhile, the protective effect of glucose metabolites was enhanced when glucose was already present in the culture. Expression of certain LDH isotypes, which catalyze the final step of the anaerobic metabolic pathway, is associated with the protective effect of pyruvate. Further investigation and study of substances which protect neurons from OGD injury may lead to development of therapeutic agents to prevent loss of neurons that do not reproduce, and may reveal a means for the protection of neural cells from brain damage.

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