Journal of Korean Medical Science
Vol 7, No. 1, 40-46 March 1992

The Effect of Hyperglycemia on Lipid Peroxidation in the Global Cerebral Ischemia of the Rat

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To investigate the influence of hyperglycemia on ischemic brain damage, we measured brain ATP, lactate and malondialdehyde (MDA) levels in global cerebral ischemic models of Wistar rats. We induced global cerebral ischemia by the 4-vessel occlusion method. After 30 or 60 min of occlusion, and after 30 min of reperfusion, we measured brain ATP, lactate and MDA levels. During the ischemic period, brain ATP levels decreased to 30-70% of sham groups both in normoglycemic and hyperglycemic groups. But during the reperfusion period, the recovery rate of ATP levels was significantly lower in the hyperglycemic than in the normoglycemic groups ($p<0.05$). After 60 min of global ischemia, brain lactate increased much more in the hyperglycemic than in the normoglycemic group, and, during reperfusion, was washed out slowly in the hyperglycemic group. The MDA level, a parameter of lipid peroxidation, increased more in the hyperglycemic group than in the normoglycemic group during reperfusion periods ($p<0.05$).

We conclude that hyperglycemia increases lactate accumulation, delays the recovery of energy metabolism, and enhances the lipid peroxidation in the transient global ischemia of rat brain. These findings may suggest the harmfulness of hyperglycemia in clinical cerebral ischemia.

Key Words: *Global Cerebral Ischemia, ATP, Lactate, MDA, Lipid Peroxidation*

INTRODUCTION

Considerable evidence exists that the brain damage induced by ischemia is enhanced by preischemic hyperglycemia, probably because the hyperglycemia exaggerates lactic acidosis during the ischemia (Myers, 1979; Siesjö, 1982 and 1984; Plum, 1983). Intracellular acidosis is considered to be a critical factor in ischemic cell damage (Kraig et al., 1985 and 1986). Several metabolic studies in animal model of transient global ischemia have suggested that high glucose levels accentuate cell damage by further enhancing anaerobic glycolysis and lactic acidosis, which result in severe intracellular acidosis incompatible with cell survival (Ginsberg et al., 1980; Welsh et al., 1980; Lassen, 1966). One possible molecular mechanism involved is enhancement of free radical formation and lipid peroxidation. Bernheim (1963) showed that lowering pH of the brain homogenate enhanced formation of thiobarbituric acid reactive (TBAR) material, and postulated that the effect of acidosis was to dissociate protein-bound iron (Barber and Bernheim, 1967). It has been shown that the production of lipid peroxides in brain homogenate was markedly enhanced by lowering of pH from 7 to 6 (Rehncrona et al., 1989; Siesjö et al., 1985; Stocks et al., 1974). Extrapolation of these results to in-vivo conditions is uncertain.

In order to investigate the effect of hyperglycemia, we measured lipid peroxidation using the thiobarbituric acid reaction and assessed the changes of brain lactate and ATP levels in global cerebral ischemic models of normoglycemic and hyperglycemic rats.

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This study was supported by the Research Fund of Seoul National University Hospital (1989).

MATERIAL AND METHOD

Classification of Experimental Groups

This study consists of 10 groups as follows: (1) normoglycemic (NG) sham group (n=10), (2) hyperglycemic (HG) sham group (n=10), (3) NG 30 min-ischemia group (n=10), (4) HG 30 min-ischemia group (n=9), (5) NG 60 min-ischemia group (n=10), (6) HG 60 min-ischemia group (n=10), (7) NG 30 min-ischemia and then 30 min-reperfusion group (n=9), (8) HG 30 min-ischemia and then 30 min-reperfusion group (n=10), (9) NG 60 min-ischemia and then 30 min-reperfusion group (n=10), (10) HG 60 min-ischemia and then 30 min-reperfusion group (n=10).

Induction of Ischemia and Subsequent Reperfusion

We used "four-vessel occlusion rat model" proposed by Pulsinelli and Brierly (1979). Ninety-eight about 20-week-old male Wistar rats weighing 250-350 g were used. Global cerebral ischemia was induced by the four-vessel occlusion (4-VO) method. In day 1, the animals were anesthetized by intraperitoneal injection of ketamine at 35-40mg/kg. Both vertebral arteries were electrocauterized at the first cervical vertebrae. The animals were allowed free access to water but food was withheld for 24 hours while the animals recovered from anesthesia. In day 2, the awake rats were restrained by brief exposure to ether. Atropine sulfate (0.1 mg) and heparin (100 I.U.) were injected intraperitoneally to prevent vago-reflex and coagulation. After recovery of consciousness, the bilateral common carotid arteries were clipped with surgical microclips to produce 4-VO. Animals that did not become unresponsive within 60 seconds after clipping were excluded from the study. This 4-VO was continued for 30 or 60 minutes. Recirculation of blood flow in reflow groups was established by releasing the clips after 30 or 60 minutes of global ischemia, and restoration of the carotid artery blood flow was verified visually. Reperfusion was allowed for 30 minutes. Body temperature was maintained at 37 ± 2°C with a rectal thermistor and heating lamp.

Induction of hyperglycemia

For the hyperglycemia study, 1.5-2.0 ml of 50% dextrose water was injected i.p. at 20-30 min before carotid clipping. Venous blood was taken for measurement of blood glucose just before carotid clipping.

Measurement of Cerebral ATP and Lactate

At various indicated times, the animals were decapitated and the brains were placed in liquid nitrogen and tissue ATP and lactate concentrations were measured.

Cerebral hemispheres were taken out rapidly and stored at -80°C for several days until analysis. The tissue was weighed and homogenized at -40°C in 20ml of a solution containing cooled 8% perchloric acid and 40% ethanol. The homogenate was centrifuged at 15,000 g for 10 min at -20°C. The pellet was diluted with 1 N NaOH for assay of protein contents by the method of Lowry et al. (1951). The supernatant was neutralized with K_2CO_3 and centrifuged at 15,000 g for 10 minutes at -20°C to remove the precipitates. The ATP concentration in the supernatant was determined by the enzymatic fluorometric method (Trautschold, 1985) and lactate by the standard enzymatic method using lactate dehydrogenase (Gawehn, 1985).

Measurement of Lipid Peroxidation

Lipid peroxidation from the brain tissue was determined by malondialdehyde (MDA) assay with a modified thiobarbituric acid (TBA) method of Masugi and Nakamura (1976). The tissue was homogenized with 10 mM ethylene diamine tetraacetate (EDTA) to chelate Fe^{++} ion under N_2 stream to protect in vitro lipid peroxidation. TBA-reactive substance (TBA-RS) was measured fluorometrically as MDA.

Methods of Statistical Analysis

Student's t-test, Mann-Whitney U test, Spearman rank correlation analysis and regression analysis were used in the statistical analysis.

RESULTS

Changes of Brain ATP Level

Table 1 shows the ATP depletion in ischemic brain in the NG and the HG condition. At 30 min of ischemia, brain ATP depleted to 2.10 ± 0.18 μmole/g wet wt. in the NG group (66.5% of NG sham group) and 2.25 ± 0.28 μmole/g wet wt. in the HG group (69.2% of HG sham group), and during 30 min of reflow the ATP levels recovered to 2.60 ± 0.42 μmole/g wet wt. in the NG group (82.3% of NG sham group) and 2.67 ± 0.29 μmole/g wet wt. in the HG group (82.2% of HG sham group). The recovery rates of 30 min-reflow after 30 min-ischemia were 15.8% in the NG group and 13% in the HG group. At 60 min of ischemia, ATP decreased markedly to 1.52 ± 0.22 μmole/g wet wt. in the NG group (47.8% of NG sham) and 1.13 ± 0.19 μmole/g wet wt. in the HG group (34.8%

Table 1. Changes in the levels of ATP, lactate and MDA in normoglycemic and hyperglycemic brains of rats during ischemia and reperfusion ‡

Ischemia (min)	Reflow (min)	ATP (μmole/g wet wt.)		Lactate (μmole/g wet wt.)		MDA (nmole/g wet wt.)	
		Normogly.	Hypergly.	Normogly.	Hypergly.	Normogly.	Hypergly.
0	0	3.16±0.41	3.25±0.40	7.78±0.46	8.01±0.88	10.87±1.63	11.15±1.23
30	0	**2.10±0.18	**2.25±0.28	10.19±1.42	*12.37±1.73	13.41±2.25	14.02±3.70
	30	2.60±0.42	*2.67±0.29	8.60±1.15	11.75±2.29	15.42±1.90	**18.62±3.81
60	0	**1.51±0.22	**1.13±0.19	**12.93±2.90	**17.97±3.23++	14.70±3.85	*17.54±3.48
	30	**1.80±0.23	**1.16±0.29	10.57±1.97	**17.59±2.55++	**19.90±4.12	**26.01±4.58†

Values are mean±SD. Significant differences are indicated (Scheffe Multiple Comparison test):
* $p<0.05$, ** $p<0.01$: vs. nonligated group (0 min group)
† $p<0.05$, †† $p<0.01$: vs. normoglycemic group
‡ See the 'Material and Method' for the experimental details.

of HG sham group), and restored to 1.80±0.23 μmole/g wet wt. in the NG group (57% of NG sham) and 1.16±0.29 μmole/g wet wt. in the HG group (35.7% of HG sham) after 30 min of reflow. The ATP recovery rates during 30 min-reflow after 60 min-ischemia were 9.2% in the NG group and 0.9% in the HG group, which showed a delayed recovery of brain ATP level during reperfusion in the HG group. The above data were summarized in Fig. 1.

Changes of Brain Lactate Level

While ATP was depleted, the lactate level in brain increased during the ischemia in both of the NG and the HG groups. As shown in Table 1, the level in the NG group during the first 30 min increased from 7.78±0.46 μmole/g wet wt. to 10.19±1.42 μmole/g wet wt. After another 30 min of ischemia, the level further increased to 12.93±2.90 μmole/g wet wt. The lactate level in the HG group also increased but the increase was significantly higher than that in the NG group; the control level was 8.01±0.88μ mole/g wet wt., and the levels at 30 min and 60 min of ischemia were 12.37±1.73 and 17.97±3.23μ mole/g wet wt. respectively in the HG group. During the reflow after 60 min-ischemia the lactate accumulation in the NG group decreased to the greater extent than in the HG group. The data above were summarized in Fig. 2.

Changes of Brain MDA Level

Changes in MDA level showed a different pattern from the above two parameters. As shown in Table 1, brain MDA increased gradually during the whole period of both ischemia and reflow. Particularly, the MDA level increased sharply during the reflow period after 60 min-schemia in both groups. But the level of MDA in the HG group was always higher at all the points of MDA assay, with the biggest difference at 30 min-reflow after 60 min-ischemia (19.90±4.12 nmole/g wet wt. and 26.01±4.58 nmole/g wet wt. in NG and HG groups, respectively, $p<0.05$). The data above were also shown in Fig. 3.

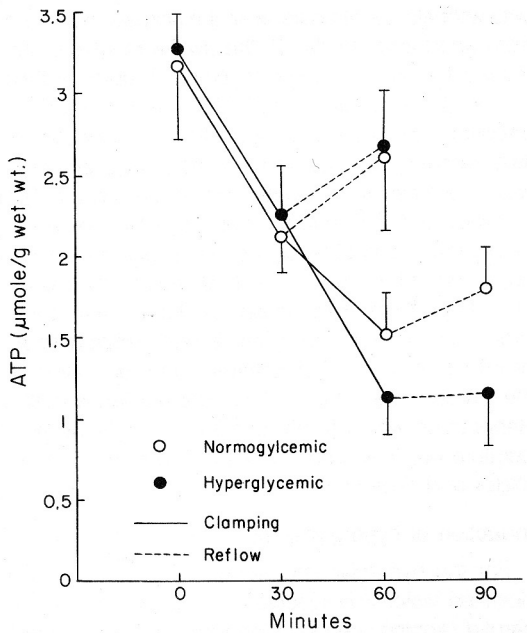

Fig. 1. Effect of hyperglycemia on the change of ATP level in the brain during ischemia and reperfusion. All the experimental conditions and data were the same as in Table 1.

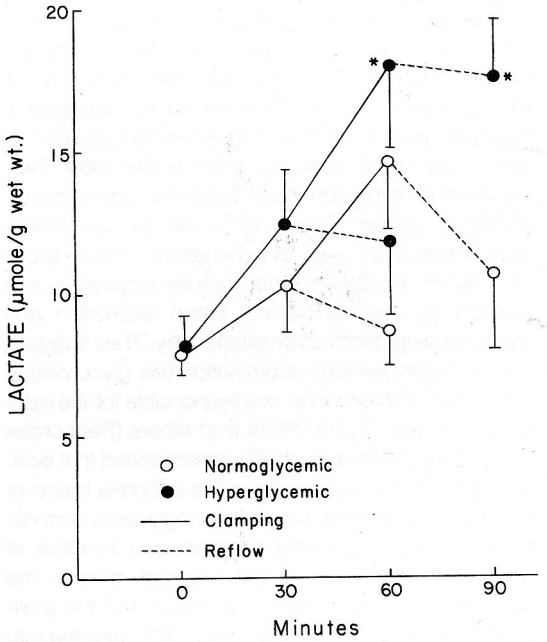

Fig. 2. Effect of hyperglycemia on the change of lactate in the brain during ischemia and reperfusion. All the experimental conditions and data were the same as in Table 1.
* vs. normoglycemic group: $p<0.01$ (student t-test)

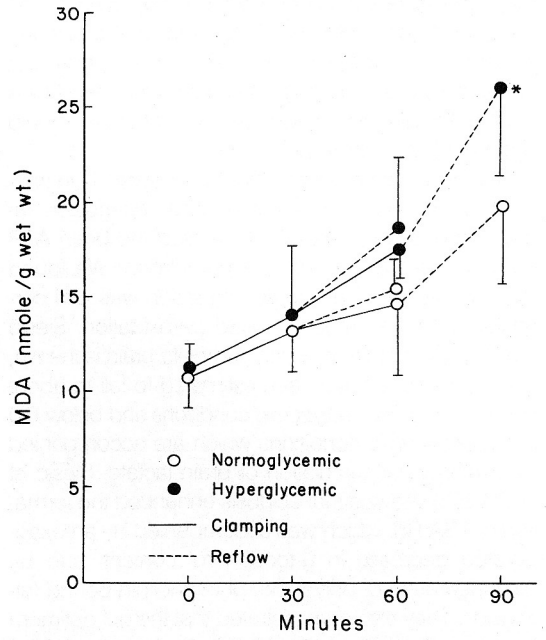

Fig. 3. Effect of hyperglycemia on brain MDA level in the brain during the ischemia and reperfusion. All the experimental conditions and data were the same as in Table 1.
* vs. normoglycemic group: $p<0.05$ (student t-test)

Relationship between Lactate and MDA Levels in the Ischemic Brain (Fig. 4)

To assess the state of correlation of lactate accumulation with the MDA rise in the ischemic brain, we performed correlation and regression analyses.

There was a good correlation between lactate and MDA levels ($r=0.64$, $p<0.001$). The regression line was showed in Fig. 4 ($Y=0.85X+6.2$).

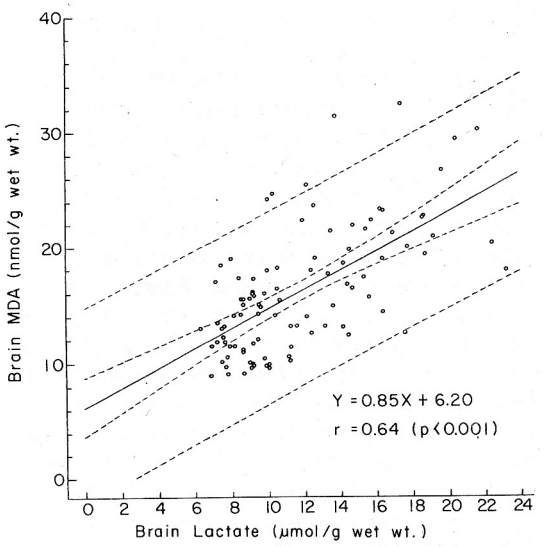

Fig. 4. Relationship between MDA and lactate levels in the brain during ischemia and repersufion ($r=0.64$, $p<0.001$).

DISCUSSION

Brain glucose concentration during cerebral hypoxia-ischemia may be one of several variables that influence the severity of ischemic brain damage. Campbell (1938) first demonstrated that if rats were fed a diet of carrots only, they survived hypoxia longer than rats fed a normal diet. Craven et al. (1950) reported similar protection against hypoxia in fasted rats and concluded that weight loss and carbohydrate depletion were responsible for the better outcome. Myers and Yamaguchi (1977) and others (Pulsinelli et al., 1980; Siemkowicz et al., 1978; Kalimo et al., 1981; Ginsberg et al., 1980; Siemkowicz et al., 1980; Rehncrona et al., 1981; Welsh et al., 1980) demonstrated that animals made hyperglycemic before cerebral ischemia suffered greater neurologic deficits, more severe morphologic

brain damage, and had poor recovery of glucose metabolism and high-energy metabolites than normoglycemic controls.

Several recent clinical and experimental data have suggested that cellular damage in cerebral ischemia is at least partly due to oxidative damage caused by free radical formation and lipid peroxidation (Flamm et al., 1978; Rehncrona et al., 1978; Bazän NG Jr., 1970; Majewska et al., 1978; Yoshida et al., 1980). This is partly supported by the finding that transient incomplete ischemia is more deleterious for brain tissue than complete cessation of cerebral blood flow (Rehncrona et al., 1978; Hossmann et al., 1973; Rehncrona et al., 1980; Nordström et al., 1976; Nordström et al., 1978). In nearly complete ischemia insufficient O_2 is available to accept electrons passed along the mitochondrial electron transport chain, leading to eventual reduction ("electron saturation") of component of this system, such as flavin adenine dinucleotide and coenzyme Q (CoQ). In the presence of small amounts of O_2, these molecules can be auto-oxidized to produce, for example, $O_2^-\cdot$. ($CoQ + O_2 \rightarrow CoQ\cdot + O_2\cdot$). The residual O_2 molecules in severely ischemic brain cannot act as electron acceptors in the "normal" fashion because oxidation-reduction ("redox") potential sufficient to favor step-wise electron transfer to them cannot be generated by such low concentrations of molecular oxygen (Demopoulos et al., 1979).

We examined the effect of hyperglycemia in an animal model that simultaneously produces severe forebrain ischemia and moderate hindbrain ischemia. Our purpose was to evaluate the effect of hyperglycemia on lipid peroxidation with the changes of ATP and lactate levels of rat brain during the global ischemia and reperfusion.

Lipid peroxidation was measured by determining with TBA using a method modified by Masugi and Nakamura (1976). This method used for measurement of TBA-RS (reactive substance) as MDA has been verified as a method protecting degradation of intact polyunsaturated fatty acids by solubilizing with sodium dodecyl sulfate (SDS). Lipid peroxidation in cerebral ischemia was observed in this study particularly when the oxygen supply was restored in the tissue by reperfusion (Fig. 3). To avoid lipid peroxidation in vitro during the process of TBA reaction, 10 mM EDTA was added to the homogenization buffer to chelate Fe^{++} and the assay procedures were carried out under 100% N_2 as described above in 'Methods'. The increase in lipid peroxides, especially detected after restoration of the blood flow following 1 hour of global ischemia, is assumed to be due to free radicals formed during this period.

Brain lactate accumulation after 1 hour global ischemia in our study was significantly higher in hyperglycemic rats than in normoglycemic ones (Fig. 2). During reperfusion, lactate wash-out was delayed in the hyperglycemic group. Excessive accumulation of lactic acid in the ischemic brain is the most likely explanation of the enhanced brain damage in hyperglycemic animals. Friede and Van Houten (1961) demonstrated in vitro that cerebellar tissue slices underwent necrobiosis when cellular respiration was blocked by cyanide but not when respiration and glycolysis were blocked simultaneously. They suggested that hydrogen ion accumulation (via glycolysis) in the cyanide-treated tissue was responsible for the cytologic changes. Myers (1979) and others (Rehncrona et al., 1981; Welsh et al., 1980) demonstrated that lactic acid accumulation is greater in the ischemic brains of hyperglycemic animals than in normoglycemic controls. Cerebral lactic acid concentration is a function of several factors, including tissue oxygen tension, the glycolytic substrate supply from blood and the brain (Ljunggren et al., 1974; Siesjö BK, 1981), and the rate of lactate efflux into the venous circulation (Zimmer et al., 1975). In the severely ischemic brain, where the blood glucose and oxygen supply approach zero and the diffusion of lactate out of the brain is negligible, the tissue lactate concentration is proportional to the cerebral stores of glycolytic substrates at the onset of ischemia (Ljunggren et al., 1974). If it is true that cerebral lactic acid damages the brain, increased blood and brain glucose stores before ischemia should augment brain damage.

This study demonstrated that hyperglycemia in transient global cerebral ischemia and reperfusion increased lipid peroxidation, decreased the brain ATP level and increased lactate accumulation. We found that the degree of lactate accumulation was well correlated with the amount of lipid peroxidation. Siesjö (1985a) observed that during complete brain ischemia, brain pH values have been estimated to fall to about pH 6.2 under normoglycemic conditions and below 6.0 in hyperglycemic conditions which are accompanied by excessive accumulation of brain lactate. Siesjö et al. (1985b) showed that acidosis enhanced the formation of TBA-RS, which was accompanied by an exaggerated decrease in α-tocopherol content, and by disappearance of polyenoic, phospholipid-bound fatty acids. They also demonstrated that the pH optimum for these effects is at pH 6.0-6.5. Bralet et al. (1991) found that acidosis of a degree encountered during ischemia markedly increased the production of TBA-

RS by brain slices. Several mechanisms may be involved in the exaltation of lipid peroxidation during acidosis (Braughler and Hall, 1989). Increase in H^+ concentration (1) accelerates the rate of dismutation of the superoxide anion $O_2^{\bullet}$ to H_2O_2 which can react with Fe^{2+} and generate the highly toxic hydroxyl radical ($OH^{\bullet}$), (2) converts $O_2^{\bullet\bullet}$ to the more reactive and more lipid soluble hydroperoxyl radical ($HO_2^{\bullet}$). Another possible effect of tissue acidosis is to stimulate free radical generation by increased dissociation of protein-bound iron, as originally suggested by Bernheim (1963). More recent data suggest that low pH can help releasing iron from its binding sites in, e.g., transferrin and ferritin (Aisen, 1979; Paques et al., 1979).

An additional, albeit inefficient, source of ATP is via substrate phosphorylation of glycolytic intermediates within the cytosol. To replace the mitochondrial function completely, glycolytic flux would need to have accelerated 18-fold over basal conditions. This may be why ATP levels depleted less in the hyperglycemic group than in the normoglycemic group at 30 minutes of global cerebral ischemia. But after 1 hour of ischemia, a higher lactate accumulation and a more vigorous generation of free radicals might be causes of a greater depletion of ATP and worse ATP recovery during reperfusion in the hyperglycemic group of this study.

Our results indicate that hyperglycemia augments lactate accumulation, lipid peroxidation, energy depletion and makes ATP recovery sluggish during reperfusion. Also, it is suggested that an excessive lactate accumulation enhances the lipid peroxidation.

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