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Short communication

Glutathione protects against hypoxic/hypoglycemic decreases in 2-deoxyglucose uptake and presynaptic spikes in hippocampal slices

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Abstract

The effects of glutathione, its analogue: YM737 (*N*-(*N*-γ-L-glutamyl-L-cysteinyl) glycine *l*-isopropyl ester sulfate monohydrate), a monoester of glutathione, and *N*-acetyl-L-cysteine on hypoxia/hypoglycemia-induced decreases in CA1 presynaptic fiber spikes and 2-deoxyglucose uptake were investigated using rat hippocampal slices. The drugs were added to normal medium for 30 min before the incubation under hypoxic/hypoglycemic conditions (20 min), and, after a 3-h washout, presynaptic potential or 2-deoxyglucose uptake in hippocampal slices was measured. Treatment with glutathione, YM737 and *N*-acetyl-L-cysteine produced an attenuation of the hypoxia/hypoglycemia-induced decrease in presynaptic fiber spikes and 2-deoxyglucose uptake. The order of potency for neuroprotective action was YM737 ≥ *N*-acetyl-L-cysteine > glutathione. The present results suggest a role for glutathione in improving hypoxia/hypoglycemia-induced dysfunction of hippocampal regions.

Keywords: Ischemia; Glutathione; Hippocampus; 2-Deoxyglucose uptake; Presynaptic potential; Neuroprotection

1. Introduction

Evidence is growing that free radicals are involved in the pathophysiology of ischemia/reperfusion injury. The hydroxyl radical has been reported to cause the decrease in the ability of synaptic potentials to generate action potentials (Pellmar and Neel, 1989; Pellmar et al., 1992). Glutathione is a thiol-containing tripeptide found in high concentrations in most cell types. As a powerful reducing agent, it plays a major role in the cellular defenses against endogenous and exogenous oxidants. One important function of glutathione is to reduce disulfide linkages and thereby repair the hundreds of sulfhydryl containing proteins vulnerable to oxidative damage. Some investigators have described changes in glutathione and its oxidant product, glutathione disulfide, during ischemia in rats. Brain ischemia is characterized by a fall in glutathione without a rise in glutathione disulfide (Slivka and Cohen, 1993). Recently Pellmar et al. (1992) reported that depletion of glutathione increased vulnerability to free radical damage of synaptic potentials and population spikes in the hippocampal CA1 regions. In addition, glutathione and cysteine have been reported to exhibit a marked protective action against glutamate-induced cytotoxicity in N18-RE-105 cells, and glutathione also reversed the reduced cellular glutathione/glutathione disulfide levels caused by glutamate (Miyamoto et al., 1989).

The glutathione analogue, YM737 (*N*-(*N*-γ-L-glutamyl-L-cysteinyl) glycine *l*-isopropyl ester sulfate monohydrate), is a monoester of glutathione in which the glycine carbonyl group of glutathione is esterified. Since such glutathione monoesters are more efficiently transported into cells than glutathione itself (Maeno et al., 1989), the administration of YM737 may induce stronger pharmacological effects than glutathione itself (Maeno et al., 1989; Yamamoto et al., 1990). Therefore, in the present experiment we investigated the effects of glutathione and YM737 on hypoxia/hypoglycemia-induced impairments of 2-deoxyglucose uptake and presynaptic fiber spikes in rat hippocampal slices. Since *N*-acetyl-L-cysteine increases glutathione levels (Issels et al., 1988), we tested the effect of *N*-acetyl-L-cysteine on hypoxia/hypoglycemia-induced neurotoxicity.

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2. Materials and methods

2.1. Slice preparation

Male Wistar rats weighing 300–400 g were used. The animals were decapitated and the brain was quickly removed. Parasagittal hippocampal slices (450 μm thick) were then prepared from the dorsal hippocampus of each animal, using a tissue chopper. The composition of the control Krebs-Ringer solution equilibrated with 95% O_2-5% CO_2 gas mixture was (in mM): NaCl 129, $MgSO_4$ 1.3, $NaHCO_3$ 22.4, KH_2PO_4 1.2, KCl 4.2, D-(+)-glucose 10.0, $CaCl_2$ 1.5. To induce hypoglycemia, the D-(+)-glucose in the incubation medium was replaced by 10 mM sucrose. The hypoxia solution was equilibrated with 95% N_2-5% CO_2 gas mixture for at least 1 h. The buffer had a pH of 7.3–7.4 and the temperature was kept at 37°C. Preparations were preincubated with normal Krebs-Ringer solution for 1 h in a recirculation submersion chamber. Our chamber design, slice transfer methods and incubation procedures have been reported previously (Shibata et al., 1992; Tanaka et al., 1994). Following a 1-h preincubation, hippocampal slices were exposed to hypoxia/hypoglycemia for 20 min. The drugs were added to normal medium 30 min before the incubation under hypoxic/hypoglycemic conditions. After a 3-h washout, the slices were removed and then incubated in normal Krebs-Ringer solution containing 0.1 μCi/ml of 2-deoxy-D-[^{14}C]glucose (specific activity, 50 mCi/mmol; Amersham) or transferred to a recording chamber to record presynaptic potentials.

2.2. Electrophysiology

Presynaptic potentials were recorded through a glass micropipette filled with normal physiological saline (DC resistance, 0.5–1 MΩ). Stimulation pulses were 0.2 Hz, 0.05 ms in duration and 0.9 mA in intensity and produced a maximal response in the normal non-hypoxic/hypoglycemic group (Tanaka et al., 1994). Extracellular recordings of presynaptic fiber spikes in the stratum radiatum of the CA1 region were made and the latency of the negative portion of the presynaptic fiber spike was fixed at 2.0 ms. Presynaptic fiber spike amplitudes were quantified as a peak-to-peak measurement between the negative peak of the fiber spike and the positivity which immediately preceded the negativity, or between the prestimulus baseline and the negative peak of the fiber spike. In previous experiments we found that there was no significant inter-slice variation in the amplitude of presynaptic fiber spikes (Shibata et al., 1992; Tanaka et al., 1994). Therefore, we averaged the amplitude of each experimental group in a non-paired comparison situation and calculated means and S.E.

2.3. 2-Deoxyglucose uptake

Incubation for 2-deoxyglucose uptake measurements was carried out at 37 ± 0.2°C. The incubation chamber was arranged to recirculate 15 ml of buffer at 4.4 ml/min with continuous bubbling of humidified 95% O_2-5% CO_2 through the buffer as it entered the chamber. The procedure for monitoring 2-deoxyglucose uptake has been reported previously (Shibata et al., 1992). Briefly, the slices were removed from the preincubation chamber, drained and then placed in the incubation chamber for 45 min. Incubations were terminated by removing the disk from the incubation chamber, rinsing with 20 ml of warm preincubated buffer, and placing it in a chamber identical to the preincubation chamber for 30 min. At the end of the wash-out period, the disk with slices was placed on dry ice to stop metabolism. The slices were then homogenized in 1 ml of phosphate buffer containing 0.5% perchloric acid, and 450 μl of the homogenate was used to determine total protein, using a Bio-Rad protein assay kit (Bio-Rad, Richmond, USA). The radioactivity in another 450 μl of the homogenate was measured in a liquid scintillation counter after being solubilized with 6 N NaOH at 60°C and then neutralized with 6 N HCl. Background dpm (30–50 dpm) was < 5% of total dpm. Background dpm was subtracted from total dpm.

2.4. Drugs

The drugs used in this study were glutathione, YM737 and *N*-acetyl-L-cysteine. Glutathione and *N*-acetyl-L-cysteine were obtained from Sigma Chemicals (USA), and YM737 was a gift from Yamanouchi Pharmaceut. (Tokyo, Japan). The drugs were dissolved into distilled water.

2.5. Data analysis

The significance of differences between groups was determined by Student's *t*-test or analysis of variance followed by Dunnett's test for individual comparisons.

3. Results

The amplitude of CA1 presynaptic fiber spikes in control hippocampal slices was 1.60 ± 0.11 mV ($n = 7$). Treatment with drugs for 50 min in normal non-hypoxic/hypoglycemic solution did not change the CA1 presynaptic potentials after a 3-h washout. The mean amplitudes of CA1 presynaptic potentials in hippocampal slices treated with glutathione (100 μM), YM737 (100 μM) or *N*-acetyl-L-cysteine (100 μM) for 50 min were 1.63 ± 0.10 mV ($n = 4$), 1.68 ± 0.18 mV ($n = 4$), or 1.57 ± 0.07 mV ($n = 4$), respectively.

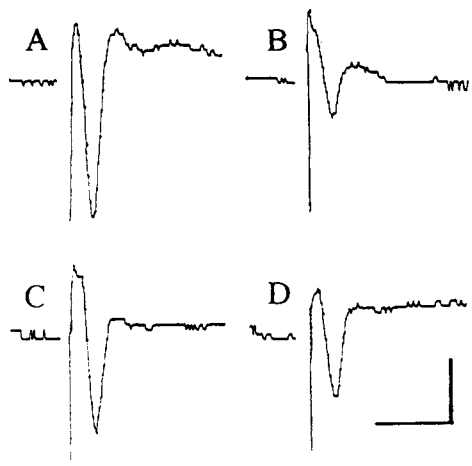

Fig. 1. Effects of glutathione, YM737 and *N*-acetyl-L-cysteine on the decrease in CA1 presynaptic potential induced by hypoxia/hypoglycemia. All observations were recorded 3 h after 20 min of combined hypoxia/hypoglycemia (ischemia). A: CA1 presynaptic potential evoked by the stimulation of Schaffer collaterals (0.05 ms, 0.9 mA) under normal conditions. B: Effect of vehicle on CA1 presynaptic potential evoked in hippocampal slices at 3 h after 20 min of ischemia. C: Effect of YM737 (100 μM) on ischemia-induced decrease in CA1 presynaptic potential. D: Effect of glutathione (100 μM) on ischemia-induced decrease in CA1 presynaptic potential. Hippocampal slices were exposed to Krebs-Ringer solution containing each of the drugs for 30 min before the induction of ischemia and during the 20-min ischemic period. Each trace is an average of 8 sweeps. Calibrations: 5 ms and 0.5 mV.

In slices exposed to 20-min hypoxia/hypoglycemia the amplitude was 0.50 ± 0.06 mV ($n = 7$) ($P < 0.01$, Student's t-test) after a 3-h washout. We examined the effect of glutathione, YM737 and *N*-acetyl-L-cysteine at a concentration of 10 and 100 μM for each drug on the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic spikes (Fig. 1 and Table 1). The hypoxia/hypoglycemia-induced decrease in amplitude was attenuated by treatment with glutathione ($F(2,10) = 4.8$, $P < 0.05$), YM737 ($F(2,12) = 22.4$, $P < 0.01$) and *N*-acetyl-L-cysteine ($F(2,11) = 28.0$, $P < 0.01$) (Table 1).

Treatment of hippocampal slices with glutathione (100 μM), YM737 (100 μM) or *N*-acetyl-L-cysteine (100 μM) for 50 min in normal non-hypoxic/hypoglycemic solution did not affect the 2-deoxyglucose uptake of hippocampal slices after a 3-h washout. 2-Deoxyglucose uptake was 242 ± 4.8 ($n = 14$) dpm/μg protein/45 min for control, 241 ± 7.3 ($n = 6$) for glutathione, 237 ± 7.5 ($n = 6$) for YM737 and 237 ± 5.8 ($n = 6$) for *N*-acetyl-L-cysteine, respectively. In previous experiments, we observed the time course of the recovery of 2-deoxyglucose uptake in hippocampal slices following 10, 15 and 20 min of hypoxia/hypoglycemia, and found that the decrease in 2-deoxyglucose uptake by slices was dependent upon the duration of hypoxia/hypoglycemia (Shibata et al., 1992). 2-Deoxyglucose uptake in control hippocampal slices was 242 ± 4.8 dpm/μg protein/45 min ($n = 14$) and that in slices exposed to hypoxia/hypoglycemia for 20 min was 123 ± 3.1 dpm/μg protein/45 min ($n = 14$) after a 3-h washout. We examined the effect of glutathione (1 μM–1 mM), YM737 (1–100 μM) and *N*-acetyl-L-cysteine (1–100 μM) on the hypoxia/hypoglycemia-induced decrease in 2-deoxyglucose uptake. The hypoxia/hypoglycemia-induced decrease in 2-deoxyglucose uptake by hippocampal slices was significantly prevented by the treatment with glutathione ($F(4,33) = 2.7$, $P < 0.05$), YM737 ($F(3,25) = 15.4$, $P < 0.01$) and *N*-acetyl-L-cysteine ($F(3,34) = 18.1$, $P < 0.01$) (Table 1). The minimum effective dose for neuroprotection against deficits of CA1 presynaptic potential and 2-deoxyglucose was 10 μM for YM737 and *N*-acetyl-L-cysteine, and 100 μM for glutathione. Thus, the order of

Table 1
Effects of glutathione, YM737 and *N*-acetyl-L-cysteine on the decrease in CA1 presynaptic spikes and 2-deoxyglucose uptake induced by 20 min of hypoxia/hypoglycemia

Treatment	Concentration (μM)	Presynaptic spikes (mV)	2-Deoxyglucose uptake (dpm/μg protein/45 min)
Normal		1.60 ± 0.11 (7)	242 ± 4.8 (14)
Hypoxia/hypoglycemia		0.49 ± 0.06 (7) [a]	123 ± 3.1 (14) [a]
+ glutathione	1	–	129 ± 9.3 (6)
	10	0.58 ± 0.03 (3)	136 ± 7.8 (6)
	100	0.66 ± 0.03 (3) [b]	146 ± 8.3 (6) [b]
	1000	–	142 ± 6.0 (6)
+ YM737	1	–	119 ± 11.0 (3)
	10	0.72 ± 0.06 (4) [b]	158 ± 9.4 (4) [c]
	100	1.12 ± 0.11 (4) [c]	166 ± 6.1 (6) [c]
+ *N*-acetyl-L-cysteine	1	–	126 ± 4.7 (3)
	10	0.69 ± 0.002 (3) [c]	147 ± 4.9 (9) [c]
	100	0.83 ± 0.03 (3) [c]	173 ± 7.3 (12) [c]

CA1 presynaptic spikes and 2-deoxyglucose uptake were measured after a 3-h washout following 20 min of hypoxia/hypoglycemia. Values shown are means ± S.E.M. Numbers in parentheses indicate the number of slices. Drugs were perfused 30 min before and throughout hypoxia/hypoglycemia periods (20 min).
[a] $P < 0.01$ vs. normal group (Student's t-test). [b] $P < 0.05$, [c] $P < 0.01$ vs. hypoxia/hypoglycemia group (Dunnett's test).

potency for neuroprotective action was YM737 ≥ *N*-acetyl-L-cysteine > glutathione.

4. Discussion

The present study demonstrated that the reductions in CA1 presynaptic fiber spikes and 2-deoxyglucose uptake in hippocampal slices induced by hypoxia/hypoglycemia were attenuated by glutathione, YM737 and *N*-acetyl-L-cysteine. Brain ischemia is characterized by a fall in glutathione without a rise in glutathione disulfide. Recently it has been reported that depletion of glutathione impaired the recovery from free radical damage of electrophysiological responses in the hippocampus (Pellmar et al., 1992). In addition, reducing agents, cysteine, dithiothreitol and glutathione, exerted a marked protective action against glutamate-induced cytotoxicity in N18-RE-105 cells (Miyamoto et al., 1989). Dithiothreitol and glutathione reversed the reduced cellular glutathione/glutathione disulfide levels caused by exposure to glutamate (Miyamoto et al., 1989). However, the actual mechanisms involved in the recovery from hypoxia/hypoglycemia treatment cannot be determined from the present experiment.

2-Deoxyglucose uptake has been reported to reflect predominantly the activity of nerve terminals (Kadekaro et al., 1987). Because glutathione is present in nerve terminals and dendritic spines, as suggested by the histochemical analysis (Slivka et al., 1987; Philbert et al., 1991), this would permit glutathione locally to influence synaptic mechanisms. In our study, YM737 caused a greater improvement of functional deficits in hippocampal slices than glutathione did. This is consistent with the finding that YM737 is more readily transported into fibroblast cells through membranes than glutathione (Maeno et al., 1989). In bilateral carotid artery-occluded rats, reduced glutathione was completely restored by administration of YM737, but not by glutathione (Noguchi et al., 1989), suggesting that YM737 is better transported into the cells. As a monoester of glutathione, therefore, the higher potency of YM737 may be, at least partly, attributed to its ability to permeate through cell membranes.

In the present study, we demonstrated that *N*-acetyl-L-cysteine had a protective action against the hypoxia/hypoglycemia-induced reduction of CA1 presynaptic fiber spikes and 2-deoxyglucose uptake. Cysteine may be produced by deacetylation of *N*-acetyl-L-cysteine. Since cysteine has a thiol group and could function as a free radical scavenger, our finding suggests that it acts in this way in the hippocampal regions. A previous study demonstrated that cysteine protected N18-Re-105 cells from cytotoxicity produced by glutamate (Miyamoto et al., 1989). In addition, cysteine and *N*-acetyl-L-cysteine were reported to inhibit the cytotoxicity to hepatocytes caused by phenylhydroquinol (Nakagawa et al., 1992). The recovery of neuronal activity is reported to be paralleled by an increase in extracellular cysteine sulfinate in the CA1 of hippocampus (Andine et al., 1991).

During ischemia and early recovery, the extracellular concentrations of the glutamate-like sulfur-containing amino acid cysteine sulfinate increased, but not as massively as those of glutamate and aspartate (Andine et al., 1991). Even though brain ischemia elevates levels of the neurotoxic amino acid, cysteine, cysteine does not cause brain damage in adult rodents (Slivka and Cohen, 1993).

In summary, YM737, an isopropyl ester of glutathione which is more readily transported through cell membranes than glutathione, improved functional deficits in ischemic hippocampal slices, presumably due to an inhibition of the lipid peroxidase response.

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