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Effects of Oxygen Free Radicals on Extracellular Glutamate Accumulation in Cultured Cells

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Exogenously applied oxygen free radical generating agent, pyrogallol, highly elevated extracellular glutamate accumulation and augmented N-methyl-D-aspartate (NMDA)-induced glutamate accumulation in cerebellar granule neuronal cells, but did not in astrocytes. Superoxide dismutase remarkably decreased the pyrogallol-induced glutamate accumulation, but either NMDA or kainate antagonists did not. In addition, pyrogallol did not affect the NMDA-induced intracellular calcium elevation. Pyrogallol partially blocked glutamate uptake into astrocytes. These results suggest that oxygen free radicals elevate extracellular glutamate accumulation by stimulating the release of glutamate as well as blocking the glutamate uptake.

Key words : Pyrogallol, Oxygen free radicals, N-Methyl-D-aspartate, Glutamate accumulation, Intracellular calcium, Cerebellar granule neuron, Astrocyte.

INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in the mammalian CNS. Glutamate is in high concentration in glutamatergic synaptic vesicles (approximately 100 mM) and presents at a concentration of 10 mM in the cytoplasm of neurons (Talor *et al.*, 1992). The glutamate levels found in rat plasma, cerebrospinal fluid and hippocampal extracellular fluid are 160, 11 and 3 μM, respectively (Lerma *et al.*, 1986). Extracellular glutamate accumulation rises almost immediately after onset of ischemia (10-50 μM) (Globus *et al.*, 1991; Matsumoto *et al.*, 1991; Shimada *et al.*, 1993). Free radicals can be generated either under hypoxia (Imaizumi *et al.*, 1984) or during postischemic reperfusion (Kirsch, 1987). Removal of glutamate from the synaptic cleft is an essential component of the transmission process at glutamatergic synapses. The glutamate is removed by reuptake into both neurons and glia by sodium dependent and high affinity transport systems (Watkins and Evans, 1981; Flott and Seifert, 1991). Hypoxia-induced cell damage and glutamate release are reduced by treatment with superoxide dismutase (SOD) as well as NMDA receptor antagonist (Cazevieille *et al.*, 1993).

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Recently, there is an interesting report suggesting that oxygen free radicals are produced upon N-methyl-D-aspartate (NMDA) receptor stimulation in cultured cerebellar granule cells, while detectable oxygen free radicals are not produced by the activation of kainate receptors or voltage-sensitive Ca^{2+} channels (Lafon-Cazal *et al.*, 1993). Nitric oxide is also generated by NMDA receptor activation via Ca^{2+}-dependent nitric oxide synthase (Garthwaite *et al.*, 1988; Bredt and Snyder, 1989). Nitric oxide (NO) is a reactive radical that can interact with oxygen free radicals ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), which is a strong oxidant that can initiate lipid peroxidation leading to cell injury and death (Lipton *et al.*, 1993).

Pyrogallol (PG), a potent generator of oxygen free radicals (Murklund and Murklund, 1974), shifts the concentration-response curve to nitric oxide to the right, but has no effect on the inhibitory response to 3-isobutyryl-1-methylxanthine (IBMX), phosphodiesterase inhibitor in muscle relaxation (Gillespie and Sheng, 1990).

The neurotransmitter function of NMDA receptor seems to be related to calcium influx and oxygen free radicals generation as well as nitric oxide generation. The aim of the present study has been to investigate the implication of oxygen free radical on the neurotransmitter functions of NMDA receptor by using PG, an oxygen free radical generating compound. To determine the activity of NMDA receptor functions, NMDA-induced extracellular glutamate accumulation

and calcium influx with PG was measured in cultured cerebellar granule cells and astrocytes.

MATERIALS AND METHODS

Cerebellar granule cell culture

Cerebellar granule cells were cultured as described (McCaslin and Morgan, 1987) with minor modifications. Briefly, 8-day-old rat pups (Sprague-Dawley, Harlan, Indianapolis, IN) were decapitated and the heads were partially sterilized by dipping them in 95% ethanol. The cerebellum was dissected from the tissue and placed in culture medium which lacks serum and bicarbonate. Dissociated cells were collected at a density of about 2×10^6 cells/ml. Growth medium (5 ml/60 mm dish) was Dulbecco's modified Eagle's medium supplemented with sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), 6% bovine calf serum (Hyclone, Logan, UT) and 6% fetal bovine serum (JRH Bioscience, Lenexa, KS). After 2 days incubation, the growth medium was aspirated from the cultures and new growth medium (5 ml/dish) containing 25 mM KCl was added with 5 M cytosine arabinoside to prevent proliferation of nonneuronal cells.

While there were many elaborate techniques for growing astrocytes in tissue culture, the simple method was used as described (McCaslin and Ho, 1994) with modification. The cerebellum was dissected from the 8-day old pups and dissociated by trituration much as described above for neuron culture. These cells could then be grown in precisely the same manner as that used for growing neurons with the exceptions of not adding KCl or cytosine arabinoside at 48 h. Without the addition of this latter chemical, the astrocytes will continue to proliferate and will eventually, usually after four to five days *in vitro*, become the predominant cell type in the cell culture dishes.

Glutamate measurement

Experiment was performed using cultures grown for 10-14 days after plating. After growing cells, growth medium was removed from the cells, and they were placed in a physiological saline HEPES (PSH) buffer containing the following mM concentrations: 135 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 40 bicarbonate, 10 glucose and 5 HEPES (pH 7.4, 300 mOsm). Cells were incubated in the presence of various dosages of compounds at 37°C for 1 h after 30 min prewashing in the dark in PSH buffer. The amount of glutamate secreted into the buffer was separated from the culture dish. The glutamate concentration was quantified by HPLC with an electrochemical detector (Bioanalytical Systems) after precolumn derivatization of sample aliquots with *o*-phthalaldehyde/2-mercaptoethanol reagent (OPA/2-ME) as described below (Ellison *et al.*, 1987). Precolumn derivatization of amino acids was performed by mixing 30 µl of the sample aliquots with 10 µl of the working OPA/2-ME reagent for 1 min before injection onto the column. The C18 column (5 µm, 4.6×150 mm, Rainin, CA) was eluted with mobile phase (pH 5.2) containing 0.1 M sodium phosphate buffer with 37 % (v/v) methanol at a rate of 1.0 ml/min.

Measurement of intracellular Ca^{2+} levels

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by ratio fluorometry as described (Grynkiewicz *et al.*, 1985; Cai and McCaslin, 1992; Oh *et al.*, 1995). The cells grown on glass cover slides were loaded with 10 µM fura-2 AM for 1 h in PSH buffer at 37°C, and then washed with PSH buffer. Cell culture slides were cut and mounted into spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) by excitation at 340 and 380 nm, and emission at 505 nm for 10 min. Baseline of $[Ca^{2+}]_i$ was measured for 60 sec before the addition of various experimental compounds. Fluorescence signal calibration was determined by addition to the cuvette of ionomycin (final concentration, 10 µM) to obtain Rmax, and EGTA (final concentration, 20 µM) to obtain Rmin. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.* (1985) using a KD of 224 nM by TM 3000 software (SPEX).

RESULTS

Oxygen free radical generating agent, PG (100 µM), markedly elevated extracellular glutamate level and NMDA-induced glutamate accumulation for 1 h incubation (control; 0.30±0.05, PG; 1.16±0.14, NMDA; 0.85±0.14 and NMDA+PG; 1.51±0.08, in µM) (Fig. 1). Oxygen free radical scavenger, SOD, alone did not induce a significant difference with control group (SOD; 0.38±0.07 µM) (Fig. 1). To determine the effects of oxygen free radicals on extracellular glutamate accumulation, PG (100 µM) with or without SOD (50 U/ml) was applied. PG-induced glutamate accumulation was strongly blocked by SOD, but not by either NMDA receptor antagonist, MK-801 (10 µM) or non-NMDA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 M) (PG 100 µM; 1.18±0.11 and PG+SOD; 0.52±0.07 in µM) (Fig. 2).

To see the possibility of the blockade of glutamate uptake by PG-induced oxygen free radicals, exogenous glutamate was applied with or without PG

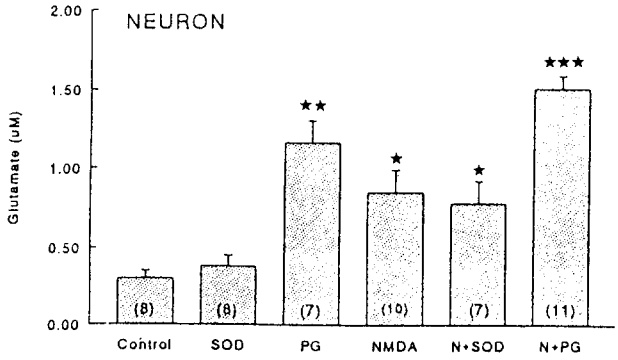

Fig. 1. Effects of oxygen free radical on the release of glutamate in cultured neuronal cells. Cerebellar granule cells were grown for 14 days in vitro and then washed with PSH buffer as described in the text. After a 30 min equilibriation period in PSH buffer, the cells were washed again and incubated for 1 h with/without NMDA (100 μM) or other compounds; superoxide dismutase (SOD, 50 U/ml), pyrogallol (PG, 100 μM). Values present the mean±SEM of mM in 3 ml of extracellular buffer. The numbers for each group are shown in columns. *p<0.05, **p<0.01, ***p<0.001 from the control by Student's t-test

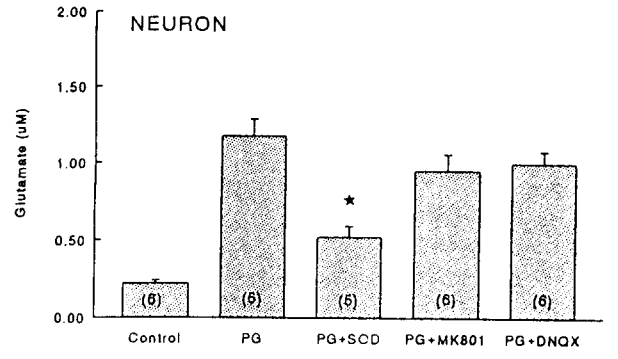

Fig. 2. The blocking effects of SOD on the oxygen free radical-induced release of glutamate in cultured neuronal cells. Experiments were performed as described Fig. 1 and text. The cells were incubated for 1 h with/without PG (100 μM) or SOD (50 U/ml), MK-801 (10 μM), DNQX (10 μM). Values present the mean±SEM of M in 3 ml of extracellular buffer. The numbers for each group are shown in columns. *p<0.05 from the PG group by an ANOVA followed with Newman-Keuls test

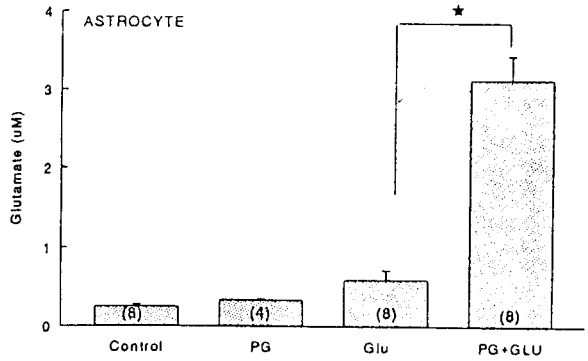

Fig. 3. The blocking effects of oxygen free radicals on glutamate uptake into astrocytes. Astrocytes were grown for 10 days in vitro and then washed with PSH buffer as described in the text. After a 30 min equilibrium period in PSH buffer, astrocytes were washed again and incubated with glutamate (10 μM) or PG (100 μM) for 1 h and residual glutamate was measured as described in the text. Values present the mean±SEM of μM in 3 ml of extracellular buffer. The numbers for each group are shown in columns. *p <0.05 by Student's t-test

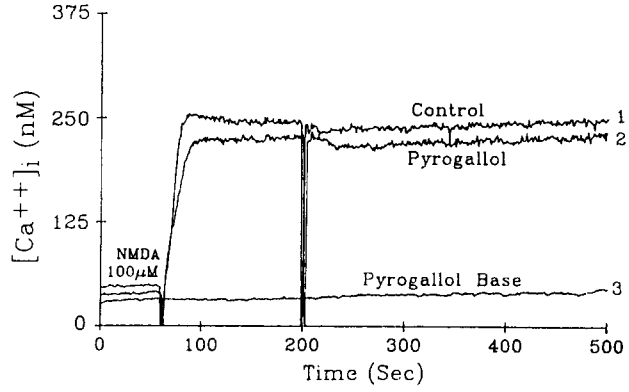

Fig. 4. Effects of PG on NMDA-induced $[Ca^{2+}]_i$ elevation. Cells were loaded with fura2-AM for 1 h and washed with PSH buffer. Intracellular Ca^{2+} levels were measured by stimulation with NMDA (100 μM) at the first break in the curve (60 sec) and buffer (control, curve 1) or PG (100 μM, curve 2) was applied at the second break (200 sec). Experiments were repeated 4 times. Elevation did not occur with PG (100 μM applied at 60 sec) alone as shown in this figure (curve 3).

in cultured astrocytes. Almost all of exogenously applied glutamate (10 μM) were uptaken into the astrocytes after 1 h incubation, but the glutamate reuptake was blocked by PG (Fig. 3); the residual glutamate concentration was 5 times elevated by addition of PG after for 1 h incubation (glutamate alone; 0.59±0.12 and glutamate plus PG; 3.13±0.31 in μM, respectively). Contrast to cerebellar granule neurons, PG itself did not induce glutamate accumulation in astrocytes. In our preliminary experiments, neither NMDA (100 μM) nor kainate (100 μM) induced glutamate accumulation during 1 h incubation in astrocytes (data not shown).

To determine the effects of oxygen free radicals on NMDA-induced $[Ca^{2+}]_i$ level, PG (100 μM) was added into the neuron after activation with NMDA or without activation. PG did not affect the basal or NMDA-induced $[Ca^{2+}]_i$ levels (Fig. 4).

DISCUSSION

Free radicals have now become accepted into one of the pathogenic factors. Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecule and which are generally very reactive. One of reactive free radicals, nitric oxide, is generated from glutamatergic systems by activation of NMDA receptors. Interestingly, ox-

ygen free radicals are also generated by NMDA receptor activation but not by kainate receptor or voltage sensitive Ca^{2+} channel activations (Lafon-Cazal *et al.*, 1993). Oxygen free radicals are thought to be generated from several sources, including coenzyme Q metabolism, catecholamine autoxidation, neutrophil respiratory burst, hemoglobin, xanthine/xanthine oxidase and arachidonic acid cascade (Braughler and Hall, 1989; Schmidley, 1990). Activated excitatory amino acid (EAA) receptors lead to the release of arachidonic acid (Dumuis *et al.*, 1988).

Oxygen free radicals can stimulate the release of EAAs from rat hippocampal slices (Pellegrini-Giampietro *et al.*, 1988) and oxygen free radical quenchers prevent the release of EAAs in an *in vitro* ischemic model (Pellegrini-Giampietro *et al.*, 1990). Also in our results, oxygen free radical generating agent, PG, markedly elevated extracellular glutamate accumulation in cultured neuronal cells (Fig. 1), but not in astrocytes (Fig. 3).

The cell viability was verified by trypan blue exclusion and by lactate dehydrogenase measurements. PG for 1 h incubation as indicated did not show cytotoxicity towards cultured cells (data not shown).

In an attempt to determine the possibility that PG-induced glutamate accumulation is secondary to the activation of EAA receptor, NMDA and non-NMDA antagonists were applied. However, these were less possible since the increased glutamate accumulation by PG was not decreased by either NMDA receptor antagonist, MK-801 or non-NMDA receptor antagonist, DNQX (Fig. 2).

There is an interesting result suggesting the role of oxygen free radicals as a neuroprotective component to NMDA receptor-mediated neuronal injury. Oxygen free radicals suppress NMDA receptor function by oxidizing the sulfhydryl residues associated with the redox modulatory site of this receptor (Aizenman *et al.*, 1990). In contrast to this suggestion, we can not see the suppressing effects of oxygen free radicals on NMDA-induced $[Ca^{2+}]_i$ level (Fig. 4). Therefore, it was presumed that these suppressing effects of oxygen free radicals were not observed since redox sites of NMDA receptors have become already oxidized by the released exogenous oxygen free radicals by NMDA receptor activations and there are no more binding sites for endogenous radicals on NMDA receptors. Several reports suggest that nitric oxide blocked NMDA receptor functions by attacking redox sites of the NMDA receptors (Lei *et al.*, 1992; Ujihara *et al.*, 1993). In our experiments, nitric oxide did not affect NMDA-induced $[Ca^{2+}]_i$ level, but nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) could block NMDA responses (unpublished data). Peroxynitrite could be generated by the reaction of nitric oxide and oxygen free radical. Therefore, the blocking effect of free radical, oxygen free radical or nitric oxide might be governed by the balance of these radicals.

In terms of extracellular glutamate accumulation, there are two possibilities; to stimulate the release of glutamate or to block the glutamate reuptake into neuron or astrocytes. Based on the result of blocking effects of free radicals on glutamate reuptake in astrocytes (Fig. 3), elevated extracellular glutamate accumulation was not purely produced from increasing of glutamate release. Furthermore, PG did not affect either basal or NMDA-induced elevation of $[Ca^{2+}]_i$ which is closely related with the release of neurotransmitters.

In conclusion, PG-induced oxygen free radicals markedly elevated extracellular glutamate accumulation in cultured cerebellar granule cells by blocking the glutamate uptake as well as stimulating the release of glutamate, while PG did not affect basal or NMDA-induced intracellular calcium elevations.

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