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Effect of *t*-butylhydroperoxide on Na^+-dependent Glutamate Uptake in Rabbit Brain Synaptosome

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The effect of an organic peroxide, *t*-butylhydroperoxide (*t*-BHP), on glutamate uptake was studied in synaptosomes prepared from cerebral cortex. *t*-BHP inhibited the Na^+-dependent glutamate uptake with no change in the Na^+-independent uptake. This effect of *t*-BHP was not altered by addition of Ca^{2+} channel blockers (verapamil, diltiazem and nifedipine) or PLA_2 inhibitors (dibucaine, butacaine and quinacrine). However, the effect was prevented by iron chelators (deferoxamine and phenanthroline) and phenolic antioxidants (*N,N'*-diphenyl-phenylenediamine, butylated hydroxyanisole, and butylated hydroxytoluene). At low concentrations (<1.0 mM), *t*-BHP inhibited glutamate uptake without altering lipid peroxidation. Moreover, a large increase in lipid peroxidation by ascorbate/Fe^{2+} was not accompanied by an inhibition of glutamate uptake. The impairment of glutamate uptake by *t*-BHP was not intimately related to the change in Na^+-K^+-ATPase activity. These results suggest that inhibition of glutamate uptake by *t*-BHP is not totally mediated by peroxidation of membrane lipid, but is associated with direct interactions of glutamate transport proteins with *t*-BHP metabolites. The Ca^{2+} influx through Ca^{2+} channel or PLA_2 activation may not be involved in the *t*-BHP inhibition of glutamate transport.

Key Words: *t*-butylhydroperoxide, Glutamate uptake, Lipid peroxidation, Na^+-K^+-ATPase activity, Rabbit brain synaptosomes

INTRODUCTION

Oxygen free radicals such as superoxide, hydrogen peroxide, and hydroxyl radical have been recognized as being involved in pathogenesis of a wide range of acute and long-term neurodegenerative brain diseases including Parkinson's disease, Huntington's disease, ischemic trauma, and seizures (McCord, 1985; Halliwell, 1989; Floyd, 1990; Traystman et al, 1991; Bendy & Lebel, 1993). Brain cell membranes have a high content of polyunsaturated fatty acids which are particularly susceptible to the peroxidative attack by reactive oxygen free radicals. In addition, iron, which promotes cytotoxic radical formation, is accumulated in specific brain regions, such as the globus pallidus and substantia nigra. On the other hand, antioxidative defense mechanisms are relatively deficient in brain cells. The brain cell contains almost no catalase and has low concentrations of glutathione, glutathione peroxidase and vitamin E (Olanow, 1993). The brain is, therefore, particularly vulnerable to the oxygen free radical attack.

Glutamate is one of the major amino acid neurotransmitters in the brain (Watkins & Evans, 1981). Several lines of evidence have suggested that an abrupt increase of glutamate in the brain extracellular space facilitates the pathogenesis of ischemic neuronal injury (Jorgensen & Diemer, 1982; Onley, 1983; Meldrum, 1985). Under normal conditions, neuronal and glial reuptake removes released glutamate from the synaptic cleft (Fonnum, 1984). Since the reuptake is mediated by the Na^+-dependent glutamate transport system at the membrane of presynaptic nerve endings, an inhibition of this transport system would

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result in an accumulation of glutamate in the synaptic cleft and thereby induce cellular injury.

Oxygen free radicals have been demonstrated to inhibit the Na^+-dependent glutamate uptake by astrocytes (Pellmar et al, 1994; Volterra et al, 1994). Similar effect of oxidants in the brain synaptosome has not been identified. This study was, thus, undertaken to characterize the effect of an oxidant *t*-butylhydroperoxide (*t*-BHP) on the glutamate uptake in rabbit brain synaptosomes. *t*-BHP has been widely employed as a model substance to study the mechanism of the cell injury resulted from an acute oxidative stress in hepatocytes (Rush et al, 1985; Starke & Farber, 1985) and renal cells (Schnellmann, 1988; Chen & Stevens, 1991). In neuronal cells, *t*-BHP has been demonstrated to induce damages of dopaminergic, cholinergic, and GABAergic neurons (Adams et al, 1994), although the underlying mechanisms have not been elucidated.

METHODS

Synaptosome preparation

Synaptosomes were prepared as described by Hajos (1975) from adult New Zealand White rabbits weighing 1.5~2.0 kg. Cerebral cortical tissue was homogenized with a Potter-Elvehjem homogenizer and was suspended at 10% (w/v) in 0.3 M sucrose. The tissue homogenate was centrifuged at 1,500 g for 10 min in Sorvall RC-5B refrigerated centrifuge. The supernatant was saved, and the pellet was resuspended in half the original volume of 0.3 M sucrose and centrifuged again at 1,500 g for 10 min. The supernatant was collected and combined with the previous supernatant. Care was taken not to include the fluffy white layer above the pellet when the supernatant was collected. The combined supernatants were centrifuged at 9,000 g for 20 min. The pellet was dispersed in 0.3 M sucrose. The suspension was layered over 0.8 M sucrose and centrifuged at 9,000 g for 25 min. Particles dispersed in 0.8 M sucrose solution were resuspended in 0.3 M sucrose and centrifuged at 20,000 g for 30 min. The pellet, which constituted the synaptosomes, was suspended in 110 mM KCl, 10 mM NaCl, 1.2 mM $CaCl_2$, 10 mM glucose and 20 mM Hepes/Tris (pH 7.4) and stored in −60°C until used.

Measurement of glutamate uptake

Synaptosomes were pretreated in the media with or without *t*-BHP at 37°C for 60 min. Uptake of glutamate by synaptosomes was measured by a rapid filtration technique as described by Chan et al (1983) with some modification. Briefly, the reaction was initiated by adding synaptosomes to the incubation medium (a 1:10 dilution of synaptosome suspension) at 37°C. The composition of the incubation medium was 10 μM [^{14}C]-L-glutamate, 115 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM Hepes/Tris (pH 7.4). At the designated times, 100 μl aliquots were taken and quickly filtered under vacuum through Millipore filters (HAWP, 0.45 μm pore size) which were soaked overnight in distilled water. The filters were then washed with 5 ml of ice-cold stop solution comprising an identical composition to the incubation medium but without substrate, and dissolved in 1.0 ml of methoxyethanol. After addition of 10 ml of scintillation cocktail, the amount of radioactivity retained in synaptosomes was determined by liquid scintillation spectrometry. Nonspecific binding of radioactive substrate to synatosomal membranes was determined by incubating vesicles in transport buffer containing 0.1% deoxycholate and radiolabelled substrates. All uptake data were corrected for nonspecific binding. Protein concentration was measured according to the method of Bradford (1976) using γ-globulin as a standard.

Measurement of synaptosomal Na^+-K^+-ATPase activity

Synaptosomes were preincubated in the media containing 0.1% deoxycholate with or without *t*-BHP at 37°C for 60 min. The ATPase activity of synaptosomes was determined by measuring inorganic phosphate (P*i*) released by ATP hydrolysis during incubation of synaptosomes with the assay medium containing 3 mM ATP (Sigma) as the substrate. The total ATPase activity was determined in the presence of 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 2 mM EDTA, and 40 mM imidazole (pH 7.4). The Mg^{2+}-ATPase activity was determined in the absence of K^+ and in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+-K^+-ATPase activity. Synaptosomes were preincubated at 37°C for 60 min

in the presence or absence of *t*-BHP, and the reaction was initiated with the addition of the synaptosomes. At the end of a 10-min incubation, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3,500 g, and inorganic phosphate (Pi) in the supernatant fraction was determined by the method of Fiske and SubbaRow (1925).

Measurement of lipid peroxidation

Lipid peroxidation of synaptosomal membrane was evaluated by measuring the amount of a thiobarbituric acid reactive compound malondialdehyde (MDA) as described by Buege and Aust (1978). Synaptosomes were pretreated with or without *t*-BHP at 37°C for 60 min. In some experiments, lipid peroxidation was initiated by incubating synaptosomes with ascorbic acid and $FeSO_4$. Synaptosomal preparations (150 μl) were added to 850 μl of 10% trichloroacetic acid containing 0.5 mM EDTA and mixed with 1.0 ml of 0.67% thiobarbituric acid and heated at 95°C for 20 min. Samples were centrifuged at 2,500 g for 15 min after cooling. The absorbance was determined at 532 nm in a spectrophotometer. Absorbance of tissue samples was compared with that of the freshly prepared malonaldehyde bis-dimethylacetal standard. Results were expressed as nmoles of MDA per mg protein.

Chemicals

[^{14}C]-L-glutamate was purchased from the Amersham International (Amersham, UK). *t*-Butylhydroperoxide (*t*-BHP), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), butacaine, dibucaine, quinacrine, verapamil, diltiazem, nifedipine, deferoxamine mesylate, 1,10-phenanthroline, malonaldehyde bis-dimethylacetal, thiobarbituric acid, ouabain, ATP, and 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (Hepes) were purchased from Sigma (St. Louis, MO, U.S.A.). *N,N'*-diphenyl-phenylenediamine (DPPD) was from Aldrich Chemicals (Milwaukee, WI). All other chemicals were the highest quality available.

Statistical analyses

The data were expressed as mean ± SE and evaluated for significance using Student's *t*-test. A probability level of 0.05 was used to establish significance.

RESULTS

Time course of glutamate uptake

Fig. 1 shows the time course of glutamate uptake by synaptosomes. Synaptosomes were preincubated in the substrate-free medium in the presence or absence of 1 mM *t*-BHP for 30 min and then incubated in the medium containing substrate for the indicated time periods. When the incubation was performed longer than 1 min, the glutamate uptake by *t*-BHP-treated synsaptosomes was significantly lower than that by control synaptosomes. Since in both groups of synaptosomes the glutamate uptake increased only during the initial 5 min period, the glutamate uptake was measured for 5 min in all subsequent experiments.

Effect of t-BHP concentration

Fig. 2 depicts the effect of *t*-BHP concentration in the preincubation medium on the glutamate uptake by synaptosomes. When synaptosomes were pretreated

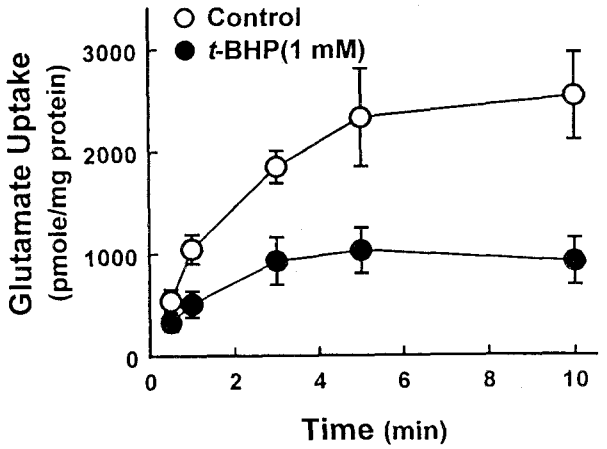

Fig. 1. Time course of the glutamate uptake by synaptosomes in the presence of an inwardly directed Na^+ gradient (100 mM). Synaptosomes were preincubated in a medium with or without 1 mM *t*-BHP for 30 min and then glutamate uptake was measured for 0.5 ~ 10 min. Data are mean ± SE of three determinations.

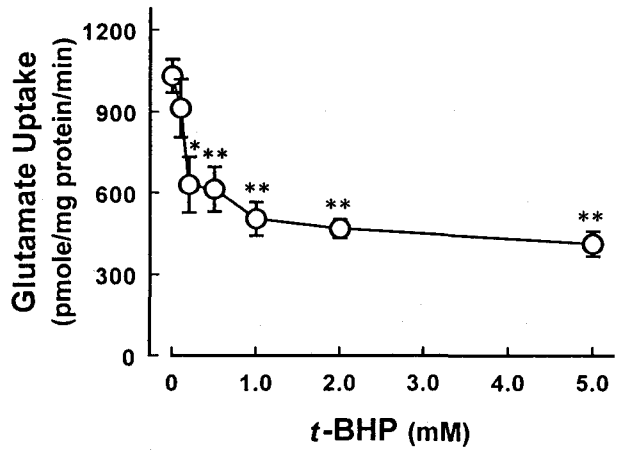

Fig. 2. Effect of various concentrations of *t*-BHP on glutamate uptake by synaptosomes. Synaptosomes were pretreated with *t*-BHP for 60 min and then glutamate uptake was determined for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). Data are mean±SE of four determinations. *p<0.05; **p<0.01 compared with the control (0 mM *t*-BHP).

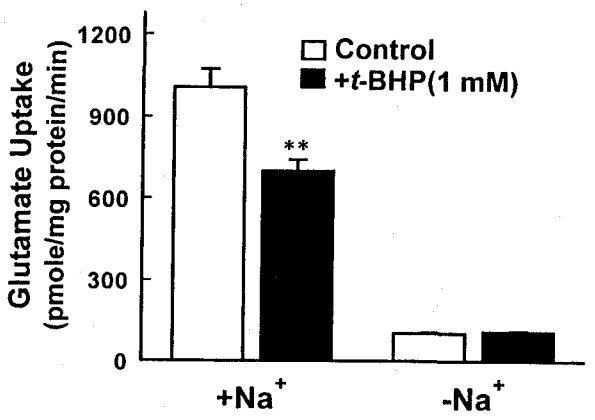

Fig. 3. Effects of *t*-BHP on Na^+-dependent and -independent glutamate uptake by synaptosomes. Synaptosomes were pretreated with 1 mM *t*-BHP for 60 min and then glutamate uptake was determined for 5 min in the medium with ($+Na^+$) or without ($-Na^+$, replaced by choline) 100 mM Na. Data are mean±SE of four determinations. **p<0.01 compared with the control in the presence of Na^+.

with 0.1~5.0 mM *t*-BHP, the glutamate uptake was exponentially decreased as the *t*-BHP concentration increased above 0.2 mM. The concentration of *t*-BHP for 50% inhibition of the glutamate uptake was approximately 1.0 mM. The glutamate uptake in the absence of Na^+ (replaced by choline) in the incubation medium was not different between the control and *t*-BHP-treated synaptosomes (Fig. 3). These results indicate that *t*-BHP impairs the Na^+-dependent glutamate transport in synaptosomes.

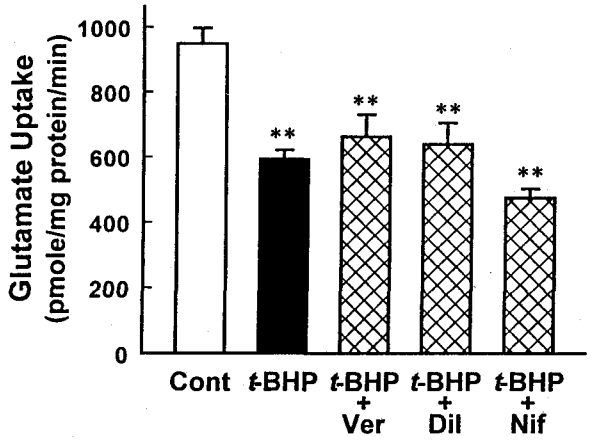

Fig. 4. Effects of Ca^{2+} channel blockers on *t*-BHP inhibition of glutamate uptake by synaptosomes. Synaptosomes were pretreated with 0.5 mM *t*-BHP in the presence or absence of 0.1 mM Ca^{2+} channel blockers for 60 min and then glutamate uptake was determined for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). Ver, verapamil. Dil, diltiazem, Nif, nifedipine. Data are mean±SE of four determinations. **p<0.01 compared with the control.

Effect of Ca^{2+} channel blockers

In order to investigate if Ca^{2+} channel blockers could protect the membrane against the *t*-BHP action, effects of three Ca^{2+} channel blockers, phenylalkylamine verapamil, benzothiapine diltiazem and 1,4-dihydropyridine nifedipine, were examined. The results summarized in Fig. 4 indicated that the inhibition of synaptosomal uptake of glutamate by *t*-BHP pretreatment was not altered by any of the three Ca^{2+} channel blockers tested. The treatment of synaptosomes with these Ca^{2+} channel blockers in the absence of *t*-BHP did not affect the glutamate uptake.

Effect of phospholipase A_2 (PLA_2) inhibitors

Fig. 5 depicts the effect of PLA_2 inhibitors on the *t*-BHP inhibition of glutamate uptake. Since previous

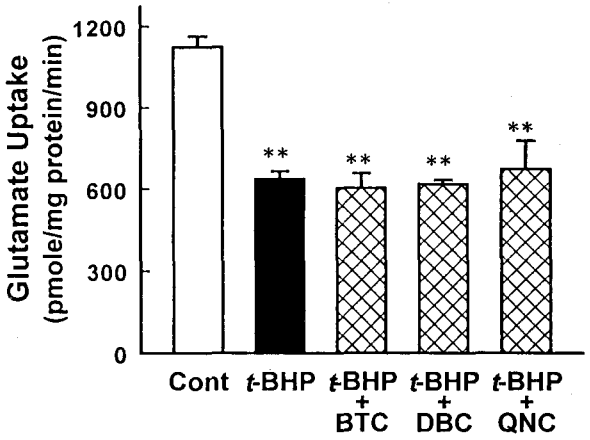

Fig. 5. Effects of PLA_2 inhibitors on *t*-BHP inhibition of glutamate uptake. Synaptosomes were pretreated with 0.5 mM *t*-BHP in the presence or absence of PLA_2 inhibitors for 60 min and then glutamate uptake was determined for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). BTC, butacaine; DBC, dibucaine; QNC, quinacrine. Data are mean ± SE of four determinations. **p < 0.01 compared with the control.

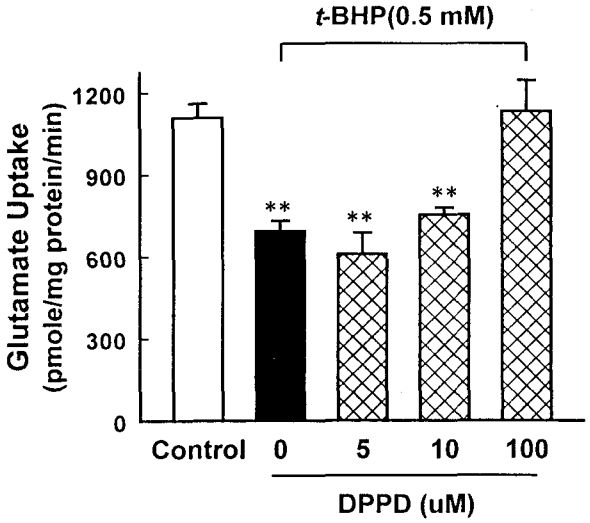

Fig. 6. Effect of DPPD on *t*-BHP-induced inhibition of glutamate uptake. Synaptosomes were pretreated with 0.5 mM *t*-BHP in the presence or absence of DPPD for 60 min and then glutamate uptake was determined for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). Data are mean ± SE of four determinations. **p < 0.01 compared with the control.

in vitro studies have shown that PLA_2 inhibitors such as butacaine (BTC), dibucaine (DBC) and quinacrine (QNC) reduce the cell injuries by oxidants and ischemia at concentrations of 30~250 μM in various cell types (Das et al, 1986; Malis & Bonventre, 1986, Pastorino et al, 1993; Bunnachak et al, 1994), we choose 250 μM as the concentration of PLA_2 inhibitors in the preincubation medium. The results depicted in Fig. 5 indicated that the inhibition of glutamate uptake by *t*-BHP was not affected by the presence of BTC, DBC or QNC in the preincubation medium. The treatment of synaptosomes with these PLA_2 inhibitors in the absence of *t*-BHP did not affect the glutamate uptake.

Effect of antioxidants

To understand the mechanism of *t*-BHP toxicity, the effect of an antioxidant DPPD in the preincubation medium was examined. The *t*-BHP inhibition of glutamate uptake was not affected by DPPD at 5 and 10 μM, but it was completely blocked by 100 μM DPPD (Fig. 6). A number of phenolic antioxidants including DPPD have been known to scavenge free radicals and thereby prevent the toxicity of oxidants (Chen & Stevens, 1991). In order to evaluate

Table 1. Effect of antioxidants on glutamate uptake in synaptosomes

	Glutamate uptake (pmole/mg protein/min)
Control	1092.52 ± 79.26
t-BHP (0.5 mM)	703.55 ± 63.76**
+ BHA (0.1 mM)	1095.71 ± 88.52
+ BHT (0.1 mM)	1049.94 ± 160.55
+ DPPD (0.1 mM)	1033.54 ± 93.25

Synaptosomes were preincubated with t-BHP in the presence or absence of antioxidants for 60 min at 37°C. Glutamate uptake was then measured for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). Data are the mean ± SE of four determinations. **p < 0.01 compared with the control.

effects of several phenolic antioxidants on the *t*-BHP toxicity, the inhibition of glutamate uptake by 0.5 mM *t*-BHP was compared with and without addition of 0.1 mM BHA, BHT or DPPD in the preincubation medium. The results summarized in Table 1 indicated that the inhibitory effect of *t*-BHP was completely

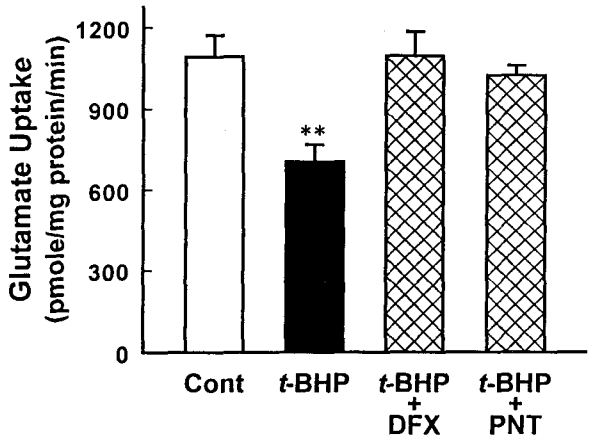

Fig. 7. Effects of iron chelators on *t*-BHP-induced inhibition of glutamate uptake. Synaptosomes were pretreated with 0.5 mM *t*-BHP in the presence or absence of iron chelators (0.1 mM) for 60 min and then glutamate uptake was determined for 5 min in the presence of an inwardly directed Na^+ gradient (100 mM). DFX, deferoxamine; PNT, phenanthroline. Data are mean ± SE of four determinations. **p < 0.01 compared with the control.

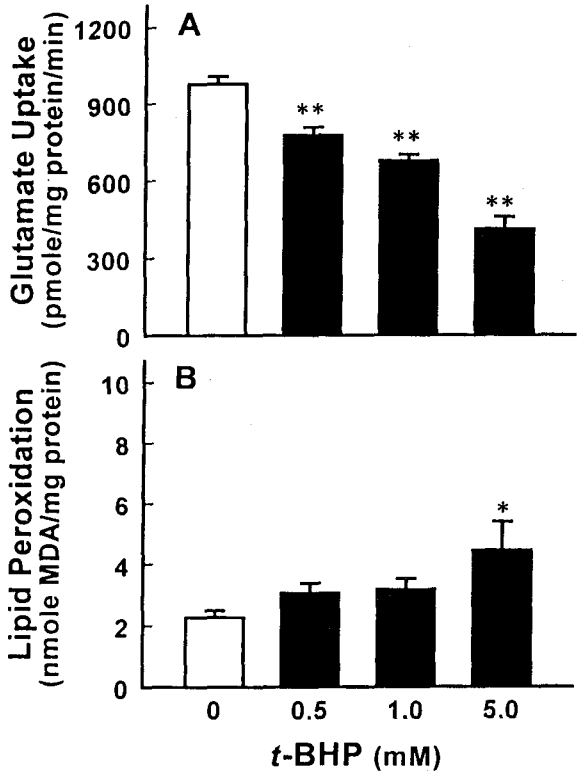

Fig. 8. Effects of *t*-BHP on glutamate uptake and lipid peroxidation in synaptosomes. Glutamate uptake (5 min) and lipid peroxidation were measured in synaptosomes pretreated with various concentrations of *t*-BHP for 60 min. Data are mean ± SE of four determinations. *p < 0.05; **p < 0.01 compared with the control.

eliminated by these antioxidants.

Role of iron in t-BHP-induced toxicity

In hepatocytes, *t*-BHP reacts with ferrous iron to produce *t*-butyl alkoxyl radical, a more potent oxidant (Masaki et al, 1989). To determine if endogenous ferrous iron contributed to the inhibition of glutamate uptake induced by *t*-BHP, the effect of *t*-BHP was examined in the presence of iron chelators, deferoxamine and phenanthroline. The results are shown in Fig. 7. When synaptosomes were preincubated with 0.5 mM *t*-BHP, the glutamate uptake was inhibited to approximately 64% of the control value. This inhibition was completely prevented when the iron chelators were included in the preincubation medium.

Role of lipid peroxidation

In the next series of experiments, we evaluated if lipid peroxidation plays a role in the *t*-BHP inhibition of glutamate uptake. Synaptosomes were pretreated with various concentrations of *t*-BHP and tested for glutamate uptake and lipid peroxidation. As depicted in Fig. 8, the glutamate uptake was significantly reduced to 79, 69 and 42% of the control at 0.5, 1.0 and 5.0 mM *t*-BHP, respectively. However, the lipid peroxidation was not altered at 0.5 and 1.0 mM *t*-BHP, and significantly increased at 5.0 mM *t*-BHP. These results suggest that the depression of glutamate uptake by *t*-BHP is not totally resulted from the lipid peroxidation.

To further determine the role of lipid peroxidation in the oxidant-induced inhibition of glutamate uptake, synaptosomes were pretreated with ascorbate/Fe^{2+} which is known to induce lipid peroxidation (Miller & Aust, 1989). As shown in Fig. 9, ascorbate/Fe^{2+} increased lipid peroxidation in a dose-dependent manner. While the formation of MDA in control synaptosomes was 2.26 ± 0.22 nmole/mg protein, the values in ascorbate/ Fe^{2+}-treated synaptosomes were 3.72 ± 0.41, 5.66 ± 0.48 and 11.70 ± 0.54 nmole/mg protein at 10/0.2, 20/0.4 and 50/1 μM ascorbate/Fe^{2+}, respectively. The glutamate uptake did not change at 10/0.2 and 20/0.4 μM ascorbate/Fe^{2+}, but it slightly inhi-

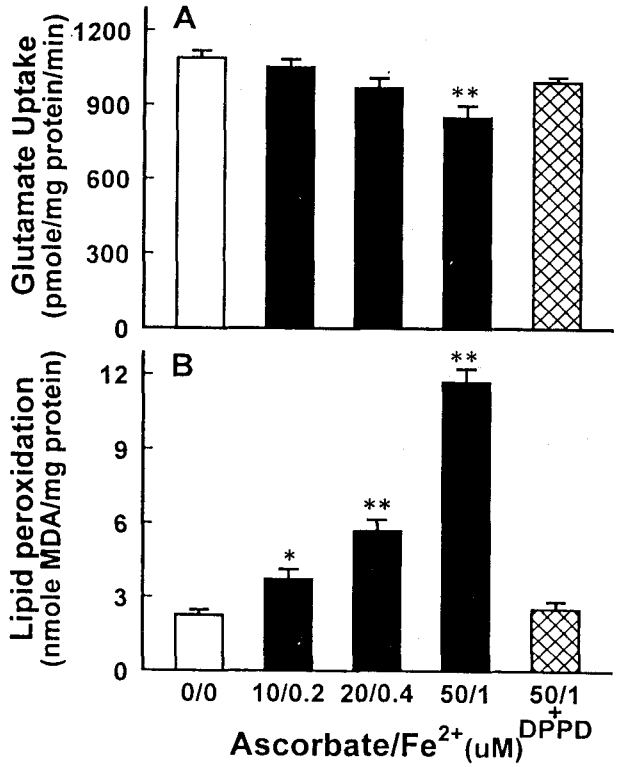

Fig. 9. Effects of ascorbate/Fe^{2+} on glutamate uptake and lipid peroxidation by synaptosomes. Glutamate uptake (5 min) and lipid peroxidation were measured in synaptosomes pretreated with various concentrations of ascorbate/Fe^{2+} for 60 min. Lipid peroxidation by 50/1 μM ascorbate/Fe^{2+} was completely protected by 100 μM DPPD. Data are mean$\pm$SE of five determinations. *$p<0.05$; **$p<0.01$ compared with the control.

bited at 50/1 μM ascorbate/Fe^{2+}. These results suggest again that the oxidant-induced inhibition of glutamate uptake in synaptosomes is not directly attributed to the lipid peroxidation. Lipid peroxidation by 50/1 μM ascorbate/Fe^{2+} was completely protected by 0.1 mM DPPD.

Effect of t-BHP on Na^+-K^+-ATPase activity

Since glutamate uptake into synaptosomes is mediated by a Na^+-dependent transport system (Iversen, 1971; Logen & Snyder, 1971; Bennet et al, 1973), the uptake is impaired by an inhibition of Na^+-K^+-ATPase (Iversen & Neal, 1968, Balcar & Johnston, 1972). Therefore, the *t*-BHP-induced depression of glutamate uptake in synaptosomes could be resulted

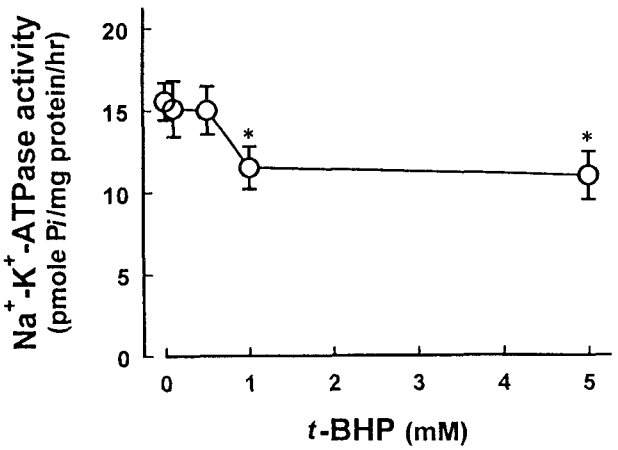

Fig. 10. Effect of *t*-BHP on synaptosomal Na^+-K^+-ATPase activity. Synaptosomes were pretreated with various concentrations of *t*-BHP for 60 min and Na^+-K^+-ATPase activity was measured for 10 min. Data are mean$\pm$SE of four determinations. *$p<0.05$ compared with the control.

from an inhibition of Na^+-K^+-ATPase activity. To examine this possibility, the effect of *t*-BHP on the synaptosomal Na^+-K^+-ATPase activity was measured. As shown in Fig. 10, *t*-BHP produced a slight, but significant, inhibition of Na^+-K^+-ATPase activity at concentrations above 1.0 mM. This indicates that the effect of *t*-BHP on the synaptosomal Na^+-K^+-ATPase activity is far less than that on the glutamate uptake, suggesting that the *t*-BHP inhibition of the glutamate uptake is not primarily associated with alterations of synaptosomal Na^+-K^+-ATPase activity.

DISCUSSION

Glutamate, a major excitatory amino acid neurotransmitter in the brain which plays a key role in learning and memory, is also known to act as a neurotoxin under certain situations such as ischemic or postischemic brain tissues (Jorgensen & Diemer, 1982; Meldrum, 1985; Olney, 1983). Thus, substances that inhibit the uptake and/or stimulate the release of glutamate would lead to a cell damage and eventually cell death.

The present study demonstrated that an oxidant, *t*-BHP, inhibited the Na^+-dependent glutamate uptake by synaptosomes isolated from the brain. The inhibition was significant at *t*-BHP concentrations above

0.2 mM (Fig. 2). By contrast, the Na^+-independent glutamate uptake was not affected by *t*-BHP as high as 1.0 mM (Fig. 3), indicating that *t*-BHP specifically inhibited the Na^+-dependent component of glutamate transport.

Since an oxidant stress on nervous tissue can induce functional impairment by increasing intracellular free Ca^{2+} (Halliwell, 1989), suppression of Ca^{2+} entry into cells may prevent the oxidant-induced cell injury. Ca^{2+} channel blockers have indeed been reported to be effective in reducing the ischemia-induced neuronal damage (Lazarewicz et al, 1990; Nishijo et al, 1995) which has been suggested to be associated with oxygen free radicals (Traystman et al, 1991). In myocytes, the cell injury caused by oxidants is effectively prevented by a Ca^{2+} channel blocker nitrendipine (Josephson et al, 1991). However, the data in the present study indicated that Ca^{2+} channel blockers could not protect the rabbit brain synaptosomal Na-dependent glutamate transport system against the *t*-BHP inhibition.

PLA_2 activation has been suggested to play an important role in excitotoxic neuronal cell injury with ischemia (Bonventre & Koroshetz, 1993). Several *in vitro* studies have also demonstrated that oxidant-induced cell injury is prevented by PLA_2 inhibitors in renal (Malis & Bonventre, 1986) and liver (Imberti et al, 1993) cells. In the present study, however, reduction in glutamate uptake by *t*-BHP was not altered by PLA_2 inhibitors (Fig. 5). These results suggest that the impairment of synaptosomal glutamate uptake by *t*-BHP was not associated with PLA_2 activation.

Lipid peroxidation has been recognized as an important mediator of certain deleterious effects of oxygen free radicals on cells. Nevertheless, the role of lipid peroxidation in the pathogenesis of cell injury with an acute oxidative stress is controversial. In renal cells, lipid peroxidation is a critical event leading to cell death by *t*-BHP (Schnellmann, 1988; Chen & Stevens, 1991), while in hepatocytes it plays no role in the acute toxicity of *t*-BHP (Rush et al, 1985). Adams et al (1994) have demonstrated that *t*-BHP-induced injury of various neurons is associated with lipid peroxidation. In the present study, antioxidants such as DPPD were effective in preventing the *t*-BHP inhibition of glutamate uptake (Table 1). This may suggest that lipid peroxidation plays an important role in the *t*-BHP inhibition of glutamate uptake. However, in the present study, *t*-BHP did not alter the lipid peroxidation (as estimated by generation of MDA) at the concentrations of 0.5 and 1.0 mM which caused a significant inhibition of glutamate uptake (Fig. 8). This suggests that the *t*-BHP inhibition of glutamate uptake was not primarily associated with peroxidation of membrane lipid. When synaptosomes were exposured to ascorbate-Fe^{2+} system to induce lipid peroxidation, the formation of MDA increased in a dose-dependent manner, and the effect was effectively prevented by DPPD (Fig. 9). Surprisingly, however, the increase in lipid peroxidation was not accompanied by a reduction in glutamate uptake. The glutamate uptake was not significantly altered until the lipid peroxidation increased to approximately 5 times the control value at ascorbate/Fe^{2+} of 50/1 μM (Fig. 9). Similarly, Rush et al (1985) have reported in isolated hepatocytes that lipid peroxidation induced by ascorbate/Fe^{2+} had no effect on the cell viability or morphology. We therefore presume that lipid peroxidation is not a major cause of the cell injury associated with oxidative stress. The protection of glutamate transport system by antioxidants against the *t*-BHP inhibition (Table 1) may be resulted from a direct interaction of antioxidant with *t*-BHP or its metabolites.

Potential mechanisms by which *t*-BHP produces cytotoxic effects were proposed by Rush et al (1985) in hepatocytes. *t*-BHP is metabolized by glutathione peroxidase to produce *t*-butyl alcohol and glutathione disulfide (GSSG) which is subsequently reoxidized to glutathione (GSH) by glutathione reductase resulting in pyridine nucleotide oxidation. Depletion of GSH and oxidation of pyridine nucleotides are associated with altered Ca^{2+} homeostasis. Alternatively, *t*-BHP reacts with ferrous iron to produce more potent oxidant *t*-butyl alkoxyl radical that can initiate lipid peroxidation. *t*-BHP free radicals may also form covalent bonds with cellular molecules, resulting in a cell injury (Rush et al, 1985). The fact that in the present study iron chelators completely prevented the *t*-BHP inhibition of glutamate uptake (Fig. 7) indicates that *t*-BHP free radicals play a critical role in mediating the toxicity. In the present study, the mechanisms by which *t*-BHP inhibited the synaptosomal glutamate uptake are not clearly identified. However, the fact that lipid peroxidation is not involved in the *t*-BHP-induced inhibition (Figs. 8 and 9) suggests the possibility that *t*-BHP impairs glutamate uptake by a

direct interaction of its metabolites with the transporter. Covalent binding between *t*-BHP intermediates and membrane proteins for glutamate transport or oxidation of the proteins by *t*-BHP metabolites could be involved.

Since the glutamate uptake in synaptosomes is driven by an inwardly directed Na^+ gradient generated by Na^+-pump, an inhibition of Na^+-K^+-ATPase activity would result in a reduction of glutamate uptake. However, the present study indicated that the effect of *t*-BHP on glutamate uptake was not mediated through an alteration of Na^+-K^+-ATPase activity.

The inhibition of glutamate uptake in the synapse would lead to an extracellular accumulation of this excitotoxic compound. An increase in extracellular glutamate in cerebral ischemia, as observed by a number of investigators (Benveniste et al, 1984; Graham et al, 1990; Globus et al, 1991), may be, in part, due to an impairment of the glutamate transport system. Since oxygen free radicals are generated in ischemic neuronal cells(Traystman et al, 1991), the results obtained from the present study suggest the possibility that oxidative stress and excitotoxicity could synergically act to produce neuronal damage.

In conclusion, *t*-BHP impaired the glutamate uptake in synaptosomes isolated from cerebral cortex. This inhibition was not changed by Ca^{2+} channel blockers or PLA_2 inhibitors, but it was completely protected by iron chelators and phenolic antioxidants. *t*-BHP-induced alterations in the glutamate uptake were not accompanied by a formation of lipid peroxidation. These results suggest that *t*-BHP-induced inhibition of synaptosomal glutamate uptake is mediated by mechanisms independent of peroxidation of membrane lipids.

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