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Title page

Lycopene depresses glutamate release through inhibition of voltage-dependent Ca\(^{2+}\) entry and protein kinase C in rat cerebrocortical nerve terminals

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**Abstract:** Lycopene is a natural dietary carotenoid which was reported to exhibit neuroprotective profile. Considering that excitotoxicity and cell death induced by glutamate are involved in many brain disorders, the effect of lycopene on glutamate release in rat cerebrocortical nerve terminals and the possible mechanism involved in such effect was investigated. We observed here that lycopene inhibited 4-aminopyridine (4-AP)-evoked glutamate release and intrasynaptosomal Ca\(^{2+}\) concentration elevation. The inhibitory effect of lycopene on 4-AP-evoked glutamate release was markedly reduced in the presence of the Ca\(_{\text{v}2.2}\) (N-type) and Ca\(_{\text{v}2.1}\) (P/Q-type) channel blocker ω-conotoxin MVIIC, but was insensitive to the intracellular Ca\(^{2+}\)-release inhibitors dantrolene and CGP37157. Furthermore, in the presence of the PKC inhibitors GF109203X and Go6976, the action of lycopene on evoked glutamate release was prevented. These results are the first to suggest that lycopene inhibits glutamate release from rat cortical synaptosomes by suppressing presynaptic Ca\(^{2+}\) entry and PKC activity.

*Key Words:* lycopene, glutamate release, presynaptic Ca\(^{2+}\) channel, PKC, synaptosome, 4-aminopyridine, cerebral cortex
Introduction

Glutamate is a major excitatory neurotransmitter in mammalian central nervous system (CNS) (Greenamyre and Porter 1994). Excessive glutamate releasing overactivates its receptors, which leads to an overload of intracellular calcium, generation of free radicals, mitochondrial dysfunction, and subsequent neuronal damage or death (Lau and Tymianski 2010; Sattler and Tymianski 2001). This glutamate-induced excitotoxicity is linked to neuropathology of acute (trauma, ischemia) and chronic (epilepsy, Alzheimer’s disease and Parkinson's disease) brain disorders (Lipton and Rosenberg 1994; Lerma et al. 2001; Rothstein 1996). Thus, inhibition of glutamate release is considered to be a potentially important mechanism for neuroprotective actions.

Lycopene, a dietary carotenoid present in tomatoes and other red fruits and vegetables, has received considerable attention for its possible role in the prevention of cancer, atherosclerosis, diabetes, and some inflammatory diseases (Böhm 2012; Cruz Bojórquez et al. 2013). In addition to these health benefits, lycopene is also thought to have brain protection. This concept stems from the observation that lycopene can penetrate the blood-brain barrier (Khachik et al. 2002), ameliorate pentylenetetrazol-induced seizures (Bhardwaj and Kumar 2016; Kumar et al. 2016), as well as attenuate ischemia- or neurotoxins-induced brain injury and memory impairment in experimental animals (Fujita et al. 2013; Lei et al. 2016; Prakash and Kumar 2013; Sachdeva and Chopra 2015). Furthermore, it was reported that dietary intake of lycopene can reduce the risks
of neurodegenerative disorders, especially Alzheimer's disease (Bun et al. 2015). Although the underlying mechanism through which lycopene exerts its neuroprotective effect has not been clearly elucidated, inhibited inflammatory processes, antioxidant activity, oxygen-free radicals scavenging, and improved mitochondrial dysfunction have been implicated (Bhardwaj and Kumar 2016; Fujita et al. 2013; Sachdeva and Chopra 2015).

Because excitotoxicity mediated by excessive glutamate release is a mechanism of neuronal injury, implicated in the pathogenesis of many acute and chronic brain disorders, we used isolated nerve terminals (synaptosomes) prepared from the rat cerebral cortex to determine if lycopene could affect glutamate release. This preparation, being devoid of the cell-body, does not suffer from any complication of interpretation produced by concomitant postsynaptic effects, and is therefore extensively used to evaluate presynaptic effects on neurotransmitter release (Andrade-Talavera et al. 2012, 2013; Lonart and Sudhof 2000; Nicholls 1993; Rodriguez-Moreno and Sihra 2013a).

Materials and Methods

Animals

Male Sprague Dawley rats (150–200 g) were purchased from BioLASCO (Taiwan Co., Ltd, Taipei, Taiwan). Animals were housed in a room maintained at 25°C with a 12 h light/dark cycle. Food and water were available ad libitum. Animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals (NAC 2011), and were approved by the
Institutional Animal Care and Use Committee at the Fu Jen Catholic University.

**Chemicals**

4-aminopyridine (4-AP), bafilomycin A1, DL-threo-β-benzylxoyaspartate (DL-TBOA), ω-conotoxin MVIIC (ω-CgTX MVIIC), dantrolene, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), bisindolylmaleimide I (GF109203X), and 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were purchased from Tocris Cookson (Bristol, UK). Fura-2-acetoxymethyl ester (Fura-2-AM) was purchased from Invitrogen (Carlsbad, CA, USA). Lycopene, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Preparation of synaptosomes**

Rats were decapitated and had their cerebral cortex removed and homogenized in 0.32 M sucrose solution. Homogenates were then submitted to low-speed centrifugation (16500 rpm, 7 min) and synaptosomes were purified from the supernatant by discontinuous Percoll-density gradient centrifugation, as previously described (Nicholls and Sihra 1986; Rodriguez-Moreno and Sihra 2004). The final synaptosomal pellets were resuspended in HEPES buffer medium (HBM) with the following composition (mM): NaCl, 140; KCl, 5; NaHCO₃, 5; MgCl₂·6H₂O, 1;
Na$_2$HPO$_4$, 1.2; glucose, 10; HEPES, 10; pH 7.4. Protein concentration was then determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.5 mg protein. Synaptosomal pellets were stored on ice and used within 4-6 h.

**Glutamate release assay**

Glutamate release was assayed by on-line fluorometry (Nicholls and Sihra 1986). Pelleted synaptosomes were resuspended in HBM containing 16 µM bovine serum albumin (BSA) and incubated in a stirred and thermostated cuvette at 37°C in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). NADP$^+$ (2 mM), glutamate dehydrogenase (50 units/ml) and CaCl$_2$ (1.2 mM) were added after 3 min. 4-AP (1 mM) was applied to evoke glutamate release. The oxidative deamination of released glutamate, leading to the reduction of NADP$^+$, was monitored by measuring NADPH fluorescence at excitation and emission wavelengths of 340 and 460 nm, respectively. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence change produced by the standard addition was used to calculate the released glutamate as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Release values quoted in the text and depicted in bar graphs represent the levels of glutamate cumulatively released after 5 min of depolarization, and are expressed as nmol/mg/5 min. Data were accumulated at 2-s intervals and cumulative data were analyzed using Lotus 1-2-3.
Determination of synaptosomal cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{C}\))

[Ca\(^{2+}\)]\(_{C}\) was measured with fura-2. Synaptosomes were resuspended (2 mg/mL) in HBM containing 16 µM BSA in the presence of 5 µM fura-2 and 0.1 mM CaCl\(_2\) and incubated at 37°C for 30 min in a stirred test tube. After fura-2 loading, synaptosomes were pelleted and resuspended in HBM containing BSA. The synaptosomal suspension was stirred in a thermostatted cuvette containing 1.2 mM CaCl\(_2\) in a Perkin-Elmer LS-55 spectrofluorimeter, and the fluorescence was monitored at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm). Data was collected at 2-s intervals. [Ca\(^{2+}\)]\(_{C}\) (nM) was calculated using the equations described by Grynkiewicz et al. (1985). Cumulative data were analyzed using Lotus 1-2-3.

Data analysis

Data are presented as mean ± SEM. Student’s unpaired t test or ANOVA was used for statistical analysis as appropriate; p values are reported throughout, and significance was set as p < 0.05.
Results

Synaptosomes were depolarized with the K$^+$ channel blocker 4-AP, which opens voltage-dependent Ca$^{2+}$ channels and induces the release of glutamate (Nicholls 1998). Figure 1A shows that 4-AP (1 mM) evoked a glutamate release of $6.9 \pm 0.1$ nmol/mg/5 min from synaptosomes incubated in the presence of 1.2 mM CaCl$_2$. Preincubation of synaptosomes with lycopene (1 µM) for 10 min did not alter the basal glutamate release but inhibited the release of glutamate evoked by 4-AP to $3.1 \pm 0.1$ nmol/mg/5 min [$t(16) = 26.1$, $P < 0.001$]. The effect of lycopene was dose-dependent, with an IC$_{50}$ of approximately 0.3 µM (Fig. 1B). Given the robust depression of glutamate release seen with 1 µM lycopene, this concentration was used in subsequent experiments to evaluate the mechanisms underlying the ability of lycopene to reduce glutamate release.

Figure 2 shows that the glutamate release evoked by 1 mM 4-AP in an extracellular Ca$^{2+}$-free solution containing 300 µM EGTA was $1.9 \pm 0.2$ nmol/mg/5 min [$F(2,12) = 308.8$; $P < 0.001$]. This Ca$^{2+}$-independent component of the 4-AP-evoked glutamate release was unaffected by 1 µM lycopene ($1.9 \pm 0.1$ nmol/mg/5 min; $P > 0.05$). DL-TBOA (10 µM), a non-selective inhibitor of all excitatory amino acid transporter subtypes, increased the 4-AP-evoked glutamate release, owing to the inhibition of reuptake of the released glutamate ($P < 0.001$). In the presence of DL-TBOA, lycopene (1 µM) still significantly reduced the 4-AP-evoked release of glutamate [$F(2,15) = 252.3$; $P < 0.001$; Fig. 2]. By contrast, bafilomycin A1 (0.1 µM), a
vesicular transporter inhibitor, inhibited the 4-AP-evoked glutamate release and prevented the inhibitory effect of lycopene \([F(2,12) = 331.4; \ P < 0.001]\). In the five tested synaptosomal preparations, no statistical difference was observed between the release after bafilomycin A1 alone and after the bafilomycin A1 and lycopene treatment \((\ P > 0.05; \ \text{Fig. 2})\). These results indicate that lycopene influences the evoked glutamate release by a decrease in vesicular exocytosis.

Figure 3 shows that the stimulation of synaptosomes with 1 mM 4-AP caused an increase in \([\text{Ca}^{2+}]_c\) levels to a plateau level \((\ P < 0.001)\). Preincubation with lycopene \((1 \ \mu\text{M})\) did not affect basal \([\text{Ca}^{2+}]_c\) levels but reduced the 4-AP-evoked increase in \([\text{Ca}^{2+}]_c\) \((t(11) = 6.9, \ P < 0.001)\). Figure 4 shows that glutamate release evoked by 1 mM 4-AP was decreased in the presence of 4 \(\mu\text{M}\) \(\omega\text{-CgTX MVIIC}\), a wide-spectrum blocker of \(\text{Ca}_{\text{v}}2.2\) (N-type) and \(\text{Ca}_{\text{v}}2.1\) (P/Q-type) channels, and prevented the inhibition of glutamate release by lycopene \((1 \ \mu\text{M})\) \([F(2,14) = 222.7; \ P < 0.001]\). In the presence of \(\omega\text{-CgTX MVIIC}\) and lycopene, the inhibition of glutamate release was not significantly different from the effect of \(\omega\text{-CgTX MVIIC}\) alone \((\ P > 0.05)\). Dantrolene \((100 \ \mu\text{M})\), a blocker of \([\text{Ca}^{2+}]_c\) release from endoplasmic reticulum ryanodine receptors, reduced the 4-AP \((1 \ \text{mM})\)-evoked glutamate release \((\ P < 0.01)\). In the presence of dantrolene \((100 \ \mu\text{M})\), the application of lycopene \((1 \ \mu\text{M})\) still effectively inhibited 4-AP-evoked glutamate release \([F(2,16) = 85.2; \ P < 0.001; \ \text{Fig. 4}]\). Similarly, CGP37157 \((100 \ \mu\text{M})\), a membrane-permeant blocker of the mitochondrial \(\text{Na}^+/\text{Ca}^{2+}\) exchanger, reduced the release of glutamate evoked by
4-AP (P < 0.01). In the presence of CGP37157 (100 µM) and lycopene (1 µM), the inhibition of glutamate release following 4-AP-depolarization was significantly different from the effect of CGP37157 alone [F(2,16) = 66.4; P < 0.001; Fig. 4]. Thus, decrease in Ca\(^{2+}\) influx mediated by Ca\(_{v}\)2.2 (N-type) and Ca\(_{v}\)2.1 (P/Q-type) channels appears to be associated with the observed lycopene-mediated inhibition of glutamate release.

Figure 5 shows that PD98059 (50 µM), a mitogen-activated/extracellular signal-regulated kinase (MEK) inhibitor, reduced 4-AP (1 mM)-evoked glutamate release (P < 0.001). In synaptosomes pretreated with PD98059, lycopene (1 µM) was still able to reduce 4-AP-evoked glutamate release [F(2,13) = 62.2; P < 0.001]. A statistical difference was observed between the release after PD98059 alone and after the PD98059 and lycopene treatment (P < 0.05; Fig. 5). H89 (100 µM), a protein kinase A (PKA) inhibitor, reduced the 4-AP (1 mM)-evoked glutamate release (P < 0.001). Similarly, in the presence of H89 (100 µM) and lycopene (1 µM), the inhibition of glutamate release was significantly different from the effect of H89 alone [F(2,13) = 147.4; P < 0.001]. GF109203X (10 µM), a protein kinase C (PKC) inhibitor, reduced the 4-AP (1 mM)-evoked glutamate release (P < 0.001). In synaptosomes pretreated with GF109203X, however, the inhibitory effect of lycopene (1 µM) on 4-AP-evoked glutamate release was prevented [F(2,15) = 448.5; P < 0.001]. No statistical difference was observed between the release after GF109203X alone and after the GF109203X and lycopene treatment (P > 0.05; Fig. 5). Similar results were also obtained with another conventional PKC inhibitor Go6976 (3 µM).
Discussion

It has been reported that lycopene can enter the brain (Khachik et al. 2002) and has neuroprotective activity in animal models of neurotoxicity such as ischemia and Alzheimer's disease (Fujita et al. 2013; Lei et al. 2016; Prakash and Kumar 2013; Sachdeva and Chopra 2015); however, its precise mechanism remains unclear. In this work we have observed that lycopene inhibited evoked glutamate release in synaptosomes isolated from rat cerebral cortex, which may explain, at least in part, for its neuroprotective effect. This is because the excitotoxicity caused by excessive glutamate has been proposed to be involved in the pathogenesis of many brain diseases (Lipton and Rosenberg 1994; Rothstein 1996). To the best of our knowledge, this study represents the first examination of the effect of lycopene on endogenous glutamate release at the presynaptic level. Several possible mechanisms for this effect are discussed as follows.

The release of glutamate produced by the depolarization of isolated nerve terminals has 2 components. A physiologically relevant \( \text{Ca}^{2+} \)-dependent component is produced by the exocytosis of synaptic vesicles containing glutamate. A second, \( \text{Ca}^{2+} \)-independent component is attributed to the reversal of the glutamate transporter (Nicholls 1998; Nicholls et al. 1987). In the present study, we found that lycopene did not affect 4-AP-evoked glutamate release in the absence of extracellular \( \text{Ca}^{2+} \). Furthermore, the observed inhibitory action of lycopene on 4-AP-evoked glutamate release was abolished by the vesicular transporter inhibitor bafilomycin.
A1, but not by the glutamate transporter inhibitor DL-TBOA. Therefore, these results suggest that the inhibition of 4-AP–evoked glutamate release by lycopene is mediated by a reduction in the Ca$^{2+}$-dependent exocytotic component of glutamate release.

Using Fura-2, this study demonstrated that lycopene significantly decreases the 4-AP–evoked increase in [Ca$^{2+}$]$_C$. Furthermore, the observed lycopene-mediated inhibition of 4-AP-evoked glutamate release was decreased from 56% to 18% after exposure to a blocker of Cav2.2 (N-type) and Cav2.1 (P/Q-type) Ca$^{2+}$ channels, but it was insensitive to the intracellular Ca$^{2+}$ release inhibitors. Although how lycopene affects presynaptic Ca$^{2+}$ channel activity remains unclear, our results suggest that the lycopene-mediated glutamate release inhibition is associated with a decreased Ca$^{2+}$ entry through the Ca$_{v}$.2.2 (N-type) and Ca$_{v}$.2.1 (P/Q-type) channels, which are known to directly participate in triggering glutamate release from the nerve terminals (Millan and Sanchez-Prieto 2002; Vazquez and Sanchez-Prieto 1997). However, the combined blockade of both Ca$^{2+}$ channel types could not eliminate the action of lycopene completely; thus, other unidentified types of Ca$^{2+}$ channels or other presynaptic pathways may be involved in the inhibition. For example, numerous receptors, such as kainate receptors and metabotropic glutamate receptors, have been shown to be present at the presynaptic level, at which they regulate voltage-dependent Ca$^{2+}$ channel function and glutamate release (Rodríguez-Moreno and Sihra 2011, 2013a; Rodríguez-Moreno et al. 1998; Sistiaga et al. 1998; Wang and Sihra 2004).
Whether a relationship exists between the lycopene-mediated inhibition of glutamate release and these receptors remains to be explored.

Ca\(^{2+}\) influx through presynaptic Ca\(^{2+}\) channels is known to activate several protein kinases associated with glutamate release in nerve terminals including MAPK, PKC, and PKA (Andrade-Talavera et al. 2013; Coffey et al. 1994; Rodriguez-Moreno and Sihra 2013b; Yamagata et al. 2005). In the present study, the PKC inhibitors efficiently antagonized the lycopene-mediated inhibition of glutamate release; nevertheless, the MAPK inhibitor or the PKA inhibitor were ineffective. Thus, the inhibitory effect of lycopene on Ca\(^{2+}\) entry observed here may lead to suppression of PKC activity and, in turn, reduces glutamate release. In fact, several synaptic proteins involved in the synaptic vesicle trafficking/recruitment and exocytosis have been found to be phosphorylated by PKC (Craig et al. 2003; Jarvis and Zamponi 2001; Morgan et al. 2005). Which downstream substrate of PKC involves in the lycopene-mediated inhibition of glutamate release remains to be determined.

In conclusion, our data shown that lycopene inhibits glutamate release from rat cortical synaptosomes by suppressing presynaptic Ca\(^{2+}\) entry and PKC activity. Even the functional role of lycopene-inhibited glutamate release explored here is not clear; such effect could be a potentially important mechanism to protect brain during excitotoxicity.

**Conflict of interest**
The authors state no conflict of interest.

Acknowledgements

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Figure Captions

**Fig. 1.** Effect of lycopene on the 4-AP-evoked glutamate release in rat cerebrocortical synaptosomes. (A) Glutamate release was evoked by 4-AP (1mM) in the absence (control) or presence of lycopene (1 µM), added 10 min before the addition of 4-AP. (B) Concentration-dependent depression of glutamate release by lycopene. Data represent mean ± SEM values of independent experiments, using synaptosomal preparations from 5 to 10 animals. ***, P < 0.001 versus control group.

**Fig. 2.** Effect of external calcium omission, the glutamate transporter blocker DL-TBOA, and the vesicular transporter inhibitor bafilomycin A1 on the lycopene-mediated inhibition of 4-AP-evoked glutamate release. Each column is mean ± SEM values of independent experiments, using synaptosomal preparations from 5 to 7 animals. ***, P < 0.001 versus control group. #, P < 0.05 versus the DL-TBOA-treated group.

**Fig. 3.** Effect of lycopene on the 4-AP-evoked increase in intraterminal Ca$^{2+}$ concentration. Each column is mean ± SEM values of independent experiments, using synaptosomal preparations from 5 animals. ***, P < 0.001 versus control group.

**Fig. 4.** Effect of Ca$_v$2.2 (N-type) and Ca$_v$2.1 (P/Q-type) channel blocker ω-CgTX MVIIC or intracellular Ca$^{2+}$ release inhibitors dantrolene and CGP37157 on the lycopene-mediated inhibition of 4-AP-evoked glutamate release. Each column is mean ± SEM values of independent experiments, using synaptosomal preparations from 5 to 7 animals. **, P < 0.01,
***, P < 0.001 versus the control group; #, P < 0.05 versus the dantrolene- or CGP37157-treated group.

**Fig. 5.** Effect of the MAPK inhibitor PD98059, PKA inhibitor H89, and PKC inhibitors GF109203X and Go6976 on the lycopene-mediated inhibition of 4-AP-evoked glutamate release. Each column is mean ± SEM values of independent experiments, using synaptosomal preparations from 5 to 6 animals. ***, P < 0.001 versus the control group; #, P < 0.05 versus the PD98059- or GF109203X-treated group.
Figure 1

A

B

Glutamate release (nmol/mg vs. Time (60 s/division))

Glutamate release (nmol/mg/5 min)

1 mM 4-AP

Lycopene (µM)

(10) (6) (5) (8) (8) (7)
**Figure 2**

- Control
- 1 μM lycopene
- 300 μM EGTA (omitting CaCl₂)
- 300 μM EGTA (omitting CaCl₂), 1 μM lycopene
- 10 μM DL-TBOA
- 10 μM DL-TBOA, 1 μM lycopene
- 0.1 μM Bafilomycin A1
- 0.1 μM Bafilomycin A1, 1 μM lycopene

Glutamate release (nmol/mg/5 min)
Figure 3

4-aminopyridine evoked increase in intraterminal Ca$^{2+}$ concentration (nM)

- Control
- 1 μM lycopene

1 mM 4-AP

(5)
Figure 4

Glutamate release (nmol/mg/5 min)

Control
1 μM lycopene
4 μM ω-CgTX MVIIC
4 μM ω-CgTX MVIIC, 1 μM lycopene
100 μM dantrolene
100 μM dantrolene, 1 μM lycopene
100 μM CGP37157
100 μM CGP37157, 1 μM lycopene

1 mM 4-AP

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Figure 5

Glutamate release (nmol/mg/5 min)

- Control
- 1 μM lycopene
- 50 μM PD98059
- 50 μM PD98059 + 1 μM lycopene
- 100 μM H89
- 100 μM H89 + 1 μM lycopene
- 10 μM GF109203X
- 10 μM GF109203X + 1 μM lycopene
- 3 μM Go6976
- 3 μM Go6976 + 1 μM lycopene

1 mM 4-AP

Statistical significance:
- *** indicates p < 0.001
- # indicates p < 0.05

Sample sizes: (5), (6)