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Molecular Basis of Vitamin E Action

TOCOTRIENOL POTENTLY INHIBITS GLUTAMATE-INDUCED pp60$^{c\text{-}Src}$ KINASE ACTIVATION AND DEATH OF HT4 NEURONAL CELLS*

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HT4 hippocampal neuronal cells were studied to compare the efficacy of tocopherols and tocotrienol to protect against glutamate-induced death. Tocotrienols were more effective than α-tocopherol in preventing glutamate-induced death. Uptake of tocotrienols from the culture medium was more efficient compared with that of α-tocopherol. Vitamin E molecules have potent antioxidant properties. Results show that at low concentrations, tocotrienols may have protected cells by an antioxidant-independent mechanism. Examination of signal transduction pathways revealed that protein tyrosine phosphorylation processes played a central role in the execution of death. Activation of pp60$^{c\text{-}Src}$ kinase and phosphorylation of ERK were observed in response to glutamate treatment. Nanomolar amounts of α-tocotrienol, but not α-tocopherol, blocked glutamate-induced death by suppressing glutamate-induced early activation of c-Src kinase. Overexpression of kinase-active c-Src sensitized cells to glutamate-induced death. Tocotrienol treatment prevented death of Src-overexpressing cells treated with glutamate. α-Tocotrienol did not influence activity of recombinant c-Src kinase suggesting that its mechanism of action may include regulation of SH domains. This study provides first evidence describing the molecular basis of tocotrienol action. At a concentration 4–10-fold lower than levels detected in plasma of supplemented humans, tocotrienol regulated unique signal transduction processes that were not sensitive to comparable concentrations of tocopherol.

Vitamin E is a generic term for tocopherols and tocotrienols that qualitatively exhibit the biological activity of α-tocopherol (1). Compared with tocopherols, tocotrienols have been poorly studied (2, 3). Tocotrienols are minor plant constituents especially abundant in palm oil, cereal grains, and rice bran that can provide a significant source of vitamin E activity. Tocotrienols differ from tocopherols by possessing a farnesyl (isoprenoid) rather than a saturated phytyl side chain. Dietary tocotrienols become incorporated into circulating human lipoproteins where they react with peroxyl radicals as efficiently as the corresponding tocopherol isomers (4, 5). Consistently, tocotrienol supplementation has been reported to influence beneficially the course of carotid atherosclerosis in humans (6). Following supplementation to humans, the level of α-tocotrienol in the plasma has been estimated to be 0.98 ± 0.8 μM (7). A possible neuroprotective property of tocotrienols was indicated in a study testing the efficacy of the tocotrienol-rich fraction from palm oil to protect against oxidative damage of rat brain mitochondria. The tocotrienol-rich fraction from palm oil was significantly more effective than α-tocopherol in protecting the brain against damage caused by exposure to ascorbate-Fe^{2+}, the free radical initiator azobis(2-amidopropane)dihydrochloride, or photosensitization. (8). At concentrations 25–50 μM, α-tocopherol is known to regulate signal transduction pathways by mechanisms that are independent of its antioxidant properties. α-Tocopherol, but not β-tocopherol having comparable antioxidant properties, inhibited inducible protein kinase C activity in smooth muscle cells (9, 10). The signal transduction regulatory properties of tocotrienols, however, are yet unknown.

ROS[1] represent a major contributor to brain damage in disorders such as epilepsy (11, 12), head trauma (13), and ischemia-reperfusion (14–16). Oxidative damage is also implicated in neurodegenerative diseases such as Huntington's (17), Alzheimer's (18), and Parkinson's. In the pathogenesis of these diseases, oxidative damage may accumulate over a period of years leading to massive neuronal loss. Glutamate toxicity is a major contributor to pathological cell death within the nervous system and appears to be mediated by ROS (19). There are two forms of glutamate toxicity as follows: receptor-initiated excitotoxicity (20) and non-receptor-mediated glutamate-induced toxicity (21). One model used to study oxidative stress-related neuronal death is to inhibit cystine uptake by exposing cells to high levels of glutamate (22). High glutamate levels block cystine uptake via the amino acid transporter Xc^- and impairs reduced glutathione (GSH) cell homeostasis. The induction of oxidative stress by glutamate in this model has been demonstrated to be a primary cytotoxic mechanism in C6 glial cells (23, 24), PC-12 neuronal cells (25, 26), immature cortical neurons cells (22), and oligodendroglia cells (27). Recently, the mitochondrial electron transport chain has been shown to be a source of ROS production during glutamate-induced apoptosis in HT22 neuronal cells, a sub-clone of HT4 cells used in the current study (21). At micromolar concentrations, antioxidants such as α-tocopherol, probucol, and α-lipoic acid have been shown to protect these cells against glutamate-induced cytotoxicity (22–24, 28, 29).

In the current study, rat hippocampal neuronal HT4 cells (30) were exposed to elevated levels of extracellular glutamate,

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[1] The abbreviations used are: ROS, reactive oxygen species; DCF, dichlorofluorescein; DCFH-DA, dichlorodihydrofluorescein diacetate; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; AAPH, 2,2′-azobis[2-amidinopropane]hydrochloride; ERK, extracellular signal-regulated kinase.

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and the ability of tocotrienols and tocopherol to protect the neuronal cells was examined. This study presents first evidence showing that at amounts 4–10-fold lower than levels of tocotrienol detected in plasma of human supplemented with the vitamin E molecule (7), α-tocotrienol has potent signal transduction regulatory properties that account for its neuroprotective function.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: L-glutamic acid monosodium salt (Sigma); DCFH-DA (Molecular Probes, Eugene, OR); 2,2′-azobis[2-amidinopropane]hydrochloride (AAPH; Polysciences Inc., PA); racemic (*d, l*) mixture of tocopherol, tocotrienols, and related esters (BASF Bioresearch, Germany). For cell culture, Dulbecco's modified Eagle's medium (Life Technologies, Inc.), fetal calf serum, and penicillin and streptomycin (University of California, San Francisco) were used, and culture dishes 100 × 15 mm (Becton Dickinson) were used.

Cell Culture—Mouse hippocampal HT4 cells, kindly provided by D. E. Koshland, Jr., University of California, Berkeley, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Confluent cells were trypsinized and subcultivated in culture dishes at a concentration of 3×10^4 cells/ml. The cells were cultured at standard conditions described above. Following 24 h of seeding, the culture medium was replaced with fresh medium supplemented with serum and antibiotic. The cells were then exposed to 10 mM L-glutamate as described previously (24, 31). No change in medium pH was observed in response to addition of glutamate. Vitamin E molecules are lipid-soluble antioxidants. The lipid peroxidation initiating peroxyl radical generator AAPH (10 mM, 24 h under standard cell culture conditions) was therefore used to expose the cells to authentic oxidative stress (32).

Vitamin E Treatment—Stock solutions (10^3 × working concentration) of tocopherols and tocotrienols were prepared in ethanol. Respective controls were treated with an equal volume (0.1%, v/v) of ethanol. The antioxidants were added to the culture dishes either 5 min before glutamate challenge or after the glutamate treatment as indicated in the respective figure legends

Determination of Cell Viability—Cell membrane integrity was detected by flow cytometry (EPICS Elite or XL, Coulter, Miami, FL) as a measure of cell viabity. For this assay the nonpermeant DNA intercalating dye propidium iodide (Molecular Probes, Eugene, OR) that is generally excluded by viable cells (33) was used. A 15-milliwatt-powered argon ion laser was used for excitation at 488 nm, and emission signal was collected at 575 nm as described earlier (34). Propidium iodide negative cells were interpreted as viable cells.

Determination of Intracellular Peroxides—Intracellular peroxides were detected using DCFH-DA as described previously (35). Following treatment with or without antioxidants and glutamate, cells were washed three times with PBS. Cells were detached from monolayer using trypsin and centrifuged (600 × *g*, 5 min). The cells were again washed with PBS and centrifuged, following that they were resuspended in PBS and incubated with DCFH-DA (25 μM) for 30 min at 37 °C. Cells were then excited with a 488 nm UV line argon ion laser in a flow cytometer (XL, Coulter, FL), and the DCF emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Determination of Intracellular Ca^{2+}—Intracellular free Ca^{2+} levels were measured using cell-permeant calcium Green-1, acetomethoxyl ester (Molecular Probes, Eugene, OR). Cells were loaded with calcium Green-1 and then were excited at 488 nm using a argon ion laser, and emission was recorded at 530 nm using a flow cytometer.

Immunoblot Analyses—For phospho-p44/42 mitogen-activated protein kinase (ERK1 and -2) immunoblots, cytosolic extract of cells treated or not treated (control) with 10 mM L-glutamate in the presence of 0.2 mM Na_3VO_4 was separated on a 10% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose, and probed with phospho-p44/42 (Thr-202/Tyr-204) E10 monoclonal antibody (New England Biolabs, Beverly, MA). This was followed by probing with appropriate horseradish peroxidase-coupled secondary antibodies. Bound antibody was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Src Overexpression—Following 18 h of seeding, HT4 cells were transfected with eukaryotic expression vector containing mouse Src (activated or kinase-dead) cDNA under the control of a cytomegalovirus promoter (Upstate Biotechnology, Inc., Lake Placid, NY). The kinase activating mutation (*src*Y529F) is a substitution of phenylalanine for tyrosine at position 529. The kinase-inactivating mutation (*src*K297R) is a substitution of arginine for lysine at position 297 (36, 37). SuperFect transfection reagent (Qiagen, Valencia, CA) was used to carry out the transfection process that lasted for 3 h. After 3 h, the transfection reagent was removed, and regular cell culture medium was added to the cells. The cells were maintained in regular culture condition for 24 h to allow for protein expression. At this point, the cells were harvested and seeded for treatment with tocotrienol and/or glutamate. After 5 h of seeding, culture medium was changed, and cells were treated as described in the legend to Fig. 4.

Determination of Protein Phosphotyrosine Profile—By using standard Western blot techniques we were not able to get a high quality resolution of bands. Instead, we labeled cellular proteins with ^{35}S, then tyrosine-phosphorylated proteins were immunoprecipitated, and autoradiography was performed as described below. Following 6 h of seeding, cells were labeled with L-[^{35}S]methionine (60 μCi/ml; NEN Life Science Products) for 12 h under standard culture conditions. To inhibit protein tyrosine phosphatase activity, cells were treated with 0.25 mM sodium orthovanadate (Sigma) for 15 min. After this, cells were either treated or not with 250 nM α-tocotrienol followed by glutamate for 1 h under standard culture conditions (as indicated in figure legends). Cells were washed with ice-cold phosphate-buffered saline, pH 7.4. Cells were lysed with 1 ml of lysis buffer (phosphate-buffered saline 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 0.1% v/v sodium dodecyl sulfate, 0.25 mM sodium orthovanadate (Na_3VO_4), 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml pepstatin). Tyrosine-phosphorylated proteins were immunoprecipitated (4 °C, overnight) from the lysate using monoclonal protein phosphotyrosine antibody (PY99; Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-agarose. The immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel electrophoresis, and the protein tyrosine phosphorylation profile was detected by autoradiography.

Determination of Src Kinase Activity—Cells were harvested and lysed in 1 ml of a lysis buffer (20 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% Nonidet P-40). The cell lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C. Aliquots of the supernatants containing 750 μg of protein were incubated for 3 h at 4 °C with 2 μg of protein A-agarose-conjugated anti-Src family kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were washed twice with the lysis buffer and twice with reaction buffer (40 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, 0.1 mM sodium orthovanadate, 1 mM NaF and 2 mM β-glycerophosphate). Kinase reactions were carried out in 30 μl of the reaction buffer containing 4 μg of acid-denatured enolase (Roche Molecular Biochemicals), 10 μM ATP, and 10 μCi of [γ-^{32}P]ATP (NEN Life Science Products) at 22 °C for 10 min. The reactions were stopped by adding 10 μl of 4× Laemmli sample buffer. The boiled samples were separated by 10% SDS-polyacrylamide gel electrophoresis, and the radioactivity incorporated into enolase was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The effect of tocotrienols on purified Src kinase activity was performed essentially as described above, except that in the kinase reaction buffer the immunoprecipitate was replaced by 500 ng (10^6 units/mg) of purified recombinant-active Src kinase (Upstate Biotechnology Inc., Lake Placid, NY).

High Performance Liquid Chromatography (HPLC)-Electrochemical Detection—To determine the amounts and uptake of tocopherols and tocotrienols in cells, HT4 cells were treated with tocopherol or tocotrienol for the duration indicated in respective figure legends. After the appropriate treatment time, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline. Washed cells were trypsinized and collected in microcentrifuge tubes and centrifuged at 3000 × *g* for 10 min. To the pellet 0.925 ml of phosphate-buffered saline containing 1 mM Na_2EDTA, 0.025 ml of butylated hydroxytoluene (10 mg/ml), and 0.5 ml of 0.1 M SDS was added. The mixture was vigorously vortexed for 15 min at 4 °C; 2 ml of ethanol was added, and the mixture was vortexed for another 2 min. Then, 2 ml of hexane was added to the mixture, and it was vortexed for another 3 min at 4 °C. The resulting mixture was extracted as described previously (38). An appropriate aliquot of the hexane extract was used for HPLC analysis. The electrochemical detector was operated with a 0.5-V potential with full recorder scale at 50 nA for quantitation of α-tocopherol and α- and γ-tocotrienols (38). Authentic compounds were used to generate standard curves.

Glutathione measurements were performed using an HPLC system coupled with a electrochemical coulometric detector (ESA, Chelmsford, MA). A C-18 column (150 × 4.6 mm, 5-μm pore size; Alltech, Deerfield,

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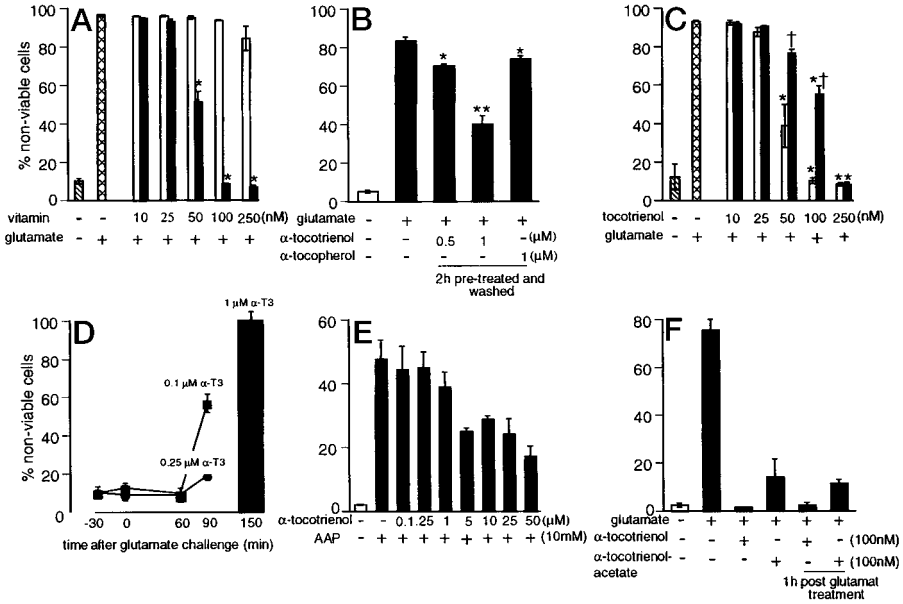

FIG. 1. **Protection against loss of cell viability by vitamin E.** *A*, where indicated, cells were treated with α-tocopherol (*open bar*) or α-tocotrienol (*solid bar*) for 5 min before glutamate challenge. *, $p < 0.001$ when compared with vitamin E non-treated and glutamate-treated group. *B*, cells were pretreated with either α-tocotrienol or α-tocopherol for 2 h following which cells were washed twice with PBS and treated with fresh medium containing 10 mM glutamate. **, $p < 0.001$; *, $p < 0.05$ when compared with vitamin E non-treated and glutamate-treated group. *C*, protection of cells against glutamate-induced death by α-tocotrienol (*open bar*) or γ-tocotrienol (*solid bar*). *, $p < 0.001$ when compared with tocotrienol non-treated and glutamate-treated group. †, $p < 0.001$ when compared with α-tocotrienol-treated group. *D*, effect of α-tocotrienol treatment at various time intervals after glutamate challenge. Cells were treated with glutamate for 12 h. At low concentrations (100 nM), tocotrienol regulated an early event in the death pathway. At higher concentrations (1 μM) tocotrienol protected even when added 2.5 h after glutamate treatment. *E*, at low concentrations (100 or 250 nM) the antioxidant property of α-tocotreinol, as determined by its ability to protect cells from AAPH-generated peroxyl-radical induced loss of cell viability, was not evident. Higher concentrations (5–50 μM) of α-tocotrienol were necessary to protect the cells. AAPH treatment was for 24 h under cell culture conditions. *F*, α-tocotrienol was more effective than α-tocotrienol acetate in protecting HT4 neuronal cells against glutamate-induced death. Cells were treated with α-tocotrienol or α-tocotrienol acetate either 5 min before glutamate treatment or 1 h after glutamate challenge as indicated.

IL) was used for glutathione separation. Glutathione levels were expressed as nanomoles/mg protein. Protein was determined using the Pierce BCA protein assay kit.

Statistics—Data are reported as mean ± S.D. of at least three experiments. Comparison among multiple groups were made by analysis of variance. $p < 0.05$ was considered statistically significant.

RESULTS

Cell Viability—Treatment of HT4 cells with elevated levels of extracellular glutamate resulted in >95% loss of cell viability within a duration of 12 h. On a concentration basis, α-tocotrienol was more effective than α-tocopherol in protecting HT4 cells against glutamate-induced cytotoxicity. A dose-dependent study of α-tocotrienol and α-tocopherol showed that at a concentration of 50 nM α-tocotrienol, but not α-tocopherol, partially protected the cells against glutamate-induced death. At 250 nM α-tocotrienol, but not α-tocopherol, provided complete protection against loss of cell viability (Fig. 1*A*). In experiments where cells treated with these forms of vitamin E, washed, and then exposed to glutamate, it was observed that pretreatment with 1 μM tocotrienol, but not tocopherol, provided significant protection (Fig. 1*B*). Comparison of the two analogues of tocotrienol, α- and γ-, showed that α-tocotrienol was more effective than γ-tocotrienol in protecting against glutamate-induced cell death (Fig. 1*C*).

To resolve temporally the site of tocotrienol action in the glutamate-induced death pathway, cells were treated with α-tocotrienol at various time points after glutamate treatment. Almost complete protection of cells was observed even when 100 nM α-tocotrienol was added at 60 min, but not 90 min, after glutamate treatment (Fig. 1*D*). At higher concentrations, *e.g.* 250 nM, however, over 80% of cells maintained viability even when α-tocotrienol treatment was added 90 min after glutamate treatment. Complete protection against loss of viability was observed when cells were treated with an excess of 5 μM even after 6 h of glutamate treatment (not shown). Micromolar amounts of α-tocotrienol was necessary to protect cells against AAPH-generated peroxyl radical-induced loss of viability. Such an antioxidant property of α-tocotrienol was not evident when cells were pretreated with 100–250 nmol of α-tocotrienol (Fig. 1*E*). Comparison of the cytoprotective efficacy of the free form of α-tocotrienol with the corresponding ester (tocotrienol acetate) showed that the free form was marginally, but significantly, more potent (Fig. 1*F*). Previously, it has been shown that vitamin E has potent iron chelation properties (39). Therefore, we sought to examine whether the protective effect of α-tocotrienol against glutamate-induced loss of viability was mediated by the metal chelation property of vitamin E. Glutamate-induced death of HT4 cells was partially inhibited by the iron chelator deferoxamine mesylate. The iron chelator regulated a late event in the death pathway because significant protection was achieved even when cells were treated with deferoxamine mesylate 4 h after glutamate treatment (Fig. 2).

Intracellular Responses—Glutamate-induced death of HT4 cells was preceded by depletion of intracellular GSH levels (Fig. 3*A*) and elevation of intracellular peroxide levels (Fig. 3*B*). Studies on the kinetics of these responses showed that GSH depletion precedes elevation of DCF fluorescence. DCF fluorescence was slightly higher after 4 h of glutamate treatment and peaked at 6 h. Although α-tocotrienol treatment did not spare glutamate-induced depletion of intracellular GSH, it completely prevented the accumulation of intracellular peroxides as measured by DCF fluorescence (Fig. 3, *A* and *B*). The accumulation of intracellular peroxides was completely prevented even if tocotrienol was treated 5 h after glutamate treatment (Fig. 3*B*). Following exposure to elevated levels of extracellular glutamate, intracellular levels of free calcium ($[Ca^{2+}]_i$) in-

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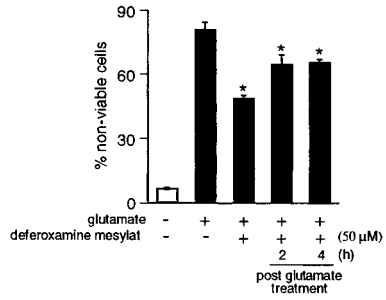

FIG. 2. **Deferoxamine mesylate partially protects HT4 cells against glutamate-induced death.** Cells were treated with the iron chelator deferoxamine mesylate either 5 min before or after glutamate treatment as indicated in the figure. *, $p < 0.05$ when compared with desferoxamine mesylate non-treated and glutamate-treated group.

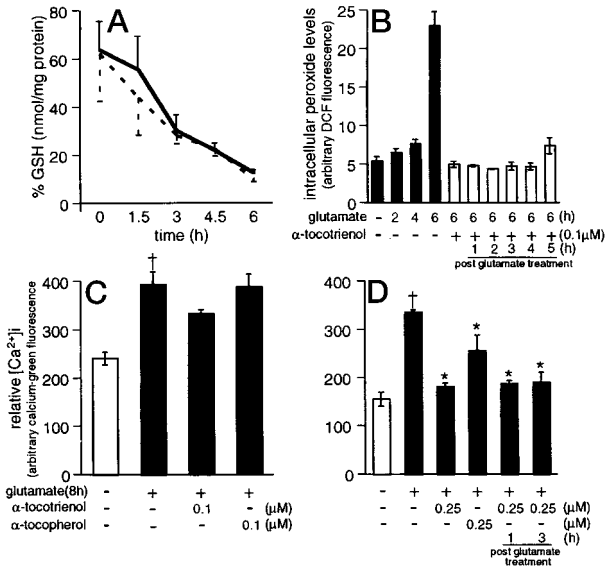

FIG. 3. **Glutamate-induced cellular oxidant and Ca^{2+} responses.** *A*, intracellular GSH levels in response to glutamate treatment. *Solid line*, control cells; *hatched line*, cell treated with 250 nM α-tocotrienol 5 min before glutamate challenge. *B*, intracellular peroxide levels. Treatment of cells with glutamate increased intracellular peroxide level as a function of time (*solid bars*). *C*, low concentration of α-tocotrienol, but not α-tocopherol, significantly attenuated glutamate-induced elevation of $[Ca^{2+}]_i$. *D*, at 250 nM, α-tocotrienol prevented glutamate-induced elevation of $[Ca^{2+}]_i$ even when treated to cells 3 h after glutamate challenge. †, $p < 0.05$ when compared with glutamate non-treated group. *, $p < 0.05$ when compared with vitamin E non-treated and glutamate-treated group.

creased (Fig. 3*C*). Increases in $[Ca^{2+}]_i$ peaked following 8 h of exposure to glutamate (not shown). Pretreatment of cells with 100 nM α-tocotrienol significantly diminished glutamate-induced elevation of $[Ca^{2+}]_i$. At this concentration, however, α-tocopherol did not show any effect (Fig. 3*C*). At 250 nM, α-tocotrienol completely prevented glutamate-induced elevation of $[Ca^{2+}]_i$. At this concentration, the efficacy of α-tocotrienol to prevent glutamate-induced perturbation of $[Ca^{2+}]_i$ homeostasis was better as compared with that of α-tocopherol (Fig. 3*D*).

Vitamin E Uptake—The availability of exogenous α-tocotrienol and α-tocopherol in HT4 cells was compared. Treatment of cells with various concentrations (100–1000 nM) of α-tocotrienol resulted in time-dependent elevation of cellular α-tocotrienol content (Fig. 4, *A–C*). Treatment of cells with 1000 nM α-tocopherol, however, did not elevate α-tocopherol level in the cells (Fig. 4*D*). Comparison of the uptake of the free and esterified forms of α-tocotrienol showed that cells more efficiently took up the free form (Fig. 4, *E* and *F*). This was clearly evident in cells treated with 250 nM of tocotrienol or tocotrienol acetate (Fig. 4*F*).

Protein Phosphorylation—Detection of the protein tyrosine phosphorylation profile in cellular extracts provided direct evidence confirming that glutamate treatment induces protein tyrosine phosphorylation only of a few proteins and that this phosphorylation process is inhibited in α-tocotrienol-treated cells (Fig. 5*A*). Examination of specific tyrosine-phosphorylated proteins revealed that glutamate induced activation of ERK1 and ERK2. This response was rapid and was sustained for 2 h as shown in Fig. 5*B*. Pretreatment of cells with 250 nM of α-tocotrienol completely abrogated glutamate-induced phosphorylation of ERK1 and ERK2 (Fig. 5*C*). Inhibition of $pp60^{c\text{-}Src}$ protein-tyrosine kinase activity by herbimycin or geldanamycin completely protected the cells against glutamate-induced loss of viability (Fig. 6, *A* and *B*). Treatment of cells with another inhibitor of protein-tyrosine kinase activity, lavendustin A, that is not specific for $pp60^{c\text{-}Src}$ did not protect against glutamate-induced cell death (Fig. 6*C*). To examine whether glutamate-induced activation of ERKs represents a key event in the execution of cell death, the effect of a specific inhibitor of the activation of mitogen-activated protein kinase kinase (PD98059) was tested. Unlike the effects of herbimycin or geldanamycin, PD98059 did not protect against glutamate-induced cell death (Fig. 6*D*). Although the $pp60^{c\text{-}Src}$ kinase inhibitor geldanamycin completely prevented glutamate-induced death of HT4 cells, this inhibitor did not influence glutamate-induced accumulation of intracellular oxidants (Fig. 6*E*). These results further support the contention that in this experimental system oxidants are not solely responsible for the execution of death.

To confirm the possible involvement of $pp60^{c\text{-}Src}$ kinase activity in glutamate-induced cell death, cells were transfected to overexpress either catalytically active or inactive forms of the protein (Fig. 7*A*). Overexpression of kinase-active $pp60^{c\text{-}Src}$ kinase sensitized the cells to glutamate-induced death. α-Tocotrienol treatment completely protected against glutamate-induced loss of cell viability even in $pp60^{c\text{-}Src}$-overexpressed cells (Fig. 7*B*). To obtain specific knowledge of the activity of $pp60^{c\text{-}Src}$ kinase in the cells, the protein was immunoprecipitated from cellular extracts, and an *in vitro* assay was performed. Glutamate treatment enhanced $pp60^{c\text{-}Src}$ kinase activity in HT4 cells, and this enhanced $pp60^{c\text{-}Src}$ kinase activity was completely inhibited by α-tocotrienol, but not α-tocopherol (not shown), treatment (Fig. 7*C*). α-Tocotrienol, however, did not influence the activity the recombinant active $pp60^{c\text{-}Src}$ kinase (Fig. 7*D*).

DISCUSSION

Previously we have observed in C6 glial cells as well as in HT4 cells that glutamate-induced death may be prevented by antioxidant treatment (24, 31). Compared with α-tocopherol, α-tocotrienol is more uniformly distributed in the membrane bilayer, more efficiently recycled from its corresponding chromanoxyl radical form, and more strongly disorders membrane lipid allowing for a better interaction of chromanols with lipid radicals (5). Because of these advantages, α-tocotrienol has better antioxidant activity than α-tocopherol (5, 8, 41). Although it is tempting to assume that the increased antioxidant activity of α-tocotrienol is responsible for its enhanced cytoprotective effect, results from experiments where cells were treated with 100 nM tocotrienol at various time points after glutamate challenge do not support the contention.

The ability of 100 nM α-tocotrienol to protect against glutamate-induced loss of cell viability was retained only if the cells were treated up to 1 h after glutamate challenge. However, at high concentrations α-tocotrienol could completely protect the cells even when treated 6 h after glutamate addition. These

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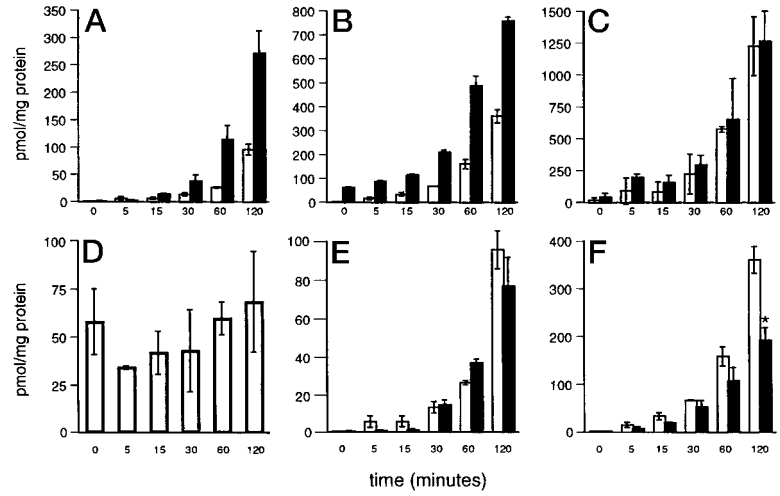

FIG. 4. **Uptake of α-, γ-tocotrienols and α-tocopherol by HT4 cells (*A–D*).** *A*, 100 nM tocotrienol; *B*, 250 nM tocotrienol; *C*, 1000 nM tocotrienol; and *D*, 1000 nM α-tocopherol. *Open bars* represent uptake of the α-form, and *solid bars* represent uptake of γ-tocotrienol. *E*, 100 nM tocotrienol (*open bar*) or corresponding acetate (*solid bar*); *F*, 250 nM tocotrienol or corresponding acetate. *, $p < 0.01$ when compared with α-tocotrienol-treated group. Exposure time in minutes is indicated along the *abscissa.*

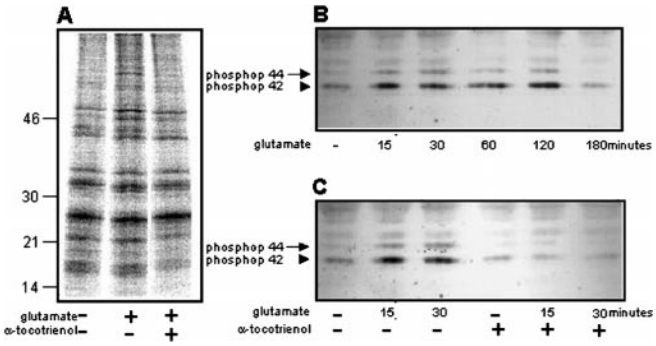

FIG. 5. *A*, protein tyrosine phosphorylation profile. α-Tocotrienol, 250 nM, 1 h; glutamate, 10 mM, 1 h. *B*, kinetics of activation of p44/42 (ERK1 and -2) mitogen-activated protein kinase in response to glutamate treatment. *C*, 250 nM α-tocotrienol added prior to glutamate treatment.

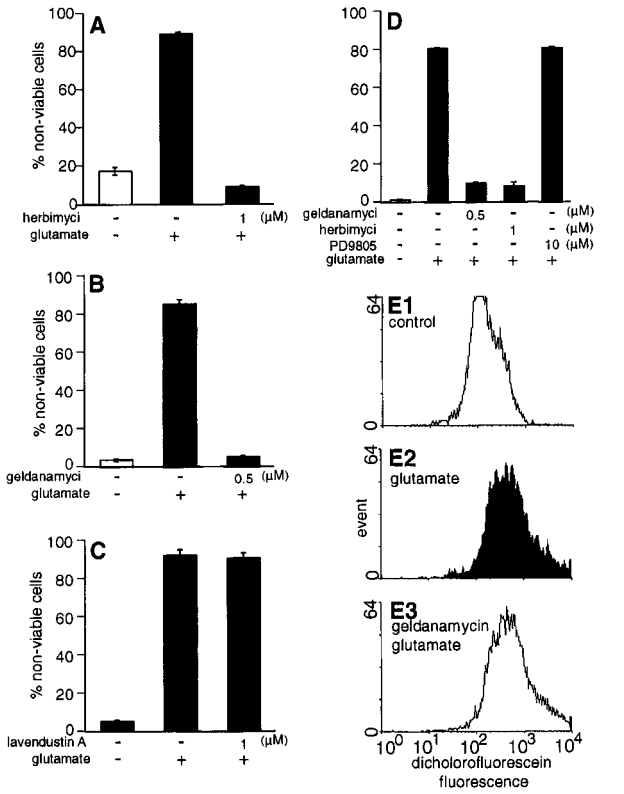

FIG. 6. **Effect of protein-tyrosine kinase inhibitors on glutamate-induced cell death (*A–D*).** *E1–E3*, histograms showing that geldanamycin-dependent protection of cells against glutamate-induced death (*B*) is not associated with lowered intracellular peroxide levels as measured by DCFH-DA after 6 h of glutamate treatment.

results indicate that the mechanism of α-tocotrienol action is dependent on the concentration of tocotrienol used. In the cascade of events leading to cell death, low concentrations of tocotrienol influenced an early event, whereas at higher concentrations tocotrienol protected cells apparently by regulating a late event. Because 1 h of glutamate treatment did not cause elevation of intracellular ROS, it seems unlikely that 100 nM of α-tocotrienol protected cells via an antioxidant mechanism. A compelling line of evidence supporting this contention is that although α-tocotrienol completely prevented intracellular peroxide accumulation even when treated several hours after glutamate exposure, it did not completely protect cell viability when added 90 min after glutamate treatment. At nanomolar concentrations α-tocotrienol does not have potent antioxidant property. Micromolar amounts of this compound was necessary to protect cells against peroxyl radical-induced loss of viability. Furthermore, trolox (the water-soluble analog of tocopherol) as well as geldanamycin completely prevented glutamate-induced cell death without decreasing glutamate-induced accumulation of intracellular peroxides (not shown). Taken together, this evidence indicates that intracellular oxidants may not play a key role in the death pathway.

Previously it has been suggested that compared with α-tocotrienol, γ-tocotrienol has more potent antioxidant properties (8). Although the differences were marginal, γ-tocotrienol tended to be less effective than α-tocotrienol in protecting the cells. These results lend further support to the hypothesis that at low concentrations the protective effect of α-tocotrienol against glutamate-induced cytotoxicity may not be related to antioxidant activity. The uptake of both α- and γ-tocotrienols by HT4 cells was clearly much better than that of α-tocopherol. It is generally believed that the chromanol nucleus of α-tocopherol is localized at the polar-hydrocarbon membrane interface whereas its phytyl chain interacts with the acyl chains of membrane phospholipids. Compared with α-tocopherol, α-tocotrienol is significantly less associated in clusters and is more uniformly distributed in the bilayer of dimyristoyl-phosaphatidylcholine liposomes (5). It is unlikely that the difference between the ability of tocotrienol and tocopherol to protect cells against glutamate challenge may be explained by differences in the uptake of these two forms of vitamin E by the cell. Although treatment of cells with 100 nM of α-tocotrienol for 5 min resulted in negligible loading of cells, these cells were completely resistant to glutamate-induced death, whereas cells loaded with the vitamin E molecule (1 μM, 2 h) were not. Furthermore,

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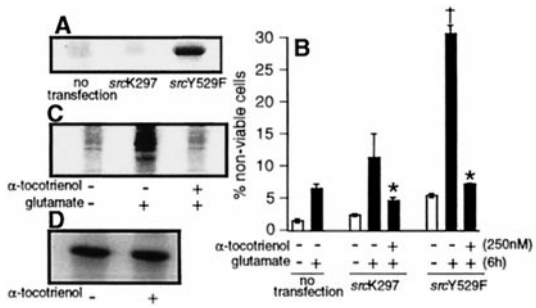

FIG. 7. *A*, Src kinase activity in cells either not transfected or transfected with either *src*K297R (encoding kinase-dead Src) or *src*Y529F (encoding kinase-active Src) plasmids. Kinase-dead refers to catalytically inactive Src kinase; kinase-active refers to constitutively active Src kinase; *B*, involvement of c-Src kinase activity in the glutamate-induced death pathway and the effect of α-tocotrienol. †, $p < 0.001$ when compared with kinase-dead c-Src group; *, $p < 0.001$ when compared with α-tocotrienol non-treated and glutamate-treated group. *C*, activity of Src-kinase immunoprecipitated from HT4 cells. α-Tocotrienol, 250 nM, 30 min; glutamate, 10 mM, 30 min. *D*, activity of recombinant active c-Src kinase. α-Tocotrienol, 250 nM.

although γ-tocotrienol was more efficiently taken up by cells than α-tocotrienol, it was less efficient than the latter in protecting the cells against glutamate-induced death. Treatment of cells with the free form of α-tocotrienol resulted in a higher concentration of free tocotrienol in the cell compared with cells treated with esterified α-tocotrienol. It is possible that such differences are because of limited esterase activity available in the membrane environment where tocotrienol acetate is likely to partition. At low concentration, the observed neuroprotective property of tocotrienol is unlikely to be mediated via iron chelation because deferoxamine mesylate appeared to protect by influencing a late event in the death pathway.

Oxidant challenge has been shown to be associated with increased $[Ca^{2+}]_i$ (44, 45) resulting from mobilization of the Ca^{2+} pool of sarcoendoplasmic reticulum (46). In glutamate-treated HT4 cells, accumulation of intracellular ROS was followed by elevated levels of $[Ca^{2+}]_i$. Such an oxidant-induced increase in $[Ca^{2+}]_i$ has been shown to contribute to cell death (47, 48). Decreased intracellular GSH resulted in calcium-mediated cell death in PC12 neuronal cells (49). Tocotrienol treatment prevented elevation of $[Ca^{2+}]_i$ despite marked depletion of intracellular GSH.

The involvement of signal transduction pathways in glutamate-induced cell death was evident. Inhibitors of the protein-tyrosine kinase activity completely prevented glutamate-induced cell death. Herbimycin and geldanamycin potently inhibited $pp60^{c\text{-}Src}$ tyrosine kinase activity (50, 51), whereas lavendustin A is an inhibitor of extracellular growth factor receptor protein-tyrosine kinase activity (52). The observation that herbimycin and geldanamycin, but not lavendustin A, prevent glutamate-induced death of HT4 neuronal cells suggested the involvement of c-Src kinase activity in the death pathway. Immunoprecipitation of tyrosine-phosphorylated protein from cellular extracts confirmed that protein tyrosine phosphorylation reactions were indeed triggered by exposure of cells to elevated levels of glutamate and that such reactions were inhibited by nanomolar concentrations of α-tocotrienol.

The involvement of $pp60^{c\text{-}Src}$ kinase activity in the death pathway was verified by experiments involving the overexpression of catalytically active or inactive Src kinase. Tocotrienol treatment completely prevented glutamate-induced death even in active c-Src kinase overexpressing cells indicating that it either inhibited c-Src kinase activity or regulated one or more events upstream of c-Src kinase activation. Further evidence supporting this contention was provided by results obtained from the determination of c-Src kinase activity in HT4 cells. SH2 and SH3 domains are known to play a central role in regulating the catalytic activity of Src protein-tyrosine kinase. High resolution crystal structures of human SRC, in their repressed state, have provided a structural explanation for how intramolecular interactions of the SH3 and SH2 domains stabilize the inactive conformation of Src (53). The observation that α-tocotrienol inhibited glutamate-induced Src activation in HT4 cells but did not influence the catalytic activity of recombinant Src suggests that α-tocotrienol inhibited events leading to glutamate-induced reorganization of the SH domains and activation of Src kinase. Many intracellular pathways can be stimulated upon Src activation, and a variety of cellular consequences can result, including morphological changes and cell proliferation. For example, the activity of c-Src kinase is implicated in the progression of breast cancer (54, 55). Mammary tumors expressing the *neu* proto-oncogene possess elevated c-Src tyrosine kinase activity (56). Markedly elevated levels of c-Src kinase activity have been detected in human skin tumors (57). Because of the key involvement of Src kinases in driving receptor-mediated oncogenesis (58), inhibitors of these kinases are being studied as candidates for anticancer drugs (59).

Further evidence suggesting that signal transduction processes related to the cell death pathway are involved in glutamate-induced cytotoxicity was obtained from the study of ERK1 and ERK2 activation. When activated, p44 and p42 mitogen-activated protein kinases (ERK1 and ERK2) are phosphorylated at specific threonine and tyrosine residues. ERK has been implicated in mediating the signaling events that precede apoptosis. ERK2 plays an active role in mediating anti-IgM-induced apoptosis of WEHI 231 B cells (61). H_2O_2 induces the activation of multiple mitogen-activated protein kinases in oligodendrocyte progenitors, and the activation of ERK is associated with oxidant-mediated cytotoxicity (62). Rapid activation of ERK, particularly ERK2, in response to glutamate treatment was completely inhibited in cells treated with α-tocotrienol suggesting that α-tocotrienol influences an early event in the glutamate-induced death pathway. In some cases Src kinase activity is required for the activation of ERK (63). Thus, it is likely tocotrienol treatment may have inhibited inducible ERK activation by down-regulating Src kinase activity.

At 25–50 μM, α-tocopherol is known to regulate signal transduction pathways by mechanisms that are independent of its antioxidant properties (9, 10). This study provides the first evidence describing a signal transduction regulatory role of tocotrienol. Protein tyrosine phosphorylation-related signal transduction pathways were observed to be involved in mediating glutamate-induced cytotoxicity. At amounts 4–10-fold lower than levels of tocotrienol detected in plasma of human supplemented with the vitamin E molecule (7), tocotrienol inhibited glutamate-induced Src kinase activation, an early event in the pathway to death. At nanomolar levels tocotrienol regulated unique signal transduction processes that were not sensitive to comparable concentrations of tocopherol.

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Molecular Basis of Vitamin E Action: TOCOTRIENOL POTENTLY INHIBITS GLUTAMATE-INDUCED pp60c-Src KINASE ACTIVATION AND DEATH OF HT4 NEURONAL CELLS

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