Neuroprotective Properties of the Natural Vitamin E α-Tocotrienol

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Background and Purpose—The current work is based on our previous finding that in neuronal cells, nmol/L concentrations of α-tocotrienol (TCT), but not α-tocopherol (TCP), blocked glutamate-induced death by suppressing early activation of c-Src kinase and 12-lipoxygenase.

Methods—The single neuron microinjection technique was used to compare the neuroprotective effects of TCT with that of the more widely known TCP. Stroke-dependent brain tissue damage was studied in 12-Lox-deficient mice and spontaneously hypertensive rats orally supplemented with TCT.

Results—Subattomole quantity of TCT, but not TCP, protected neurons from glutamate challenge. Pharmacological as well as genetic approaches revealed that 12-Lox is rapidly tyrosine phosphorylated in the glutamate-challenged neuron and that this phosphorylation is catalyzed by c-Src. 12-Lox-deficient mice were more resistant to stroke-induced brain injury than their wild-type controls. Oral supplementation of TCT to spontaneously hypertensive rats led to increased TCT levels in the brain. TCT-supplemented rats showed more protection against stroke-induced injury compared with matched controls. Such protection was associated with lower c-Src activation and 12-Lox phosphorylation at the stroke site.

Conclusion—The natural vitamin E, TCT, acts on key molecular checkpoints to protect against glutamate- and stroke-induced neurodegeneration. (Stroke. 2005;36:e144-e152.)

Key Words: nutrition ■ pathophysiology ■ vitamin

Vitamin E is a generic term for tocophersols and tocotrienols.1 Compared with tocophersols, tocotrienols have been poorly studied.2–4 The current work is based on our striking evidence that in neuronal cells, nmol/L concentrations of α-tocotrienol (TCT), but not α-tocopherol (TCP), blocked glutamate-induced death.5–7 These studies from our laboratory presented first evidence showing that at amounts 4- to 10-fold lower than the levels of TCT detected in plasma of human supplemented with the vitamin E molecule,8 TCT has potent signal transduction regulatory properties that account for its neuroprotective function. Of importance, this striking property was exhibited by a nutrient known to be safe for human consumption. We have reported that glutamate-induced c-Src activation is a key executioner of glutamate-induced neuronal death and that such activation is sensitive to nanomolar concentrations of TCT.5 The significance of our report was further enhanced by a later publication demonstrating that indeed Src deficiency or blockade of Src activity in mice provides cerebral protection after stroke.9 Next, we observed that glutamate-induced 12-lipoxygenase (12-Lox) activation is a critical piece of the signaling path that kills neurons.7 Glutamate-inducible c-Src and 12-Lox were identified as 2 key cytosolic targets of TCT within the neuron. In the current work, we used a microinjection approach to assess the threshold of TCT necessary to protect a neuron against glutamate and to test whether the targets of TCT are indeed cytosolic. Next, we sought to establish whether there is a direct connection between c-Src and 12-Lox in the neurodegenerative pathway. Finally, we examined the significance of 12-Lox and of oral TCT in stroke-induced neurodegeneration in vivo.

Materials and Methods

Materials

The following materials were obtained from the source indicated. l-Glutamic acid monosodium salt; arachidonic acid; dimethyl sulfoxide; l-buthionine-[S,R]-sulfoximine; (Sigma); baicalein; 5,6,7,-Trihydroxyflavone (BL15; Oxford Biomedical Research); herbimycin A, geldanamycin, PP2, PP3 (EMD Biosciences); Fluro-Jade B (Chemicon International); TCT (90%, Carotech); Tocomin (50% tocotrienol, Carotech); 12-Lipoxygenase from porcine leukocytes (Biomol Research Laboratories Inc). For cell culture, Dulbecco's Modified Eagle Medium, Minimum Essential Medium, fetal calf serum and penicillin and streptomycin were purchased from Invitro...
Cell Culture

Mouse Hippocampal HT4 Neurons
Mouse hippocampal HT4 cells were provided by D.E. Koshland Jr, University of California at Berkeley. Cells were grown in Dulbecco’s modified Eagle’s medium as described previously. Primary Cortical Neurons
Cells were isolated from the cerebral cortex of rat fetuses (Sprague-Dawley, day 17 of gestation; Harlan, Indianapolis, Ind) as described. After isolation from the brain, cells were counted and seeded on culture plates at a density of 2 to 3×10^5 cells/35-mm plate. Cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 40 μM cystine, and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml amphotericin). Cultures were maintained at 37°C in 5% CO2 and 95% air in a humidified incubator. All experiments were carried out 24 hours after plating.

Treatmen With Neurotoxic Agents
Immediately before experiments, the culture medium was replaced with fresh medium supplemented with serum and antibiotics. Glutamate (10 mM) was added to the medium as an aqueous solution. No change in the medium pH was observed in response to the addition of glutamate. Other agents used to induce death in neuronal cells are described in the pertinent figure legends.

Vitamin E Treatment
Stock solutions (10^-3 M) of TCT were prepared in ethanol. Respective controls were treated with equal volume (0.1%, v/v) of ethanol. TCT was added to the culture dishes 5 min before glutamate. No change in the medium pH was observed in response to the addition of glutamate. Other agents used to induce death in neuronal cells are described in the pertinent figure legends.

Determination of Cell Viability
The viability of cells was assessed by measuring lactate dehydrogenase (LDH) leakage from cells to media 24 hours after glutamate treatment using in vitro toxicology assay kit from Sigma Chemical Co. The protocol has been described in detail in a previous report. In brief, LDH leakage was determined using the following equation: LDH leakage = LDH activity in the cell culture media/total LDH activity (ie, LDH activity of cells in monolayer + LDH activity of detached cells + LDH activity in the cell culture media).

Microinjection of Vitamin E
HT4 cells (0.2×10^5/plate) were grown in 35 nm plates for microinjection 24 hours before injection. Microinjection was performed using a micromanipulator Femtotet B 5247 and Injectman NI 2 (Eppendorf) with a 50 hPa of pressure and 0.1-second time setting. The glass micropipettes (Sterile Femtotips II, Eppendorf) used for injection were 0.5 μm inner and 0.7 μm outer diameter. Primary cortical neurons (2.5 to 3.0×10^5/plate) were grown in 35 nm plates for microinjection 24 hours before injection. Microinjection was performed using a micromanipulator Femtotet B 5247 and Injectman NI 2 (Eppendorf) with a 50 hPa of pressure and 0.1-second time setting. The compensation pressure during injection was 40 hPa. The glass micropipettes (Sterile femtotip I, Eppendorf) used for injection were with 0.5 μm inner and 1 μm outer diameter. Microinjection process that lasted for 24 hours. The cells were then harvested and sealed for treatment with glutamate. To inhibit protein tyrosine phosphatase activity, cells were treated with 0.15 mmol/L sodium orthovanadate (Sigma) for 15 min. After this, cells were either treated or not with 250 nmol/L TCT or 1 μmol/L herbimycin A followed by challenge with either glutamate or BSO and arachidonic acid for different time intervals under standard culture conditions as indicated in the respective figure legends. During harvest, cells were washed with ice-cold phosphate-buffered saline (pH 7.4) and lysed with 0.25 ml of lysis buffer according to manufacture instructions (Cell Signaling Technology, Inc). Immunoblots were performed using anti-phosphotyrosine antibody followed by Western Blot for 12-Lox.

Determination of 12-Lipoxygenase Tyrosine Phosphorylation in Src Transfected Cells
Following 18 h of seeding, HT4 cells were transfected with an eukaryotic expression vector containing mouse Src (dominant negative, activated or kinase-dead) cDNA under the control of a cytomegalovirus promoter (Upstate Biotechnology, Inc). Dominant-negative (DN) c-Src (K296R/Y528F) mutant has 2 mutations, an arginine for lysine substitution (K296R) in the ATP binding pocket of the tyrosine kinase domain that abolishes phosphotransferase activity, and a phenylalanine for tyrosine substitution (Y528F) that abolishes phosphorylation, thereby preventing intramolecular interaction between the C-terminus and the SH2 domain of c-Src. The kinase activating mutation (Y529F) is a substitution of phenylalanine for tyrosine at position 529. The kinase-inactivating mutation (K297R) is a substitution of arginine for lysine at position 297. Lipofectamine 2000 reagent (Invitrogen) was used to carry out the transfection process that lasted for 24 hours. The cells were then harvested and seeded for treatment with glutamate. To inhibit protein tyrosine phosphatase activity, cells were treated with 0.15 mmol/L sodium orthovanadate (Sigma) for 15 min. After this, cells were either treated or not with 10 μmol/L TCT or 1 μmol/L herbimycin A followed by challenge with either glutamate or BSO and arachidonic acid for different time intervals under standard culture conditions as indicated in the respective figure legends. During harvest, cells were washed with ice-cold phosphate-buffered saline, pH 7.4 and lysed with 0.25 ml of lysis buffer according to manufacture instructions (Cell Signaling Technology, Inc). Data substantiating the efficiency of Src gene transfer in these cells has been reported previously. Tyrosine phosphorylation on 12-Lox was detected as described above.

Assay for c-Src–Dependent Phosphorylation of 12-Lipoxygenase in Vitro
12-Lox (12.5 μL=60 μg) and 125 μmol/L ATP (0.5 μL) were mixed with 5 μL of kinase assay buffer (25mmol/L Tris-HCl, pH 7.5, 10 mmol/L MgCl2, 0.1 mmol/L sodium orthovanadate, 2 mmol/L DTT, 5mmol/L β-glycerophosphate) per reaction. The reaction was started with the addition of recombinant c-Src (6U/ reaction). The reaction was stopped with 5-lectrophoresis buffer, boiled for 5 min, subjected to 10% SDS-polyacrylamide gel electrophoresis, and probed with phosphate antibodies mouse mAb (P-Tyr-100, Cell Signaling Technology Inc). A negative control included membranes stripped and reprobed with anti-12-Lox polyclonal antiserum (Cayman Chemical Co, Inc). The kinase-inactivating mutation (K297R) is a substitution of arginine for lysine at position 297. Lipofectamine 2000 reagent (Invitrogen) was used to carry out the transfection process that lasted for 24 hours. The cells were then harvested and seeded for treatment with glutamate. To inhibit protein tyrosine phosphatase activity, cells were treated with 0.15 mmol/L sodium orthovanadate (Sigma) for 15 min. After this, cells were either treated or not with 10 μmol/L TCT or 1 μmol/L herbimycin A followed by challenge with either glutamate or BSO and arachidonic acid for different time intervals under standard culture conditions as indicated in the respective figure legends. During harvest, cells were washed with ice-cold phosphate-buffered saline, pH 7.4 and lysed with 0.25 ml of lysis buffer according to manufacture instructions (Cell Signaling Technology, Inc). Data substantiating the efficiency of Src gene transfer in these cells has been reported previously. Tyrosine phosphorylation on 12-Lox was detected as described above.

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Determination of 12-Lipoxygenase Phosphorylation
For immunoprecipitation, HT4 cells (0.7×10^6 cells/plate) were seeded in 100 mm plates and primary cortical neurons (2 to 3×10^5 cells/plate) were seeded in 35 mm plates. To inhibit protein tyrosine phosphatase activity, cells were treated with 0.15 mmol/L sodium orthovanadate (Sigma) for 15 min. After this, cells were either treated or not with 250 nmol/L TCT or 1 μmol/L herbimycin A followed by challenge with either glutamate or BSO and arachidonic acid for different time intervals under standard culture conditions as indicated in the respective figure legends. During harvest, cells were washed with ice-cold phosphate-buffered saline (pH 7.4) and lysed with 0.25 ml of lysis buffer according to manufacture instructions (Cell Signaling Technology, Inc). Immunoblots were performed using anti-phosphotyrosine antibody followed by Western Blot for 12-Lox.
Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique previously described. To ensure greater solubility of arachidonic acid and to minimize the use of ethanol in the assay medium, the potassium salt of arachidonic acid was freshly prepared by mixing arachidonic acid with 0.1 M KOH (1:1) and used within 30 min of preparation. The final assay mixture (total volume of 1 ml) contained 10 μmol/L of AA (10 μl from a 1 mmol/L stock) and 2 units of 12-Lox (porcine leukocyte enzyme, nonphosphorylated or phosphorylated) in 100 mmol/L Tris-HCl buffer (pH 7.4). Wherever required, 10 μmol/L of TCT (1 μl from a 10 mmol/L stock) was also included in the reaction mixture. The mixture was then gently mixed and reaction was started adding the enzyme. At the end of the first min of incubation at 37°C, absorbance of the reaction mixture was measured at 234 mmol/L (as an index of formation of conjugated dienes), against appropriate blanks prepared under identical conditions, using a Shimadzu model UV-2401PC spectrophotometer. The activity of 12-Lox was calculated from the absorbance values as n moles/min using the ε of 2.52×10⁶ mol/L⁻¹ cm⁻¹ and normalized as % control.

**Mouse Stroke Model**

12-Lox knockout (B6.129S2-Alox15tm1Fun) and the corresponding background C57BL6/J mice were obtained from Jackson Laboratory, MI as described previously.7 Transient focal cerebral ischemia was induced in 8 to 10 week old male mice by middle cerebral artery (MCA) occlusion. The mice were anesthetized with 1% to 1.5% halothane in oxygen-enriched air delivered through a facemask. Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique previously described.15,16 Briefly, a 6-0 nylon monofilament was inserted into the internal carotid artery, via the external carotid artery. Then the filament tip (~1.0 mm length and 0.25 mm width) was positioned for occlusion at a distance of 6 mm beyond the internal carotid artery-pterigoalipatine artery bifurcation. We observed that this approach results in a 60% to 70% drop in cerebral blood flow as measured by laser Doppler (DR14, Moor Instruments). Once the filament was secured, the incision was sutured and the animal was allowed to emerge from the anesthesia in its home cage. After 60 min of occlusion, the animal was briefly re-anesthetized with halothane in oxygen-enriched air, and reperfusion was initiated via withdrawal of the filament. This surgical protocol typically results in a core infarct limited to the parietal cerebral cortex and caudate putamen of the right hemisphere. Throughout the surgery, rectal temperature was maintained at 37.0–3.5°C through the use of a homeothermic blanket system. Mice were given a 0.5 ml subcutaneous injection of lactated Ringer solution at the conclusion of the surgical procedure, and returned to their home cage for 72 hours.15,16

**Determination of Infarct Volume**

Brains were rapidly removed, placed in a −70°C freezer for 2 min, and then sectioned into 5 2-mm-thick coronal sections. Sections were incubated for 15 min in 2,3,5-triphenyltetrazolium (TTC), with rotation every 2 min to allow uniform tissue staining. The TTC solution was maintained at 37°C throughout the staining process. Following staining, the sections were fixed in 10% buffered formalin solution. The brain slices were photographed using an Inquiry software (Loats Associates, Inc). The images were used to determine infarct size as a percentage of the contralateral hemisphere after correcting for edema, as previously described.15,16 The infarct extended from caudate putamen into surrounding cortex, and was visible in 4 of 5 slices of the brain from control wild-type mice.

**Spontaneously Hypertensive Rat Stroke**

Studies I and II

Spontaneously hypertensive rats (SHR) were obtained from Harlan, Indianapolis. These inbred albino rats have been derived from a nucleus colony obtained from the National Institutes of Health, Bethesda, Maryland.

**Study I**

Spontaneously hypertensive rats (n=32; male; 4 weeks old, Harlan, Indianapolis, IN) were randomly divided into 2 groups: control and supplemented groups. All rats were maintained in vitamin E-deficient laboratory chow (TD88163; Harlan) from 4 to 17 weeks of age until stroke was performed. The control group was orally gavaged with vitamin E-stripped corn oil with volume matching the mean volume of the supplement in the test group. The supplement, Tocomin 50%, contained 50% TCT and was provided by Carotech Sdn Bhd. Stock supplement solution (0.3 g Tocomin per ml) was made in vitamin E-stripped corn oil. The test group was orally gavaged with the supplement oil at a dosage of 1g Tocomin per kg body weight. The supplementation was done daily (5d/wk) for 8 weeks. Stroke was performed at 12 weeks of age 20 to 24 hours after the last supplementation.

**Study II**

SHR (n=42; male; 4 weeks old, Harlan) were randomly divided into 2 groups: control and supplemented. All rats were maintained in vitamin E-deficient chow (TD88163; Harlan) from 4 to 17 weeks of age until stroke was performed. The control group was orally gavaged with vitamin E-stripped corn oil with volume matching the mean volume of the supplement in the test group. Stock solution of TCT supplement solution (0.06 g TCT per ml) was made in vitamin E-stripped corn oil. The test group was orally gavaged with the supplement oil at a dosage of 50 mg TCT kg body weight. Incorporation of orally supplemented vitamin E to the brain is a slow process. Longer supplementation period improves bioavailability of vitamin E to the brain.6,33 Compared to the protocol of study I where supplementation was performed for 8 weeks, the duration of supplementation in study II was increased to 13 weeks (5d/wk). Stroke was performed at 17 weeks of age 20 to 24 hours after the last supplementation. For both studies, rats were maintained under standard conditions at 22±2°C with 12:12 hours dark: light cycles. All animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) of the Ohio State University, Columbus, Ohio.

**Spontaneously Hypertensive Rat Stroke**

Male SHR weighing 250 to 350 g were fasted for 24 hours before surgery. Because pre-ischemic hyperglycemia may increase infarct size,14 animals were fasted in an attempt to control for glucose levels. SHR were chosen because infarction can be reliably produced in this species with little variability in infarct size.17 We have substantial experience with this system.35-37 Halothane (1.5% to 2.0%), mixed with oxygen and nitrogen, was delivered through a nose cone using a flow regulator. The tail artery was cannulated with a polyethylene catheter to monitor blood pressure and to obtain blood samples for assessing physiological variables. Permanent focal neocortical ischemia was produced by tandem right common carotid artery (CCA) and MCA occlusion as described.11 Core body temperature was maintained at 37°C throughout the procedure with a heat lamp connected to a rectal thermometer. PaO2 was maintained above 80 mm Hg during the surgical procedure and mean arterial pressure was maintained above 90 mm Hg, the lower limit of autoregulation in SHR36 by adjusting the halothane concentration. Immediately after CCA/MCA occlusion, all wounds were sutured closed and the animals were allowed to recover from anesthesia. The Institutional Laboratory Animal Care and Use Committee have approved all procedures. A single investigator did complete surgery for each experiment over a 2-week period on rats delivered from a single shipment for each of the two studies (Harlan Sprague-Dawley, Inc, Indianapolis, Ind).
Arterial blood pressure was monitored throughout the surgical procedure and then checked 2 to 4 hours after surgery when animals were recovered from the anesthesia. Arterial blood PaO₂, PaCO₂, pH, glucose, and hematocrit were measured just after tail artery cannulation and repeated just before MCA occlusion. Rectal temperatures were recorded at 2 to 4 hours after CCA/MCA occlusion. Hematocrit was measured again during decapitation. When the CCA/MCA occlusion surgery was done under the controlled conditions as described above, none of the monitored physiological variables were predictive of infarct size. Despite this fact, to avoid variations in infarct size potentially attributable to physiologic alterations, we had decided that rats with post CCA/MCA occlusion PaO₂ < 70 mm Hg, mean arterial blood pressure < 100 mm Hg, rectal temperature > 39°C or a > 5% drop in hematocrit would be excluded from the study. Specifically, regarding the temperature exclusion criteria Morikawa et al reported no differences in infarct size in rats maintained at 36°C for 2 hours after MCA occlusion versus those kept at 39°C. Furthermore, no association between infarct size and temperature was seen for rats in the 36°C and 39°C groups.\textsuperscript{39}

For harvest of brains, animals were anesthetized with halothane and decapitated 24 hours after CCA/MCA occlusion in all experiments. Because infarct volume provides an objective numeric value, and it was chosen as the primary outcome measure. Functional scoring systems were not used because of the subjective nature of assessment and the arbitrary numeric value assignment.\textsuperscript{40} Infarct volume was measured 24 hours after MCA occlusion because infarct margins are maximally delineated by this time.\textsuperscript{39-45}

**12-Lipoxygenase Phosphorylation in Brain Tissue**

Rat brain tissues (100 to 150 mg) from study II were used to detect 12-Lox phosphorylation. For extraction, brain tissues were pulverized in liquid nitrogen and then homogenized in lysis buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/ml leupeptin and 1mmol/L PMSF) using a teflon homogenizer. After homogenization, tissue lysate was centrifuged at 15 000 rpm at 4°C for 20 min. After the first spin, supernatant was collected and centrifuged again at 15 000 rpm at 4°C for an additional 20 min. The clear supernatant was collected and protein concentration determined using BCA protein reagent. Immobilized phospho-tyrosine mouse mAb (P-Tyr-100, Cell Signaling Technology Inc) was used to immunoprecipitate tyrosine-phosphorylated proteins (20μg protein per sample). The immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel electrophoresis, and probed with anti–12-Lox polyclonal antiserum (Cayman Chemical Inc).

**Histological Analyses of Spontaneously Hypertensive Rat Brain**

Brains were rapidly removed from the cranium. A small portion of the noninfarcted occipital lobe was removed for vitamin E analysis and the remainder of the brain was placed in neutral buffered 10% formalin for a minimum of 3 to 4 weeks. For study II, 6 rats per group were used for histological examinations. Brains were dehydrated and embedded in paraffin. Coronal sections, 10 μm thick, were cut at 500 μm intervals. The sections were stained with hematoxylin and eosin (H&E).

**Study I**

Each brain section was magnified using a photographic lens and the infarct area traced onto paper. Each drawing was then retraced onto a digitizing tablet interfaced to a computer (Matrix v2.0), which computed infarct areas for each segment.

**Study II**

Digital photograph of each brain section were taken using a camera attached to microscope. Snappy version 1.0 was used to capture photographs of brain sections. Infarct area from digital photographs was measured using WoundMatrix software version 2.0 as described previously.\textsuperscript{44} Total infarct volume was calculated by summing the infarcted areas of sequential sections and by multiplying the sum with the thickness of sections. Image analysis for the experiment was done by a technician blinded to the study group. Intra-observer variability using this method on two separate occasions was excellent (product moment coefficient of correlation \(r=0.98, n=12\)). All results were analyzed for statistical significance using a two-tailed Student \(t\) test.

**Immunohistochemistry**

Sections of infarct site and contralateral control site of the poststroke brain were collected in OCT or formalin for different immunohistochemical analyses.

**Fluoro-Jade B Staining**

Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable method for staining degenerating neurons and their processes. Compared to conventional methodologies, Fluoro-Jade is a sensitive and more definitive marker of neuronal degeneration.9,10

To determine the neuronal degeneration, frozen brain sections (10 μm) were stained using Fluoro-Jade procedure.\textsuperscript{16} Fluorescence image analyses: tissue sections were analyzed by fluorescence microscopy (Axiovert 200M). Image analysis software (Axiovision 4.3, Zeiss, Germany) was used to quantify fluorescence intensity (fluorescent pixels) of Fluoro-Jade positive areas (expressed as pm² area).

**pp60Src and Phospho-SRC Staining**

Formalin-fixed brain tissue were embedded in paraffin and sectioned. The sections (4 μm) were deparaffinized and stained with mouse monoclonal antibody to Src (Upstate Cell Signaling Solutions) and rabbit polyclonal antiserum antiphospho-Src (Upstate Cell Signaling Solutions).

**Vitamin E Extraction and Analysis**

Vitamin E extraction and analysis of rat brains were performed as described previously using an HPLC-coulometric electrode array detector (Coularray Detector, model 566, with 12 channels; ESA Inc). This system enables the simultaneous detection of TCTs and TCps in the same run as described by us previously.\textsuperscript{6,19}

**Statistics**

Bar graphs represent mean±SD. Difference between 2 means was tested by Student \(t\) test. A value of \(P<0.05\) was interpreted as a significant difference. Comparisons among multiple groups were made by ANOVA. \(P<0.05\) was considered statistically significant.

**Results**

Microinjection studies led to the observation that 10⁻¹⁹ mol of cytosolic, but not nuclear, TCT into a HT4 neuron protects from subsequent glutamate-induced insult (Figure 1A, B). This observation is consistent with our previous reports identifying that the intracellular targets of TCT in the neuron lies in the cytosol.\textsuperscript{5,7} Figure 1B demonstrates that after 24 hours of glutamate treatment, all cells (Figure 1A), except the one microinjected with TCT, died and perished leaving the TCT-injected lone cell to survive. When repopulated with healthy cells, the surviving cell continued to live as evident by the green calcine vital stain for live cells. The reddish stain in the calcine-positive cell at the center of the field marks the injected cell (Figure 1C). Consistent with our previous report demonstrating that TCP does not share the neuroprotective effects of nanomolar TCT, we observed that microinjection of TCP failed to protect HT4 neurons as well as primary neurons (not shown) against glutamate (Figure 1F–I). The protective...
effect of subattomole quantity of TCT was applicable to primary neurons as well (Figure 1J, K).

Reduced glutathione (GSH) plays a significant role in resisting neurodegeneration. Neurons are rich in arachidonic acid, which substantially exacerbates neurotoxicity caused by lowered cellular GSH. In this model of neurodegeneration, pharmacological inhibitors of both c-Src as well as of 12-Lox protected HT4 neurons (Figure 2A, B). These results suggested a direct interaction of c-Src and 12-Lox in executing neuronal death. We hypothesized that 12-Lox is subject to tyrosine phosphorylation...
by c-Src activated in response to glutamate challenge. Experiments directed at testing the hypothesis revealed that glutamate induces rapid tyrosine phosphorylation in 12-Lox (Figure 2C, F). Such phosphorylation was sensitive to pharmacological inhibitors of c-Src as well as to TCT (Figure 2E). Tyrosine phosphorylation of neuronal 12-Lox was also observed in response to treatment of cells with GSH-depleting buthionine sulfoximine and arachidonic acid. Such phosphorylation was inhibited in the presence of nanomolar TCT (Figure 2D). Glutamate-induced 12-Lox phosphorylation was more pronounced in the kinase-active (Y529F) c-Src overexpressing cells than in wild-type (pUSE) or kinase-dead c-Src (K297R) overexpressing cells (H). Cells were activated with 10 mmol/L glutamate for 15 min.

Pursuant to our previously reported finding that 12-Lox-deficient neurons are resistant to glutamate-induced death and that 12-Lox represents a key target for TCT action,7 we observed that 12-Lox-deficient mice were resistant to stroke injury (Figure 4). The compelling neuroprotective effects of TCT in vitro led us to question the significance of this nutrient in vivo. In SHR stroke study 1, experimental stroke outcome data revealed that the Tocomin-fed rats tended (P=0.057, Figure 5E) to have reduced injury after stroke than the control group. The study was repeated with a modified experimental design, including a more pure form of the supplement, longer supplementation period, and a larger sample size. The supplementation increased brain TCT level without significantly changing the brain TCP (Figure 5C, D). The brain is known to retain its vitamin E levels effectively under conditions of dietary vitamin E deficiency.22 Consistently, we did not observe any significant drop in the TCP level of the brain of the control rats maintained on vitamin E deficiency (Figure 5C). Thus, other antioxidant systems such as the GSH system in the brain are not likely to be affected under the conditions of our study.

TCT-supplemented rats were protected against stroke-induced brain injury (Figure 5F). Analysis of the brain tissue collected from the stroke site as well as from the contralateral nonstroke site of the brain demonstrated that although phospho-12-Lox was not detected in the nonstroke site, 12-Lox was clearly tyrosine-phosphorylated in the tissue from the stroke site. Such stroke-associated phosphorylation of 12-Lox was lower in the brain of rats supplemented with TCT (Figure 5G).

The histochemical application of Fluoro-Jade results in a simple, sensitive, and reliable method for staining degenerating neurons and their processes.23 More prominent Fluoro-Jade staining in brain sections from control rats compared with sections from supplemented rats (Figure 6) was consistent with...
stroke-induced brain injury results shown in Figure 5F. Previously, we have reported that activation of c-Src represents a key mechanism that contributes to neurodegeneration.5 Y416 represents a major autophosphorylation site in c-Src activation loop that is also responsible for c-Src activation.24 Here we provide first in vivo evidence indicating that stroke is associated with c-Src activation at the injury site (Figure 6). Stroke-associated c-Src activation was partly suppressed in TCT supplemented rats (Figure 6).

Discussion

TCTs differ from TCPs by possessing a farnesyl (isoprenoid) rather than a saturated phytyl side chain. The unsaturated side chain of TCT allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver.25 Micromolar amounts of TCT, not TCP, have been shown to suppress the activity of hydroxy-3-methylglutaryl coenzyme A reductase.26,27 Our finding that cytosolic, but not nuclear, TCT is neuroprotective is in line with our previous observations characterizing that the molecular targets of TCT in the neuron are cytosolic.5,7 Furthermore, the results showing that at subattomole levels TCT, but TCP, are neuroprotective is consistent with our previous reports claiming that at low doses, the neuroprotective property of TCT is not shared by TCP.5 Efforts to elucidate the mechanisms underlying the neuroprotective properties of TCT led to the finding that glutamate-induced rapid c-Src activation is prevented in the presence of nanomolar concentrations of TCT. This function of TCT was not shared by TCP.5 The significance of TCT as inducible c-Src inhibitor in neuronal cells was further enhanced by a subsequent study reporting that Src deficiency or blockade of Src activity in mice provides cerebral protection after stroke.9 Next, we identified 12-Lox as another TCT-sensitive molecular checkpoint that proved to be critical in executing death of neurons in response to glutamate and other GSH-lowering agents.7 After glutamate challenge, 12-Lox was rapidly activated and migrated from the cytosol to the membrane.7 Here, we tested the efficacy of inhibitors of both c-Src as well as 12-Lox in models of cell death other than glutamate-induced but related to GSH-lowering. Based on past experience with such experimental systems,5,7 we challenged cells with either BSO alone or with a combination of BSO and arachidonic acid. TCT protected the neurons under both challenging conditions. Of interest, inhibitors of c-Src as well as of 12-Lox protected the cells against both challenging conditions. These observations led us to question whether 12-Lox may act as a substrate for c-Src. Results presented here suggest that 12-Lox is subject to rapid tyrosine phos-
phorylation in neuronal cells challenged with glutamate or GSH-lowering agents. Such phosphorylation is rapid and coincides with the timeline of c-Src activation.\(^5,11\) Inhibitors of c-Src abrogated such inducible 12-Lox tyrosine phosphorylation, supporting the notion that c-Src may directly phosphorylate 12-Lox in challenged neurons. To test this hypothesis, we used genetic approaches of overexpressing kinase-active, kinase-dead, or dominant-negative c-Src in neuronal cells. Current findings from cell biology studies as well as from the study of c-Src and 12-Lox in cell-free systems indicate that in response to challenge by glutamate or GSH-lowering agents, c-Src is rapidly activated and phosphorylates 12-Lox.

In support of a central role of 12-Lox in glutamate-induced neurodegeneration, we have previously reported that inhibitors of 12-Lox prevent death of neuronal cells caused in response to glutamate or GSH-lowering agents.\(^7\) Our case for 12-Lox as a critical mediator of glutamate-induced neurodegeneration was strengthened by the finding that compared with neurons from corresponding wild-type mice, cortical neurons from 12-Lox-deficient mice are resistant to glutamate-induced death.\(^7\) Our current findings demonstrate that 12-Lox deficiency protects against stroke injury. This builds a compelling case to look at 12-Lox as a therapeutic target for the management of stroke-related injury in the brain. Functional outcomes in in vivo stroke models have been proposed to be valuable while building the case for clinical trials to test the effect of any neuroprotective agent.\(^28\) Although the incorporation of cognitive and sensorimotor functional outcome assessment represents an important step forward in stroke research, reports of MCAO-induced behavioral deficits often conflict.\(^29\) The effect of TCT on stroke-induced changes in functional outcome remains to be investiga-

Figure 5. Oral TCT supplementation protected against stroke-induced injury and 12-lipoxygenase phosphorylation in the brain of spontaneously hypertensive rats. The effect of oral supplementation on brain TCP (A and C) and TCT (B and D). *\(P<0.01\) significantly different compared with the corresponding control group, Infarct volume (E and F) in response to stroke. (G) TCT supplementation lowered 12-lipoxygenase phosphorylation in the stroke-site brain tissue of SHR. The brain tissue sample was harvested 24 hours after stroke from a predictable area of infarct core around the occluded vessel. This includes the primary motor cortex and the primary somatosensory cortex. (H) Histologic analyses of poststroke infarct zone cortical section (Figure 6) revealed lower count of Fluoro-jade positive (FJ+) neurons in tocotrienol supplemented rats. *\(P<0.05\). These results represent the image analysis data of sections (A1, B1, and C1) shown in Figure 6. Study I, \(n=16\); Study II, \(n=21\).

Figure 6. Histologic analyses of poststroke infarct zone cortical sections. Fluoro-Jade (FJ) stain. In the contralateral hemisphere of the brain from control rats (A1), there was hardly any FJ-positive neuron. The ipsilateral hemisphere of the brain from TCT supplemented rats (C1) contained fewer (see Figure 5H) FJ-positive cells compared with such cells in the ipsilateral hemisphere of the brain from control rats (B1). Src and phospho-Src staining. In the nonstroke site of the brain section, Src was present (A2) but not in a phosphorylated form (A3). Stroke did not influence Src expression but clearly induced Src phosphorylation and activation. Such stroke-induced Src activation was less in the brain sections of TCT-supplemented rats compared with control rats that were subjected to stroke (C3 vs B3). For all sections shown, brain was harvested 24 hours after stroke. Bar=100 \(\mu\)m.
gated. In glutamate-challenged neurons, TCT effectively modulates both 12-Lox as well as c-Src activity to favor survival. This study demonstrated that oral TCT supplementation may protect against stroke in vivo.

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