

Characterization of the potent neuroprotective properties of the natural vitamin E α -tocotrienol

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Abstract

The natural vitamin E tocotrienols possess properties not shared by tocopherols. Nanomolar α -tocotrienol, not α -tocopherol, is potentially neuroprotective. On a concentration basis, this finding represents the most potent of all biological functions exhibited by any natural vitamin E molecule. We sought to dissect the antioxidant-independent and -dependent neuroprotective properties of α -tocotrienol by using two different triggers of neurotoxicity, homocysteic acid (HCA) and linoleic acid. Both HCA and linoleic acid caused neurotoxicity with comparable features, such as increased ratio of oxidized to reduced glutathione GSSG/GSH, raised intracellular calcium concentration and compromised mitochondrial membrane potential. Mechanisms underlying HCA-induced neurodegeneration were comparable to those in the path implicated in glutamate-induced neurotoxicity. Inducible

activation of c-Src and 12-lipoxygenase (12-Lox) represented early events in that pathway. Overexpression of active c-Src or 12-Lox sensitized cells to HCA-induced death. Nanomolar α -tocotrienol was protective. Knock-down of c-Src or 12-Lox attenuated HCA-induced neurotoxicity. Oxidative stress represented a late event in HCA-induced death. The observation that micromolar, but not nanomolar, α -tocotrienol functions as an antioxidant was verified in a model involving linoleic acid-induced oxidative stress and cell death. Oral supplementation of α -tocotrienol to humans results in a peak plasma concentration of 3 μ M. Thus, oral α -tocotrienol may be neuroprotective by antioxidant-independent as well as antioxidant-dependent mechanisms.

Keywords: antioxidant, lipoxygenase, mitochondria, neurodegeneration, stroke, tyrosine kinase.

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Vitamin E is a generic term for tocopherols and tocotrienols which qualitatively exhibit the biological activity of α -tocopherol (Sen *et al.* 2004). Compared with tocopherols, tocotrienols have been poorly studied (Traber and Packer 1995; Traber and Sies 1996; Sen *et al.* 2004, 2006). It is clear, however, that members of the vitamin E family are not redundant with respect to their biological functions. α -Tocotrienol, γ -tocopherol and δ -tocotrienol have emerged as vitamin E molecules with functions in health and disease that are clearly distinct from that of α -tocopherol (Hensley *et al.* 2004; Sen *et al.* 2006). At concentrations of 25–50 μ M, α -tocopherol uniquely regulates specific signal transduction pathways by mechanisms that are independent of its antioxidant properties (Boscoboinik *et al.* 1994). Micromolar amounts of tocotrienol, but not tocopherol, suppress the activity of hydroxy-3-methylglutaryl co-enzyme A reductase (Pearce *et al.* 1992; Pearce *et al.* 1994). Tocotrienols possess anticancer and cholesterol-lowering properties that are often not exhibited by tocopherols (Theriault *et al.* 1999; Packer *et al.* 2001; Schaffer *et al.* 2005; Sen *et al.* 2006).

Tocotrienols differ from tocopherols by possessing a farnesyl (isoprenoid) rather than a saturated phytyl side-chain. We have reported that, at nanomolar concentrations, α -tocotrienol, but not α -tocopherol, prevents stroke-related

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Abbreviations used: BCA, bicinchoninic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; carboxy- H_2 DCFDA, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; $\Delta\psi$, membrane potential; FOX1, ferrous oxidation in xylenol orange; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HCA, homocysteic acid; HPETE, hydroperoxy-eicosatetraenoic; LDH, lactate dehydrogenase; Lox, lipoxygenase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TCP, α -tocopherol; TCT, α -tocotrienol; TMRM, tetramethylrhodamine methyl ester.

neurodegeneration (Sen *et al.* 2000; Khanna *et al.* 2003). On a concentration basis, this finding represents the most potent of all biological functions exhibited by any natural vitamin E molecule. Our recent work has demonstrated the efficacy of α -tocotrienol in protecting against stroke-induced injury to the brain *in vivo* (Khanna *et al.* 2005b). Orally supplemented tocotrienols are transported to vital organs including the brain and spinal cord (Khanna *et al.* 2005a). The unsaturated side-chain of tocotrienol allows more efficient penetration into tissues that have saturated fatty layers, such as the brain and liver (Suzuki *et al.* 1993). Following supplementation in humans, micromolar concentrations of α -tocotrienol have been detected in blood plasma (O'Byrne *et al.* 2000; Khosla *et al.* 2006). The maximal α -tocotrienol concentrations in supplemented individuals averaged approximately 3 μM in blood plasma, 1.7 μM in low-density lipoprotein, 0.9 μM in triglyceride-rich lipoprotein and 0.5 μM in high-density lipoprotein (Khosla *et al.* 2006). Thus, the peak plasma level corresponded to 12–30-fold more than the concentration of α -tocotrienol required to completely prevent stroke-related neurodegeneration (Sen *et al.* 2000; Khanna *et al.* 2003).

The murine HT hippocampal neural cell line, lacking the intrinsic excitotoxicity pathway, represents an useful model with which to characterize redox-sensitive pathways involved in neurotoxicity (Sen *et al.* 2000; Schubert and Piasecki 2001; Tan *et al.* 2001; Dargusch and Schubert 2002; Khanna *et al.* 2003, 2005b). In this study, we sought to develop an understanding of the antioxidant-independent and -dependent neuroprotective properties of α -tocotrienol by using two different triggers of neurotoxicity, homocysteic acid (HCA) and linoleic acid. Pathways for neurodegeneration induced by HCA are akin to those induced by glutamate (Sagara *et al.* 2002; Baydas *et al.* 2005). In contrast, linoleic acid is known to directly induce oxidative stress (Greene and Hammock 1999; Turpeinen *et al.* 1999; Sasaki *et al.* 2003; Reiterer *et al.* 2004; Terrasa *et al.* 2005).

Materials and methods

Materials

L-HCA, dimethyl sulfoxide, xylene orange, ammonium ferrous sulfate and sorbitol were from Sigma (St Louis, MO, USA); baicalein, 5,6,7-trihydroxyflavone (BL15) from Oxford Biomedical Research, Oxford, MI, USA; herbimycin A and geldanamycin from EMD Biosciences (San Diego, CA, USA); linoleic acid from Nu-Chek Prep (Elysian, MN, USA); and α -tocotrienol and α -tocopherol from Carotech Bhd (Chemor, Perak, Malaysia). For cell culture, Dulbecco's modified Eagle's medium, minimum essential medium, fetal calf serum, penicillin and streptomycin were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Culture dishes were obtained from Nunc (Roskilde, Denmark).

Cell Culture

Mouse hippocampal HT4 neural cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2 as described previously (Tirosh *et al.* 1999, 2000; Sen *et al.* 2000, 2004; Khanna *et al.* 2003). Immediately before experiments with neurotoxic agents, the culture medium was replaced with fresh medium supplemented with serum and antibiotics. L-HCA (1 mM) was added to the medium as an aqueous solution (Sen *et al.* 2000; Khanna *et al.* 2003). HCA was diluted from 100-fold concentrated solutions that were adjusted to pH 7.5. For linoleic acid (18 : 2) treatment, stock solutions ($10^3 \times$ working concentration) were prepared in ethanol. Linoleic acid was dissolved in serum before adding to cells. Respective controls were treated with an equal volume of ethanol (0.1%, v/v) in serum. For vitamin E treatment, stock solutions ($10^3 \times$ working concentration) of α -tocotrienol and α -tocopherol were prepared in ethanol. Respective controls were treated with an equal volume of ethanol (0.1%, v/v). In cases of pretreatment, α -tocotrienol or α -tocopherol was added to the culture dishes 5 min before exposure to HCA or linoleic acid.

Primary cortical neurons were isolated from the cerebral cortex of rat feti (Sprague–Dawley, day 17 of gestation) as described previously (Murphy *et al.* 1990; Khanna *et al.* 2003). After isolation from the brain, cells were counted and seeded on culture plates at a density of $2\text{--}3 \times 10^6$ cells per well of six-well plates (Murphy *et al.* 1990). Cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 40 μM cystine and antibiotics (100 $\mu\text{g/mL}$ streptomycin, 100 U/mL penicillin and 0.25 $\mu\text{g/mL}$ amphotericin). Cultures were maintained at 37°C in 5% CO_2 and 95% air in a humidified incubator. All experiments were carried out 24 h after plating (Khanna *et al.* 2003).

Determination of cell viability

The viability of cells in culture was assessed by measuring the leakage of lactate dehydrogenase (LDH) (Han *et al.* 1997b) from cells into the medium 18–24 h following HCA or linoleic acid (18 : 2) treatment using the *in vitro* toxicology assay kit from Sigma. The protocol has been described in detail in previous reports (Han *et al.* 1997a, 1997b). In brief, LDH leakage was determined using the following equation:

$$\% \text{ total LDH leaked} = (\text{LDH activity in the cell culture medium} / \text{total LDH activity}) \times 100$$

where total LDH activity is the sum of LDH activities in the cell monolayer, detached cells and the cell culture medium (Sen *et al.* 2000; Khanna *et al.* 2003, 2005b). The approach has been used to measure necrotic (Steiner *et al.* 2000; Lopez *et al.* 2003) as well as apoptotic (Liu *et al.* 2003; Kajta *et al.* 2004) death of neurons. The LDH release approach has been used to study neurotoxicity caused by HCA (Parsons *et al.* 1998) as well as by oxidative stress (Kraus *et al.* 2005).

c-Src overexpression

Following 18 h of seeding, HT4 cells were transfected with an eukaryotic expression vector containing mouse Src (activated or

kinase dead) cDNA under the control of a cytomegalovirus promoter (Upstate Biotechnology, Inc., Lake Placid, NY, USA). 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 (Sen *et al.* 2000; Khanna *et al.* 2005b). Lipofectamine 2000 (Invitrogen) was used to carry out the transfection. After 3 h of lipofectamine treatment, the transfection reagent was replaced with regular cell culture medium. The cells were maintained under regular culture conditions for 24 h to allow protein expression. At this point, the cells were harvested and seeded for treatment with tocotrienol, BL15 and/or HCA. After 12 h of seeding, the culture medium was changed, and cells were treated as described in the relevant figure legend. To assess the level of c-Src expression, HT4 cells were harvested 24 h after transfection, seeded for 12 h, and collected for analysis of protein expression by Western blotting. The protein concentrations were determined using bicinchoninic acid (BCA) protein reagents. Samples (20 µg protein/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% gel) and probed with anti-c-Src (Upstate Cell Signaling Solutions, Lake Placid, NY, USA). To evaluate the loading efficiency, membranes were stripped and reprobed with β -actin antibody (Sigma).

12-Lipoxygenase (12-Lox) overexpression

This procedure was performed as described previously (Khanna *et al.* 2003). Following 18 h of seeding, HT4 cells were transfected with plasmid pcDNA3.1 + 12-Lox (ResGen; Invitrogen) or empty pcDNA3.1 using Lipofectamine 2000 transfection reagent (Invitrogen). The experiment was performed as described above for c-Src overexpression. After 12 h of seeding, the culture medium was changed, and cells were treated as described in the relevant figure legend. To assess the level of 12-Lox protein expression, HT4 cells were harvested 24 h after transfection, seeded for 12 h and harvested for extraction of protein. The sample protein concentrations were determined using BCA protein reagents. Samples (10 µg protein/lane) were separated by SDS–PAGE (10% gel) and probed with 12-Lox polyclonal antiserum (Cayman Chemical Co., Inc., Ann Arbor, MI, USA). To evaluate the loading efficiency, membranes were stripped and reprobed with β -actin antibody (Sigma).

Small interfering RNA (siRNA) delivery and analysis of genes and proteins

Cells (0.1×10^6 cells/well in a 12-well plate) were seeded in antibiotic-free medium for 24 h before transfection. DharmaFECT™ 1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO, USA) was used to transfect cells with 100 nM siRNA pool (Dharmacon RNA Technologies) for 72 h according to manufacturer's protocol. As a control, siControl non-targeting siRNA pool (mixture of four siRNAs, designed to have at least four mismatches with the corresponding mouse gene) was used. A transfection efficiency of >90% was achieved. Cells were harvested and seeded for treatment with α -tocotrienol, BL15 and/or HCA as indicated in figure legends. After 12 h of seeding, the culture medium was changed and the cells were treated as described in the figure legends. For quantification of mRNA and protein expression, samples were collected after 72 h of siRNA transfection. Total RNA was isolated from cells using the Absolutely RNA® miniprep kit

(Stratagene, La Jolla, CA, USA). The abundance of mRNA for c-Src and 12-Lox was quantitated using real-time PCR. The double-stranded DNA-binding dye SYBR green-I was used. The following primer sets were used: m_glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-ATGACCACAGTCCATGCCATCACT-3'; m_GAPDH reverse, 5'-TGTGAAGTCGACGAGACAACCT-3'; m_12-Lox forward, 5'-ACCCCTACTACATCCAGGCTTCCA-3'; m_12-Lox reverse, 5'-TGACATCCGCCCTATATGCTGAA-3'; m_c-Src forward, 5'-TCCACACCTCTCCGAAGCAA-3'; and m_c-Src reverse, 5'-CATGCTGATGGCCTGTGTCA-3'.

After protein extraction, protein concentrations were determined using BCA protein reagents. Samples (20 µg protein/lane) were separated by SDS–PAGE (10% gel) and probed with c-Src and 12-Lox antibodies. To evaluate the loading efficiency, membranes were probed with β -actin antibody (Khanna *et al.* 2005b).

Reduced (GSH) and oxidized (GSSG) glutathione assay

GSH and GSSG were detected simultaneously in HT4 cell acid lysates using an HPLC coulometric electrode array detector (CoulArray Detector, model 5600 with 12 channels; ESA Inc., Chelmsford, MA, USA) as described previously (Sen *et al.* 2002; Khanna *et al.* 2003, 2005b). The CoulArray detector employs multiple channels set at specific redox potentials (Roy *et al.* 2002). Data were collected using channels set at 600, 700 and 800 mV. The samples were snap-frozen and stored in liquid nitrogen until HPLC assay. Sample preparation, composition of the mobile phase, and specification of the column used have been previously reported (Sen *et al.* 2000, 2002).

Determination of intracellular free Ca^{2+} level ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ was measured using cell-permeant calcium green-1, acetomethoxyl ester (Molecular Probes, Eugene, OR, USA), as described previously (Tirosh *et al.* 2000). Following different treatments, cells were washed three times with phosphate-buffered saline (PBS). Cells were detached from monolayers using trypsin, and centrifuged at 600 g for 5 min. After another wash, the cells were resuspended in PBS and loaded with the acetomethoxyl ester of calcium green-1 (1 µM) for 20 min at room temperature (22°C). For the detection of intracellular fluorescence, the fluorochrome-loaded cells were excited using a 488-nm argon ion laser in a flow cytometer. The emission was recorded at 530 nm. Data were collected from at least 10 000 cells per sample.

Visualization of reactive oxygen species (ROS) in live cells

The Image-iT™ live green ROS detection system (Molecular Probes) was used to visualize ROS in live HT4 cells. This new assay approach is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy- H_2DCFDA) (Armeni *et al.* 2004; Minami *et al.* 2005). The non-fluorescent carboxy- H_2DCFDA permeates live cells and is deacetylated by non-specific intracellular esterases. In the presence of non-specific ROS, the reduced fluorescein compound is oxidized and emits bright green. Fluorescence microscopy (Axiovert 200M; Zeiss, Oberkochen, Germany) was performed to capture images of nuclei (blue fluorescence; Hoechst 33342) and oxidized fluorescein.

Determination of lipid hydroperoxides

For lipid hydroperoxide measurement, the ferrous oxidation in xylenol orange (FOX1) assay was used (Jiang *et al.* 1992; Bleau

et al. 1998). After 4 h of treatment with or without linoleic acid, cells were washed and collected in PBS. Cells were homogenized on ice and the homogenate was used to measure lipid hydroperoxides.

Measurement of mitochondrial membrane potential ($\Delta\psi$)

Mitochondrial $\Delta\psi$ was measured using the fluorescent lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM) (Molecular Probes), which accumulates within mitochondria in a potential-dependent manner (Reid *et al.* 2005). Following 24 h of seeding, cells were exposed to the desired concentrations of HCA or linoleic acid for the specified time period. The cells were then resuspended in Hanks' balanced salt solution and stained with 4 μ M TMRM for 15 min at 37°C in the dark. The cells were washed twice with ice-cold PBS, and the fluorescence was measured using a fluorometer (Cytofluor II; PerSeptive Biosystems, Inc., Framingham, MA, USA) at excitation and emission wavelengths of 530 and 590 nm.

Statistical analyses

Data are reported as mean \pm SD of at least three experiments. Comparisons between multiple groups were made by ANOVA. $p < 0.05$ was considered statistically significant.

Results

Treatment of the HT4 neural cell line with HCA resulted in overt toxicity within the first 24 h of exposure. Such HCA-induced death of HT4 cells was completely prevented in cells pretreated with nanomolar concentrations of α -tocotrienol. At comparable doses, α -tocopherol failed to protect the cells against HCA-induced toxicity, demonstrating increased neuroprotective ability of α -tocotrienol compared with its better known isoform α -tocopherol (Fig. 1a). Counting of cells after staining with trypan blue revealed that 95% of the cells were viable under control conditions. Treatment of cells with HCA for 24 h resulted in the death of 91% of cells (Fig. 1a). At nanomolar concentrations α -tocotrienol decreased, albeit not completely, HCA-induced loss of cell viability even when added 8 h after the HCA challenge (Fig. 1b). At this late stage, micromolar concentrations of α -tocotrienol had a more significant protective effect (Fig. 1b). These results point towards a difference in the mechanisms of action of nanomolar and micromolar concentrations of α -tocotrienol.

Neurotoxicity caused by HCA was significantly prevented by pharmacological inhibitors of c-Src kinase and 12-Lox, suggesting the possible involvement of these pathways in the neurodegenerative process (Fig. 1a). To establish the significance of c-Src kinase in HCA-induced neurotoxicity, a genetic approach was employed. HT4 cells were transfected with activated (Y529F) c-Src, kinase-dead (K297R) c-Src, or the corresponding empty vector (pUSE). Such transfection significantly increased c-Src protein expression (Fig. 2a). To test whether overexpression of active c-Src exacerbates HCA-induced neurotoxicity, we chose to study cells treated with HCA for 12 h. At this time point, HCA induced loss of viability of very few cells, offering the opportunity to

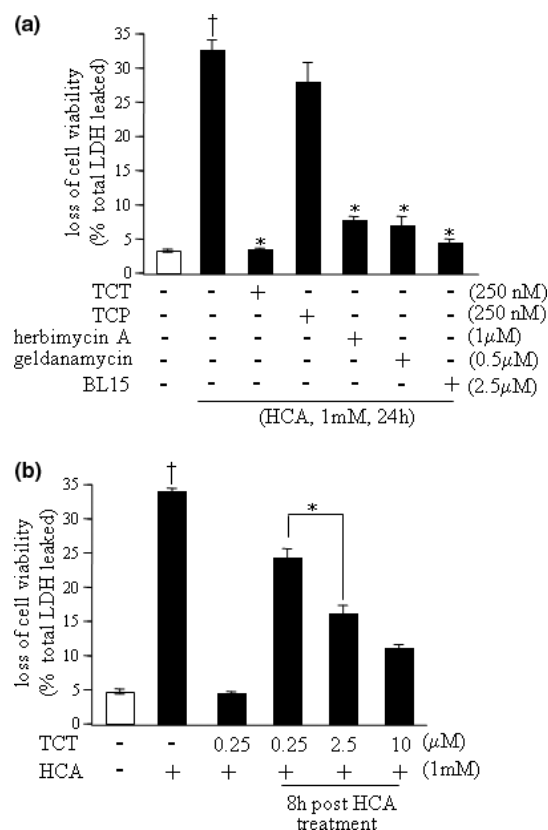


Fig. 1 Protection against HCA-induced toxicity. (a) HT4 cells were either treated or not with α -tocotrienol (TCT), α -tocopherol (TCP), BL15, herbimycin or geldanamycin (as indicated) for 5 min and challenged with HCA (1 mM) for 24 h. Micromolar but not nanomolar concentrations of TCT protected cells when TCT was given 8 h after the HCA challenge. HT4 neuronal cells were either treated or not with TCT for 5 min before or 8 h after HCA. Viability was measured 24 h after the HCA challenge. Values are mean \pm SD. * $p < 0.05$; † $p < 0.05$ versus control (no HCA) group. (ANOVA).

investigate exacerbation of toxicity. Akin to control cells transfected with empty vector pUSE, cells overexpressing kinase-dead (K297R) c-Src exhibited marginal toxicity in response to HCA treatment. Overexpression of kinase-active (Y529F) c-Src, however, clearly accelerated HCA-induced cell death (Fig. 2b). HCA-induced death in controls as well as in cells overexpressing active c-Src was completely prevented by nanomolar α -tocotrienol (Fig. 2b). These findings indicate that c-Src executes HCA-induced neurotoxicity, and that nanomolar α -tocotrienol is capable of preventing HCA-induced death under basal conditions and also that exacerbated in the presence of excess active c-Src. To test whether c-Src participates directly in HCA-induced neural cell death, we adopted the knock-down approach. c-Src knock-down was performed using siRNA (Fig. 3). Cells with c-Src knock-down were observed to be significantly resistant to HCA-induced loss of viability (Fig. 3).

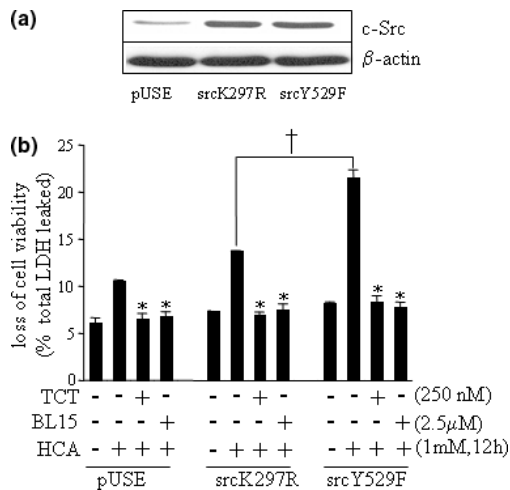


Fig. 2 Involvement of c-Src kinase activity in HCA-induced neuronal death and its sensitivity to α -tocotrienol. (a) Western blot demonstrating overexpression of kinase-dead (K297R) and kinase-active (Y529F) c-Src protein in HT4 neuronal cells. pUSE, empty plasmid control. (b) Active (Y529F), but not kinase-dead (K297R), c-Src accelerated HCA-induced cell death. TCT (0.25 μ M) and BL15 (2.5 μ M) protected against the toxic effects of active c-Src. Values are mean \pm SD. * p < 0.05 versus group treated with HCA alone; † p < 0.05 (ANOVA).

BL15, a pharmacological inhibitor of 12-Lox, prevented neurotoxicity caused by HCA in the presence of excess active c-Src (Fig. 2b). These findings suggest that the ability of c-Src to execute death in HCA-challenged neural cells is dependent on the activity of 12-Lox. We therefore tested the significance of 12-Lox in HCA-induced cell death. To address this issue, 12-Lox was overexpressed in HT4 cells (Fig. 4a). Compared with control cells transfected with the empty vector pcDNA3.1, cells overexpressing 12-Lox were sensitized to HCA-induced death (Fig. 4b). HCA-induced death of control cells as well as those overexpressing 12-Lox was completely prevented by nanomolar α -tocotrienol and by BL15 (Fig. 4b). To test the significance of 12-Lox as a trigger of HCA-induced neural cell death, we performed studies with knock-down cells. 12-Lox knock-down was performed using siRNA (Figs 5a and b). Cells with 12-Lox knock-down were significantly resistant to HCA-induced loss of viability (Fig. 5c). These findings firmly establish that 12-Lox is a central executioner of HCA-induced neurotoxicity and that nanomolar α -tocotrienol is capable not only of preventing HCA-induced death but also that exacerbated in the presence of excess 12-Lox.

HCA-induced neurotoxicity has been associated with oxidant insult. Cellular oxidant load rapidly manifests in the form of glutathione oxidation and loss (O'Donovan and Fernandes 2000; Sen 2000). Thus, we sought to monitor the levels of GSH and GSSG in cells challenged with HCA. HCA caused rapid oxidation and loss of cellular GSH.

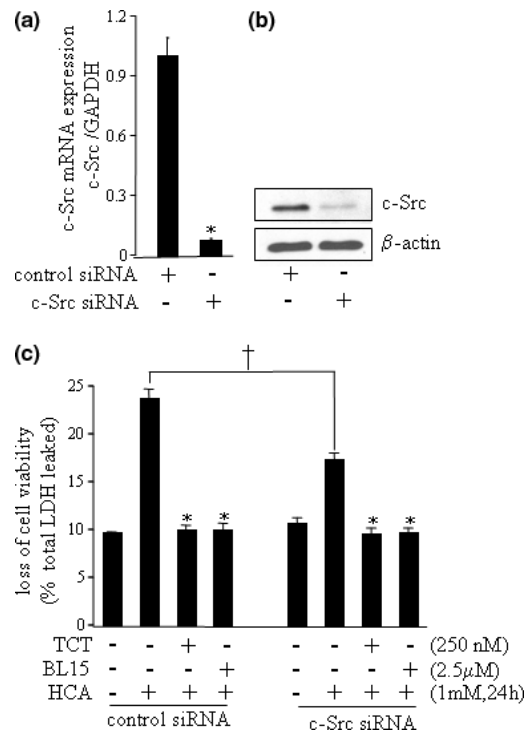


Fig. 3 Knock-down of c-Src attenuated HCA-induced cell death. Transfection with c-Src siRNA decreased c-Src mRNA (a; * p < 0.05 versus control siRNA group, ANOVA) and protein (b) expression. (c) c-Src knock-down attenuated HCA-induced loss of cell viability. The results of BL15 and TCT treatment are shown in these siRNA-transfected cells as positive control for complete protection. Values are mean \pm SD. * p < 0.05 versus corresponding group treated with HCA alone; † p < 0.05 (ANOVA).

Significant changes were already apparent at 2 h after HCA treatment. The changes became more prominent at 6 h after challenge of HT4 cells with HCA (Fig. 6). The GSSG/GSH ratio serves as a marker of oxidative stress (Sen 2000; Rahman *et al.* 2005). HCA induced a significant increase in the cellular GSSG/GSH ratio. Although nanomolar α -tocotrienol completely prevented HCA-induced cell death (Figs 1–5), such defense was not associated with protection against loss or oxidation of GSH (Fig. 6).

The Image-iT™ assay system (Molecular Probes) enables the detection of ROS in live cells (Maurer *et al.* 1999; Konorev *et al.* 2000). Using this approach, it was possible to visualize the build-up of intracellular ROS after challenge of cells with HCA. Overt accumulation of intracellular ROS was detected in part at 4 h. The response was more overt at 8 h (Fig. 7). Micromolar α -tocotrienol completely prevented HCA-induced accumulation of intracellular ROS (Fig. 7). These results support the antioxidant efficacy of micromolar, but not nanomolar, concentrations of α -tocotrienol.

Treatment of cells with HCA resulted in a marked increase in $[Ca^{2+}]_i$ and simultaneous loss of mitochondrial $\Delta\psi$. Both

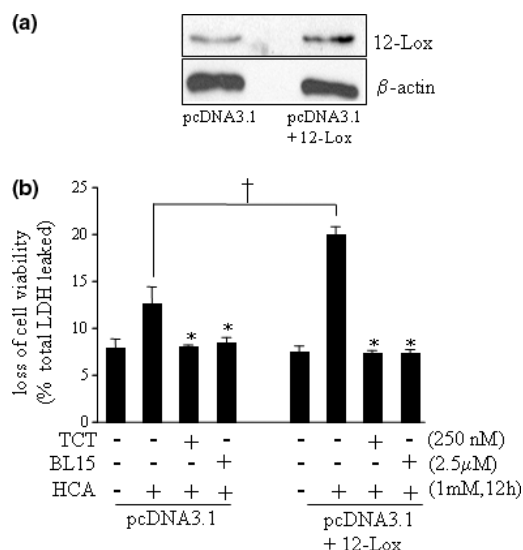


Fig. 4 Involvement of 12-Lox in HCA-induced neuronal death and its sensitivity to α -tocotrienol. (a) Western blot demonstrating overexpression of 12-Lox in HT4 cells. pcDNA3.1, empty plasmid control; pcDNA3.1 + 12-Lox, 12-Lox expression vector. (b) 12-Lox overexpression accelerated HCA-induced cell death. Both TCT (0.25 μ M) and BL15 (2.5 μ M) protected against the toxic effects of 12-Lox. Values are mean \pm SD. * p < 0.05 versus group treated with HCA alone; † p < 0.05 (ANOVA).

cellular responses were observed in the late phase of HCA treatment. The changes were not significant up to 4 h after HCA challenge, but at 8 h after HCA treatment changes in $[Ca^{2+}]_i$ and $\Delta\psi$ were highly significant (Fig. 8). Of note, at this time point no change in cell viability was observed in response to HCA treatment. Thus, the observed changes in $[Ca^{2+}]_i$ and mitochondrial $\Delta\psi$ were not merely a consequence of cell death. Both of these adverse cellular responses were prevented by the presence of nanomolar α -tocotrienol (Fig. 8). These observations indicate that oxidation and loss of GSH, as noted under the given experimental conditions, was not a critical determinant of cell survival. Nanomolar α -tocotrienol did not seem to protect against HCA-induced death simply as an antioxidant. Nanomolar α -tocotrienol, however, was effective in preventing HCA-induced perturbations in $[Ca^{2+}]_i$ and mitochondrial $\Delta\psi$.

The ω -6 polyunsaturated fatty acid linoleic acid induced neurotoxicity at a threshold dose of 50 μ M (Fig. 9a). Overt toxicity was induced by linoleic acid in 12 h, but maximal effects were noted at 24 h (Fig. 9b). In this system of neurodegeneration, known to be primarily caused by oxidative stress (Greene and Hammock 1999; Turpeinen *et al.* 1999; Sasaki *et al.* 2003; Reiterer *et al.* 2004; Terrasa *et al.* 2005), nanomolar α -tocotrienol failed to protect. In contrast to the situation observed with HCA, both α -tocotrienol and α -tocopherol exhibited comparable protective effects at micromolar concentrations (Fig. 9c). Linoleic acid-induced neurotoxicity was associated with oxidative

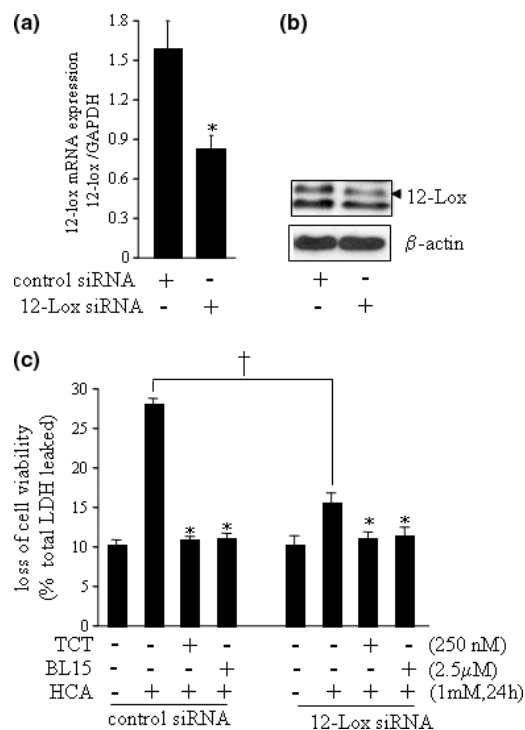


Fig. 5 Knock-down of 12-Lox attenuated HCA-induced nerve cell death. Transfection with 12-Lox siRNA decreased 12-Lox mRNA (a; * p < 0.05 versus control siRNA group) and protein (b) expression. (c) 12-Lox knock-down attenuated HCA-induced loss of cell viability. The results of BL15 and TCT treatment are shown in these siRNA-transfected cells as positive control for complete protection. Values are mean \pm SD. * p < 0.05 versus corresponding group treated with HCA alone; † p < 0.05 (ANOVA).

stress. Raised levels of ROS were detected in linoleic acid-treated live cells. Micromolar, but not nanomolar, α -tocotrienol completely prevented such build-up of ROS (Fig. 10a–d). These observations again indicate that α -tocotrienol is not an effective antioxidant at nanomolar concentrations. Additional evidence along this line was generated by estimating the levels of lipid hydroperoxides in cells treated with linoleic acid. Exposure of cells to linoleic acid resulted in a clear increase in lipid hydroperoxide levels as measured using the FOX1 assay. This oxidative stress response was not sensitive to nanomolar α -tocotrienol. Micromolar α -tocotrienol, however, significantly prevented linoleic acid-induced formation of lipid hydroperoxides (Fig. 10e). Analysis of cellular glutathione status confirmed that micromolar, but not nanomolar, α -tocotrienol serves as an antioxidant. Linoleic acid induced loss of GSH, increased GSSG and increased the GSSG/GSH ratio. All of these changes were sensitive to micromolar α -tocotrienol, but not to nanomolar α -tocotrienol (Figs 11a–c). In the linoleic acid system of neurotoxicity, which relies primarily on oxidative stress, cell death was associated with increased $[Ca^{2+}]_i$ and compromised mitochondrial $\Delta\psi$. These adverse cellular

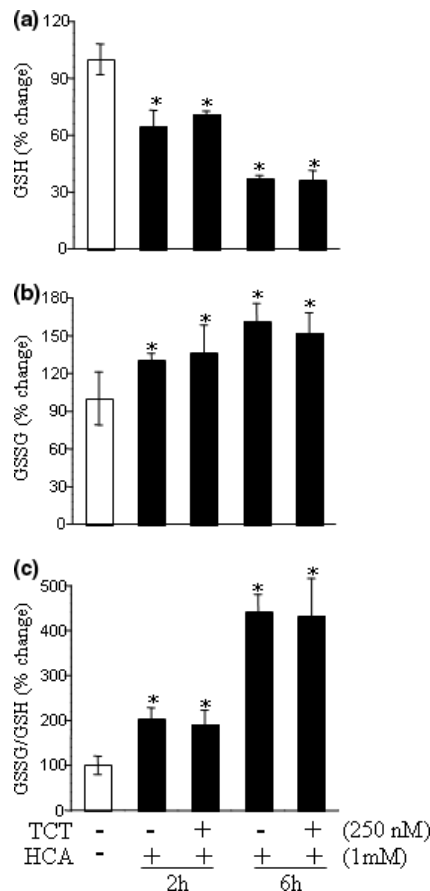


Fig. 6 HCA-induced cellular glutathione oxidation. (a) HT4 cell GSH levels, (b) cellular GSSG levels, and (c) cellular GSSG/GSH ratio as a marker of oxidative stress. Values are mean \pm SD. * p < 0.05 versus cells not treated with HCA (ANOVA).

responses were sensitive to micromolar, but not nanomolar, α -tocotrienol (Figs 12a and b).

Finally, we sought to verify whether the protective effects of nanomolar α -tocotrienol against HCA and that of micromolar α -tocotrienol against linoleic acid holds good in primary neurons. The study of immature primary cortical neurons resulted in findings that were highly consistent with the results from the HT4 neural cell line. Although nanomolar α -tocotrienol protected cells against HCA, it failed to protect neurons against linoleic acid. Neuronal cell death caused by linoleic acid was successfully attenuated by micromolar α -tocotrienol (Fig. 13).

Discussion

Hyperhomocysteinemia is a risk factor for vascular and neuronal lesions often observed with concomitant high levels of HCA. HCA, an oxidized metabolite of homocysteine, induces neurotoxicity (Hasegawa *et al.* 2005). HCA, an endogenous agonist for glutamatergic NMDA receptors, is predominantly localized in glial cells (Benz *et al.* 2004). In

contrast to homocysteine, HCA induces calcium influx into neurons, with characteristics of an excitotoxic glutamatergic agonist at raised concentrations (Sommer *et al.* 2004). HCA disrupts calcium homeostasis and induces degeneration of neurons *in vivo* (Adalbert *et al.* 2002). Recently it has been demonstrated that glutamate induces the release of HCA from astrocytes following the stimulation of ionotropic and metabotropic L-glutamate receptors (Benz *et al.* 2004). In this work, a previously established model of HCA-induced neurotoxicity was investigated (Khanna *et al.* 2003; Ryu *et al.* 2003). Our findings show that c-Src facilitates HCA-induced neurotoxicity. c-Src is heavily expressed in the brain (Soriano *et al.* 1991) and in human neural tissues (Pyper and Bolen 1989). Differentiating rodent neurons are known to express high levels of c-Src. Initially, c-Src was identified as being important in growth cone-mediated neurite extension and synaptic plasticity (Maness *et al.* 1988) and in neuronal differentiation (Ingraham *et al.* 1989). Targeted disruption of c-Src, however, did not cause any abnormality in the brain (Soriano *et al.* 1991). Our laboratory presented the first evidence demonstrating that c-Src plays a central role in executing glutamate-induced death of HT4 neural cells (Sen *et al.* 2000). Consistent with this, it was demonstrated in a subsequent report that Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. Thus, Src has emerged as a key intermediate and novel therapeutic target in the pathophysiology of cerebral ischemia (Paul *et al.* 2001). Recently it has been confirmed that the Src family kinase inhibitor PP2 reduces focal ischemic brain injury (Lennmyr *et al.* 2004). This study presents the first evidence for the involvement of c-Src in HCA-induced neurotoxicity.

Lipoxygenases, mainly 5-Lox, 12-Lox and 15-Lox, are named for their ability to insert molecular oxygen at the 5-, 12- or 15-carbon atom of arachidonic acid, forming a distinct hydroperoxy-eicosatetraenoic (HPETE) acid (Yamamoto 1992). 12-Lox produces 12(S)-HPETE which is further metabolized into four distinct products: an alcohol [12(S)-hydroxyeicosatetraenoic acid (HETE)], a ketone (12-keto-eicosatetraenoic acid, or two epoxy alcohols (hepoxilin A3 and B3). Immunohistochemical studies revealed the occurrence of 12-Lox in neurons, particularly in hippocampus, striatum and olivary nucleus, as well as in glial and cerebral endothelial cells (Nishiyama *et al.* 1992, 1993). Using immature cortical neurons and HT neural cells, it has been shown that a decrease in intracellular GSH triggers the activation of neuronal 12-Lox, which leads to the production of peroxides, the influx of Ca^{2+} and ultimately to cell death (Li *et al.* 1997). We have previously reported a central role of 12-Lox in glutamate-induced death of primary neuronal cultures by demonstrating that 12-Lox-deficient neurons are resistant to glutamate-induced toxicity (Khanna *et al.* 2003). Furthermore, we have demonstrated recently that 12-Lox-deficient mice are substantially protected against damage to

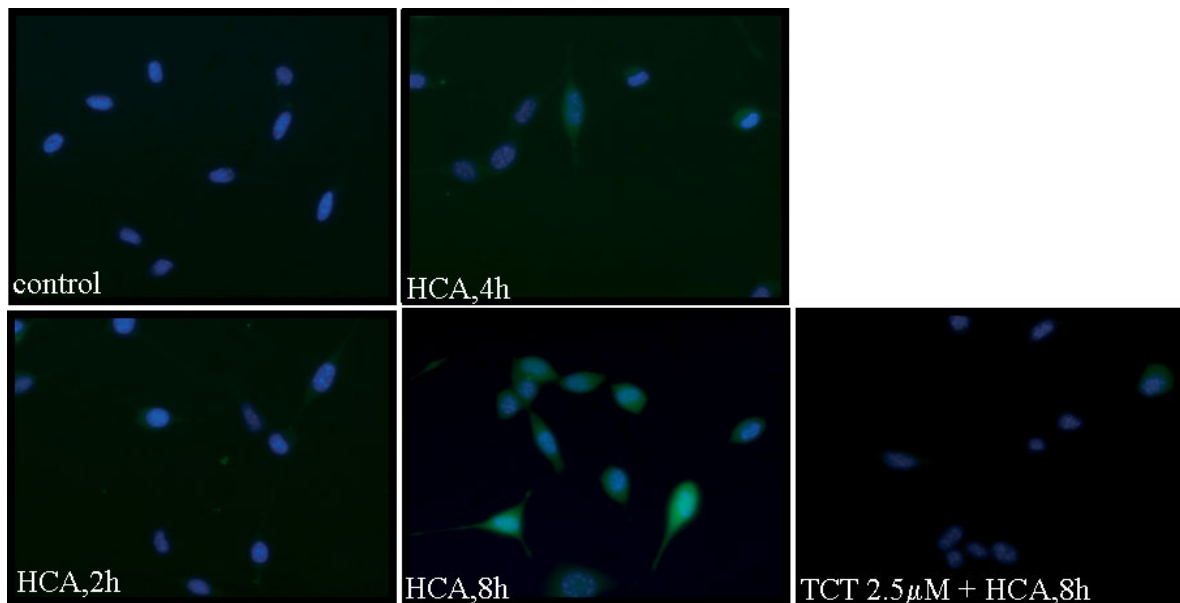


Fig. 7 HCA-induced increase in cellular ROS. ROS production in live cells was visualized using the Image-iT™ system from Molecular Probes. After 8 h, most of the cells were ROS positive (green). Pretreatment of cells with micromolar TCT (2.5 μ M) completely eliminated the green ROS signal. Nuclei are stained blue. For references to color please see the web version of this article.

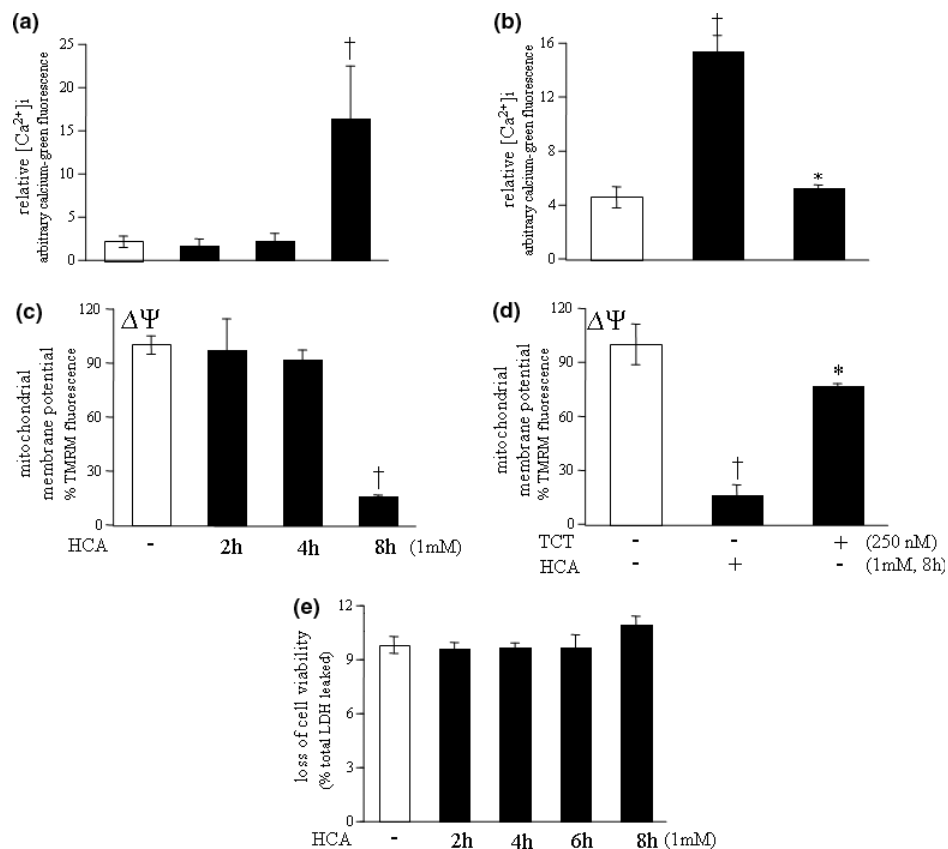


Fig. 8 HCA-induced changes in $[Ca^{2+}]_i$ and mitochondrial $\Delta\Psi$. Values are mean \pm SD. * $p < 0.05$ versus cells treated with HCA alone; † $p < 0.05$ versus control (open bar) (ANOVA). (e) There was no significant change in cell viability during the time frame studied.

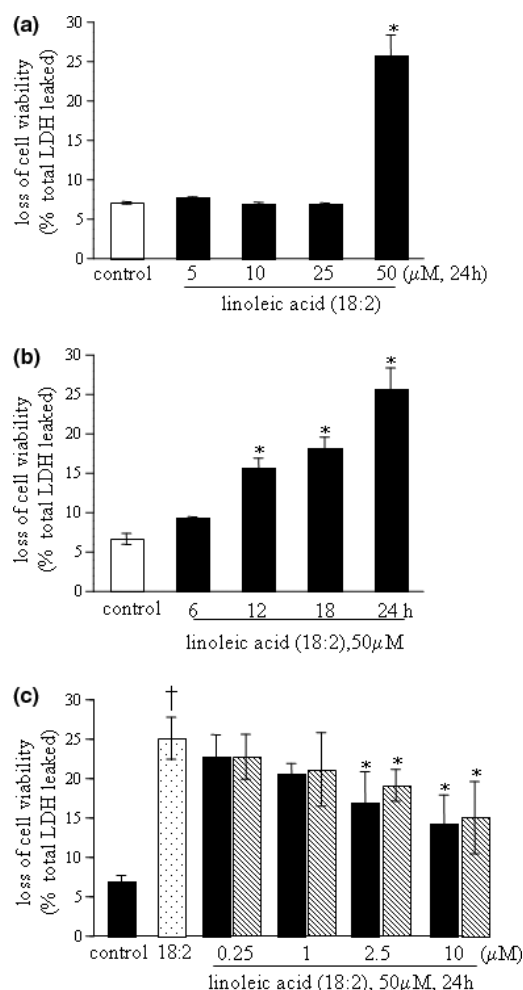


Fig. 9 Linoleic acid-induced neurotoxicity. (a) Effect of various doses of linoleic acid (18:2); * $p < 0.05$ versus control. (b) Time course of toxicity; * $p < 0.05$ versus control. (c) Micromolar, but not nanomolar, concentrations of vitamin E protected against linoleic acid-induced toxicity. Solid bar, α -tocotrienol; hatched bar, α -tocopherol. * $p < 0.05$ versus cells challenged with 18:2 alone; † $p < 0.05$ versus control. Values are mean \pm SD. Statistical analysis was by ANOVA.

the brain tissue caused by stroke *in vivo* (Khanna *et al.* 2005b). This work presents the first evidence depicting 12-Lox as a central player in HCA-induced neurotoxicity.

Involvement of c-Src and 12-Lox represents the hallmark of glutamate-induced neurotoxicity (Sen *et al.* 2000; Khanna *et al.* 2002, 2003, 2005b). Thus, involvement of c-Src and 12-Lox in HCA-induced neurotoxicity may be viewed as a parallel between the two death pathways. This observation is consistent with previous reports indicating that HCA and glutamate kill neurons by comparable mechanisms (Sagara *et al.* 2002; Baydas *et al.* 2005). We previously found that glutamate-induced neurotoxicity involves an early signaling component and a late oxidant-insult component. c-Src and 12-Lox represent central players in the early signaling

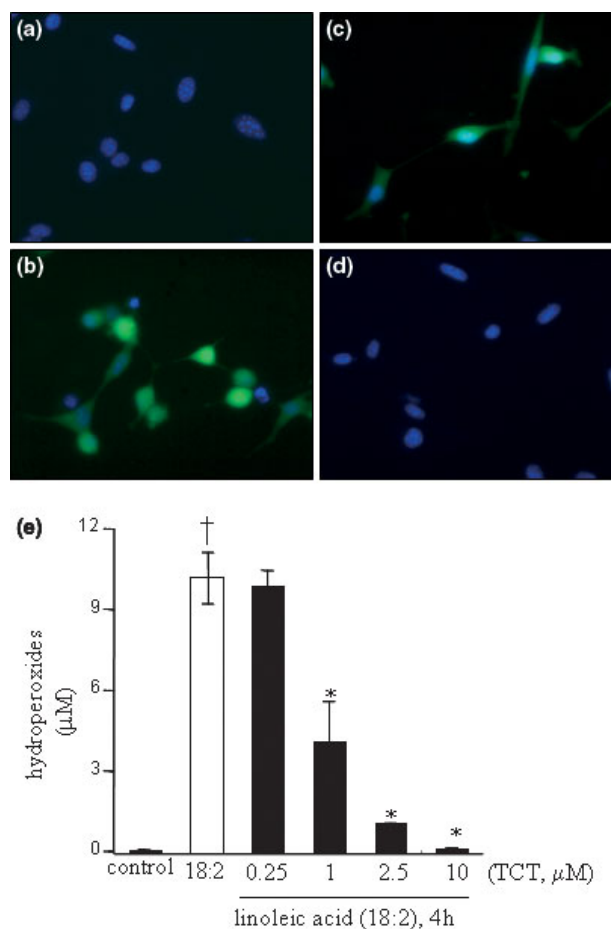


Fig. 10 Linoleic acid-induced oxidative stress. (a–d) ROS production in live cells was visualized using the Image-iT™ system from Molecular Probes. (a) Control (to b, passage and time matched); (b) linoleic acid (50 μ M, 4 h); (c) linoleic acid (50 μ M, 4 h) pretreated (5 min) with 250 nM α -tocotrienol; (d) linoleic acid (50 μ M, 4 h) pretreated with 2.5 μ M α -tocotrienol. (e) Lipid hydroperoxide levels assayed using the FOX1 method. HT4 cells were pretreated with the specified concentration of α -tocotrienol for 5 min before exposure to linoleic acid (50 μ M). After 4 h of linoleic acid exposure, cells were harvested to measure hydroperoxides. Values are mean \pm SD. * $p < 0.05$ versus group treated with 18:2 alone; † $p < 0.05$ versus control (not treated with 18:2) (ANOVA). For references to color please see the web version of this article.

component. Inducible activation of both of these signaling mediators is inhibited by nanomolar α -tocotrienol (Sen *et al.* 2000; Khanna *et al.* 2003). The results of this study show that, although nanomolar α -tocotrienol is capable of protecting neurons from HCA-induced neurotoxic signaling, it does not have potent antioxidant functions. This is supported by the current observation that nanomolar α -tocotrienol protects only when treatment is given early on. Micromolar α -tocotrienol was required to safeguard the neural cells when the treatment was performed 8 h after glutamate treatment, a time point when oxidant levels in the cell are high.

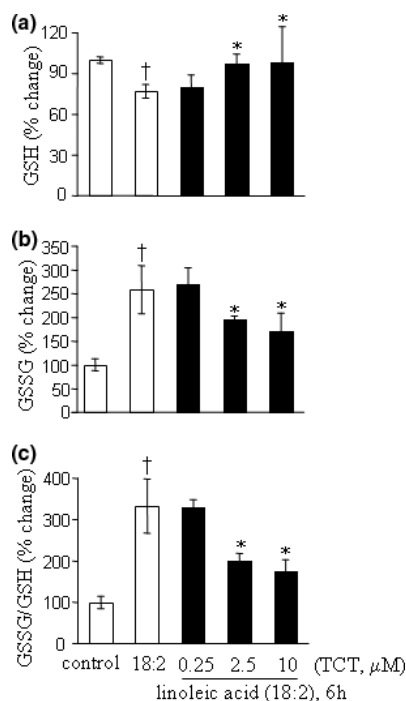


Fig. 11 Linoleic acid-induced cellular glutathione oxidation and loss. (a) Cellular GSH levels, (b) cellular GSSG levels, and (c) cellular GSSG/GSH ratio as a marker of oxidative stress. Values are mean \pm SD. * p < 0.05 versus cells treated with linoleic acid alone; † p < 0.05 versus control (not treated with 18 : 2) (ANOVA).

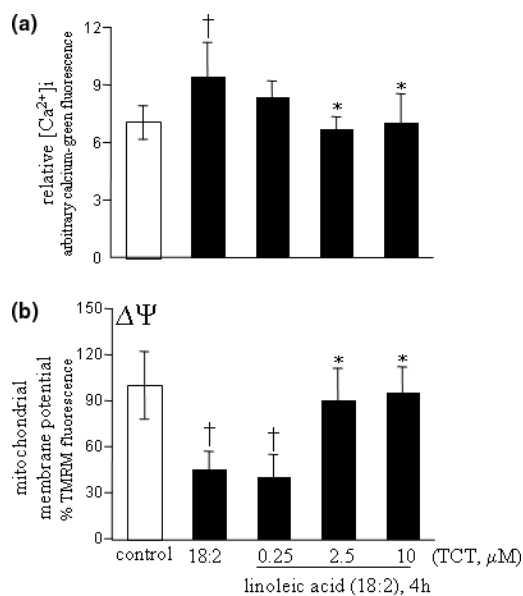


Fig. 12 Linoleic acid-induced changes in $[Ca^{2+}]_i$ and mitochondrial $\Delta\psi$. Values are mean \pm SD. * p < 0.05 versus cells treated with 18 : 2 alone; † p < 0.05 versus control (open bar) (ANOVA).

To further test the hypothesis that nanomolar α -tocotrienol functions via an antioxidant-independent mechanism whereas micromolar α -tocotrienol serves as an antioxidant,

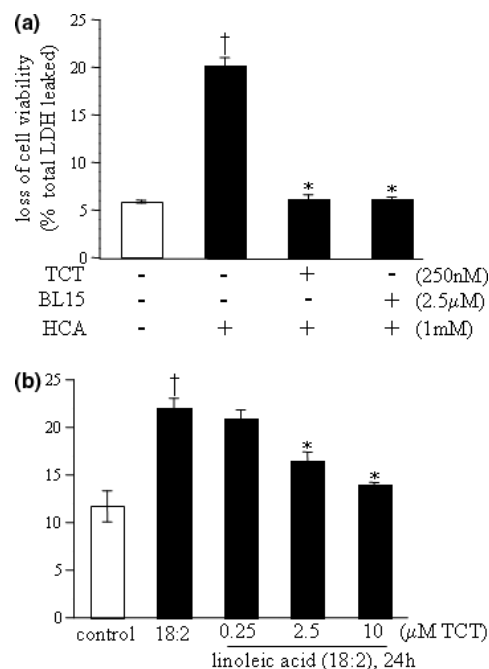


Fig. 13 Protective effects of α -tocotrienol in primary cortical neurons. (a) Neurons were either treated or not with α -tocotrienol or BL15 for 5 min and challenged with HCA (1 mM) for 24 h. (b) Neurons were treated or not with different doses of TCT as indicated for 5 min and then challenged with linoleic acid (25 μ M) for 24 h. Values are mean \pm SD. * p < 0.05 versus corresponding group treated with HCA or linoleic acid alone; † p < 0.05 versus corresponding control (open bar) (ANOVA).

we investigated linoleic acid-induced neurotoxicity. Unsaturated fatty acids rapidly accumulate during ischemia and participate in inducing irreversible brain injury, especially because they are highly susceptible to peroxidation when the tissue is reoxygenated (Zaleska and Wilson 1989). Linoleic acid represents the main polyunsaturated fatty acid in mammals (Spiteller 2001). Linoleic acid induces oxidative stress (Reiterer *et al.* 2004) and a diet rich in linoleic acid has been shown to increase oxidative stress *in vivo* in humans (Turpeinen *et al.* 1999). Oxidized derivatives of linoleic acid induce oxidative stress (Goodfriend *et al.* 2004; Lee *et al.* 2005; Terrasa *et al.* 2005) and kill PC12 cells (Sasaki *et al.* 2003). Consistent with these findings, in the present study it was found that exposure to linoleic acid resulted in oxidant insult and subsequent neurotoxicity. In this experimental system micromolar, but not nanomolar, α -tocotrienol protected the neurons. These findings indicate that micromolar, but not nanomolar, α -tocotrienol functions as an antioxidant to protect neurons. Given that the peak plasma concentration of α -tocotrienol is in the range of 3 μ M after oral supplementation in humans (Khosla *et al.* 2006), α -tocotrienol may be expected to exert its neuroprotective effects *in vivo* by both antioxidant-independent as well as antioxidant-dependent

mechanisms. Indeed, oral supplementation of α -tocotrienol has been observed to protect against stroke in spontaneously hypertensive rats *in vivo* (Khanna *et al.* 2005b).

Taken together, although both HCA and linoleic acid caused neurotoxicity with comparable features such as increased GSSG/GSH, raised $[Ca^{2+}]_i$ and compromised mitochondrial $\Delta\psi$, some fundamental differences in the neurodegenerative pathway were noted. Mechanisms underlying HCA-induced neurodegeneration were comparable to those in the path implicated in glutamate-induced neurotoxicity. Inducible activation of c-Src and 12-Lox represented early events in that neurodegenerative pathway. The implication of these data for excitotoxicity is not yet clear. Nanomolar α -tocotrienol inhibited both HCA-induced responses, conferring neuroprotection to the HCA-challenged neurons. Oxidative stress ensued as a late event in HCA-induced neurotoxicity. Micromolar, not nanomolar, α -tocotrienol protected the neural cells by virtue of its antioxidant function. This property was shared by α -tocotrienol and α -tocopherol. Importantly, although pretreatment with nanomolar α -tocotrienol completely protected against HCA-induced cell death, loss and oxidation of cellular GSH was not prevented. Thus, nanomolar α -tocotrienol did not function as an antioxidant. In keeping with this, nanomolar α -tocopherol, which is known to have antioxidant properties comparable to those of α -tocotrienol, did not protect. The observation that micromolar, but not nanomolar, α -tocotrienol functions as an antioxidant was verified in the experimental model involving linoleic acid-induced oxidative stress and cell death. Oral supplementation of α -tocotrienol to humans results in a peak plasma concentration of 3 μ M. Thus, it is prudent to conclude that orally supplemented α -tocotrienol may protect neurons from toxic insults by antioxidant-independent as well as -dependent mechanisms.

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