



Published in final edited form as:

Stroke. 2011 August ; 42(8): 2308–2314. doi:10.1161/STROKEAHA.110.608547.

Natural Vitamin E α -Tocotrienol Protects Against Ischemic Stroke by Induction of Multidrug Resistance-Associated Protein 1

Han-A Park, PhD, Natalia Kubicki, BS, Surya Gnyawali, PhD, Yuk Cheung Chan, PhD, Sashwati Roy, PhD, Savita Khanna, PhD, and Chandan K. Sen, PhD

Departments of Surgery, Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, Ohio 43210

Abstract

Background and Purpose— α -Tocotrienol (TCT) represents the most potent neuroprotective form of natural vitamin E that is Generally Recognized As Safe (GRAS) certified by US FDA. This work addresses a novel molecular mechanism by which α -TCT may be protective against stroke *in vivo*. Elevation of intracellular GSSG triggers neural cell death. Multidrug resistance-associated protein 1 (MRP1), a key mediator of GSSG efflux from neural cells, may therefore possess neuroprotective functions.

Methods—Stroke-dependent brain tissue damage was studied in MRP1 deficient mice and α -TCT supplemented mice.

Results—Elevated MRP1 expression was observed in glutamate challenged primary cortical neuronal cells and stroke-affected brain tissue. MRP1 deficient mice resulted in larger stroke-induced lesion recognizing a protective role of MRP1. *In vitro*, protection against glutamate-induced neurotoxicity by α -TCT was attenuated under conditions of MRP1 knockdown suggesting a role of MRP1 in α -TCT-dependent neuroprotection. *In vivo* studies demonstrated that oral supplementation of α -TCT protected against murine stroke. MRP1 expression was elevated in the stroke affected cortical tissue of α -TCT-supplemented mice. Efforts to elucidate the underlying mechanism identified MRP1 as a target of miR-199a-5p. In α -TCT supplemented mice, miR-199a-5p was downregulated in stroke affected brain tissue.

Conclusions—This work recognizes MRP1 as a protective factor against stroke. Furthermore, findings of this study adds a new dimension to the current understanding of the molecular bases of α -TCT neuroprotection by identifying MRP1 as a α -TCT-sensitive target and by unveiling the general prospect that oral α -TCT may regulate microRNA expression in stroke-affected brain tissue.

Keywords

Antioxidant; vitamin E; microRNA; glutathione

Address correspondence to: Prof. Chandan K Sen, 512 Davis Heart & Lung Research Institute, 473 West 12th Avenue, The Ohio State University Medical Center, Columbus, Ohio 43210, Tel. 614 247 7658, Fax. 614 247 7818, Chandan.sen@osumc.edu.

Disclosures
None.

Introduction

Vitamin E is a generic term for tocopherols (TCPs) and tocotrienols (TCTs). Although TCPs have been widely studied for decades, the significance of α -TCT as the most potent neuroprotective forms of natural vitamin E has been uncovered recently. We have reported that nanomolar concentrations of α -TCT, not α -TCP, prevent stroke-associated neurodegeneration¹⁻². α -TCT protects neural cells by two major mechanisms including inhibition of inducible c-Src and 12-lipoxygenase pathways^{1,3}. Recently, we have reported a key role of intracellular GSSG in causing neural cell death⁴. Under normal physiological conditions, in most tissues GSSG represents 1% of the total glutathione in the cell. However, under conditions of oxidative stress, reduced glutathione (GSH) is rapidly oxidized to GSSG. Once intracellular GSSG is formed, it may be recycled to GSH in the presence of reducing equivalents which are known to be depleted in the face of oxidant insult. Excessive cellular GSSG is toxic and therefore pumped out at the expense of ATP. We have demonstrated that inefficient clearance of intracellular GSSG may trigger neural cell death. These observations highlight the significance of cellular GSSG clearing systems in neuroprotection especially in the context of stroke where oxidative stress is overt⁴.

Multidrug resistance-associated protein 1 (MRP1) plays a key role in clearing intracellular GSSG⁵. MRP1 is an integral membrane glycoprophosphoprotein abundantly expressed in the brain. We sought to test the hypothesis that under conditions of oxidant insult, improved clearance of intracellular GSSG by augmented MRP1 function results in neuroprotective outcomes. Furthermore, this study tested orally supplemented natural vitamin E α -TCT for its ability to enhance MRP1 function in the context of stroke-induced brain injury.

Materials and Methods

For specific details, see online supplement.

Primary cortical neurons

Cells were isolated from the cerebral cortex of rat feti (Sprague-Dawley, day 17 of gestation; Harlan, Indianapolis, IN) (see online supplement).

siRNA delivery and analysis of genes

DharmaFECTTM1 transfection reagent was used to transfect cells with 100nmol/L siRNA pool (Dharmacon RNA Technologies, Lafayette, CO) (see online supplement).

Delivery of microRNA(miR) mimic and inhibitor

DharmaFECTTM 1 transfection reagent was used to transfect cells with miRIDIAN rno-miR-199a-5p mimic or inhibitor (Dharmacon RNA Technologies) (see online supplement).

pGL3-MRP1-3'fUTR luciferase reporter assay

(see online supplement)

Quantification of miR expression

miR fraction was isolated using miRVanaTM miRNA isolation kit (Ambion, Austin, TX). miR-199a-5p levels were quantified using Taqman Universal Master Mix (Applied Biosystems, Forster City, CA) (see online supplement).

Western blot

Samples (40–50 μ g of protein / lane) were separated on a 4–12% SDS-polyacrylamide and probed with anti-MRP1 (1:20 dilution, Enzo Life Sciences, Plymouth Meeting, PA) (see online supplement).

Cell viability assay

(see online supplement)

Immunocytochemistry

(see online supplement)

Calcein clearance assay

After MRP1 siRNA transfection, primary cortical neurons were treated with glutamate. Calcein-AM (5 μ mol/L, Invitrogen, Carlsbad, CA) was loaded to the cells for 30min and loss of cellular fluorescence was analyzed (see online supplement).

Mouse stroke model

Transient focal cerebral ischemia was induced in 8 week old MRP1 deficient (n=20, male, Taconic, Hudson, NY) or background FVB mice (n=15, male, Taconic) by middle cerebral artery occlusion (MCAO) (see online supplement).

MRI

T2-weighted imaging was performed on stroke-affected mice. Imaging experiments were carried out using a 11.7T MR system comprised of a vertical bore magnet (Bruker Biospin, Ettlingen, Germany) (see online supplement).

 α -Tocotrienol supplementation

C57BL/6 (5-week old, male, Harlan, Indianapolis) mice were randomly divided into two groups, control (n=18) and supplemented (n=23). The control group was orally gavaged with vitamin E stripped corn oil with volume matching the mean volume of α -TCT supplement group. The test group was orally gavaged with α -TCT (Carotech, Malaysia) in vitamin E-stripped corn oil at a dosage of 50 mg/kg body weight for 13 weeks as reported previously¹. MCAO was performed at 20 to 24h after the last supplementation. After 48h of MCAO, T2-weighted images were recorded. Mice suffering from surgical complications (*e.g.* hemorrhage or death) during MCAO were excluded. Immediately after imaging, tissues from control and stroke-affected hemispheres of control (n=9) and α -TCT supplemented (n=10) mice were harvested. Mice were maintained under standard conditions at $22\pm 2^\circ\text{C}$ with 12:12 dark: light cycles. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Ohio State University.

HPLC-electrochemical detection

(see online supplement)

mRNA expression assay from laser-captured microdissected somatosensory cortex of brain sample

Laser microdissection and pressure catapulting (LMPC) was performed on coronal slices of mouse brains using the microlaser system from PALM Microlaser Technologies AG. (see online supplement).

Histology

Coronal slices of cortical sections were stained with rabbit polyclonal antibody to MRP1 (1:200, Abbiotec, San Diego, CA), 0.0001% Fluoro-JadeR C (Millipore, Billerica, MA) or 4-hydroxynonenal (4-HNE, 1:1000, Enzo Life Sciences, Plymouth Meeting, PA) (see online supplement)

Statistics

Data are reported as mean \pm SD. Differences between means were tested using Student's *t*-test or one-way ANOVA with Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Neuroprotection of α -tocotrienol was compromised in MRP1 knockdown in primary cortical neurons

In neural cells, glutamate toxicity is associated with oxidant insult resulting in rapid elevation of intracellular GSSG. We were therefore led to test the hypothesis that under such conditions of GSSG loading, GSSG efflux mechanisms such as MRP1 would be augmented as a defense response in favor of neuronal survival. We observed that extracellular glutamate challenge may induce MRP1 expression in both primary cortical neurons (Figure 1A) as well as HT4 neural cells (Figure S1A).

Previously we have reported that α -TCT is potentially neuroprotective against a number of insults including GSSG-induced cell death^{3-4,6}. To test whether such neuroprotective property of α -TCT depends on MRP1 in neural cells, cellular GSSG clearance was studied utilizing a MRP1 knockdown approach (Figure 1B & S1 B-D). Higher MRP1 activity resulted in enhanced clearance of calcein from preloaded neurons. Glutamate challenge upregulated MRP1 function. Such improved clearance of calcein in glutamate challenged cells was blunted by MRP1 knockdown demonstrating specificity of the assay for MRP1 (Figure 1C). The ability of α -TCT to protect against glutamate challenge was compromised following MRP1 knockdown in primary cortical neurons (Figure 1D).

MCAO-induced brain injury was exacerbated in MRP1 deficient mice

Transient focal cerebral ischemia was induced by MCAO in MRP1 deficient mice or corresponding background FVB mice. Both FVB and MRP1 deficient mice showed comparably decreased levels of MCA-area blood flow during occlusion (not shown). Interestingly, MRP1 deficient mice showed a larger hemispherical infarct volume than that noted in FVB mice (Figure 2A). Increased abundance of MRP1 protein was observed in the infarct hemisphere of FVB mice (Figure 2B). MCAO-induced brain injury increased tissue GSSG levels in both MRP1 deficient as well as in FVB mice. Notably, MRP1 deficient mice contained 1.6-fold higher tissue levels of GSSG in the infarct hemisphere compared to that detected in background mice (Figure 2C). These data indicate that loss of MRP1 function impairs GSSG clearance during stroke resulting in increased brain injury. Histochemical studies depicted that increased MRP1 positive cells were selectively localized in the infarct hemisphere of FVB mice (Figure 3A). Both MRP1 deficient as well as FVB mice presented positive Fluoro-Jade immunofluorescence staining in the stroke-affected cortex supporting the incidence of neurodegeneration. Importantly, the infarct hemisphere of MRP1 deficient mice exhibited a higher level of neurodegenerative outcome (Figure 3B).

miR-199a-5p silences MRP1

To test whether MRP1 expression is subject to post-transcriptional gene silencing by microRNA (miR), we first performed *in silico* studies using databases such as Target Scan

(www.targetscan.org), Miranda (www.microrna.org) and PicTar (www.pictar.org). miR-199a-5p emerged as a major candidate miR which is likely to target MRP1 exhibiting a single conserved binding site to the 3' untranslated region (3'UTR) of MRP1 transcript. Next, we turned towards biological validation of MRP1 as a target of miR-199a-5p. In cultured cells, miR-199a-5p levels were modulated by delivery of miR-199a-5p mimic or miR-199a-5p inhibitor (Figure 4A-B & Figure S2A). miR-199a-5p silenced MRP1 in both primary (Figure 4C-D) as well as HT4 cells (Figure S2B-C). To determine whether MRP1 is a direct target of miR-199a-5p in HT4 neural cells, a fragment of the 3'UTR of MRP1 mRNA containing the putative miR-199a-5p binding sequence cloned into a firefly luciferase reporter construct was used. This construct was co-transfected with a control renilla luciferase reporter construct into cells along with miR-199a-5p mimic or inhibitor. miR-199a-5p mimic significantly decreased luciferase activity while miR-199a-5p inhibitor significantly increased the activity of the miR-199a-5p firefly luciferase reporter recognizing MRP1 as a direct target of miR-199a-5p (Figure S2D).

Oral α -tocotrienol protected against stroke via MRP1 upregulation

To test the neuroprotective effects of α -TCT against stroke *in vivo*, randomly divided mice were gavaged with either vitamin E stripped corn oil or α -TCT (50mg/ kg body weight) for 13 weeks. MCAO was performed one day after the last supplementation. MCAO affected brains were harvested 48h after reperfusion (Figure S3A). α -TCT supplementation significantly increased brain α -TCT level (Figure S3B) without changing α -TCP levels (Figure S3C). MCAO limited blood flow in α -TCT supplemented as well as control groups comparably (Figure S3D). However, MCAO-induced hemispherical infarct volume was significantly attenuated in α -TCT supplemented group (Figure 5A).

To test whether the neuroprotective properties of α -TCT against stroke *in vivo* was dependent on the ability of α -TCT to upregulate MRP1, cortical tissue elements were microdissected from infarct as well as contralateral non-infarct sites using a LMPC system (Figure S4). Notably, α -TCT supplementation induced MRP1 but lowered miR199a-5p level in the infarct hemisphere (Figure 5B-D). Immunohistochemical studies demonstrated higher abundance of MRP1 positive cells at the infarct hemisphere of α -TCT-supplemented mice (Figure 6A). α -TCT supplementation attenuated stroke-induced neurodegeneration (Figure 6B). Stroke induced lipid peroxidation as evident by elevated levels of 4-HNE-positive cells in the infarct tissue. Consistent with other observations indicating a beneficial influence of α -TCT supplementation, 4-HNE-positive cells were fewer in the infarct hemisphere of α -TCT supplemented mice (Figure 6C).

Discussion

Following a number of failed clinical trials testing the conventional form of vitamin E, α -TCP⁷⁻⁸, interest in naturally occurring forms of vitamin E such as γ -TCP as well as the TCT family is sharply rising. Meta-analyses of clinical trials testing the efficacy of vitamin E in human health suffer from a blind spot because they fail to recognize that α -TCP, the only form of vitamin E tested in such trials, represent only a fraction of the natural vitamin E family⁷⁻⁸. Because TCPs and TCTs have unique functional properties it is important to limit title claims to the specific form of vitamin E studied. Neuroprotection by α -TCT at nanomolar concentration represents the most potent functional property of the entire vitamin E family⁶. Mechanisms explaining such property include inhibition of inducible c-Src as well as 12-lipoxygenase activity in stroke-related neurodegenerative settings^{1,9}.

Stroke associated ischemic insult is known to compromise ATP production as well as depletes the reducing equivalent pool of the brain. This, in turn, compromises the function of ATP-dependent ion pumps as well as the ability of the brain to manage oxidant insult. As a

result, on one hand K^+ efflux and Ca^{2+} influx are increased in response to membrane depolarization. On the other hand, excessive GSSG builds up within the cell. Both elevated intracellular Ca^{2+} as well as GSSG are directly implicated in cell death signaling¹⁰⁻¹¹. Our previous work highlights the critical significance of elevated and trapped cellular GSSG in executing neural cell death⁴.

MRP1 represents a major GSSG clearing system in neural cells¹². The current work is the first to recognize MRP1 as a potential therapeutic target for stroke. During stroke-related oxidative insult, GSSG is rapidly formed but its redox cycling to GSH is severely arrested. GSSG reductase activity is impaired following stroke¹³. In addition, ischemia-reperfusion depletes NADPH impairing all reductase functions dependent on this reducing equivalent¹⁴. Therefore, a sharp rise in brain tissue GSSG/GSH ratio occurs following stroke⁴. In the current work we note that such elevation of intracellular GSSG is associated with the induction of MRP1 perhaps as an adaptive survival response. This contention is consistent with previous reports demonstrating upregulation of MRP1 in response to oxidant insult as an adaptive response defending cell survival¹². Mechanisms of multidrug resistance and clinical outcomes in response to manipulation of MRP1 expression have been extensively studied in the context of various types of cancers. However, the significance of MRP1 in brain-related pathologies other than cancer is poorly developed. Currently, the limited information available consistently demonstrates that under pathological conditions known to be associated with oxidant insult, *i.e.* Alzheimer's and epilepsy, MRP1 expression is elevated in the brain¹⁵⁻¹⁶. Our observation that stroke related injury to the brain is exacerbated in MRP1 deficient mice establishes a key significance of MRP1 in determining stroke outcomes.

The current study identified MRP1 as a biologically validated target of miR-199a-5p. This constitutes first evidence that MRP1 is subject to post-transcriptional gene silencing by miRs. This finding provided a great segue to address the significance of miRs in stroke¹⁷. Antagomir miR-497 treatment has been recognized for its potential to attenuate injury related to acute ischemic stroke¹⁸. The miR-200 family and miR-182 has been found to be upregulated in ischemic pre-conditioning¹⁹. Although the significance of miR-199a-5p in stroke has not been studied, miR-199a-5p is recognized as a hypoxia-sensitive miR²⁰. Thus, the hypoxia component of stroke-related ischemia may be responsible for downregulating miR-199a-5p following stroke²¹.

Conclusions

Findings of this study adds a new dimension to the current understanding of the molecular bases of α -TCT neuroprotection by identifying MRP1 as a α -TCT-sensitive target and by unveiling the general prospect that oral α -TCT may regulate microRNA expression in stroke-affected brain tissue. Neuroprotective as well as hypocholesterolemic properties of α -TCT make it a good candidate for nutrition-based intervention in people at high risk for stroke. Transient ischemic attack (TIA), or mini-stroke, serves as a sentinel warning sign for high-risk stroke patients²². Prophylactic stroke therapy therefore provides an opportunity for intervention in TIA patients prior to a major stroke event. Outcomes of the current study warrant clinical assessment of α -TCT in TIA patients. Furthermore, α -TCT is a nutrient that is certified by the US FDA to be Generally Recognized As Safe (GRAS; GRN307) and not a drug with potential side effects. Thus, α -TCT may be considered as a preventive nutritional countermeasure for people at high risk for stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This study was supported by NIH grant NS42617.

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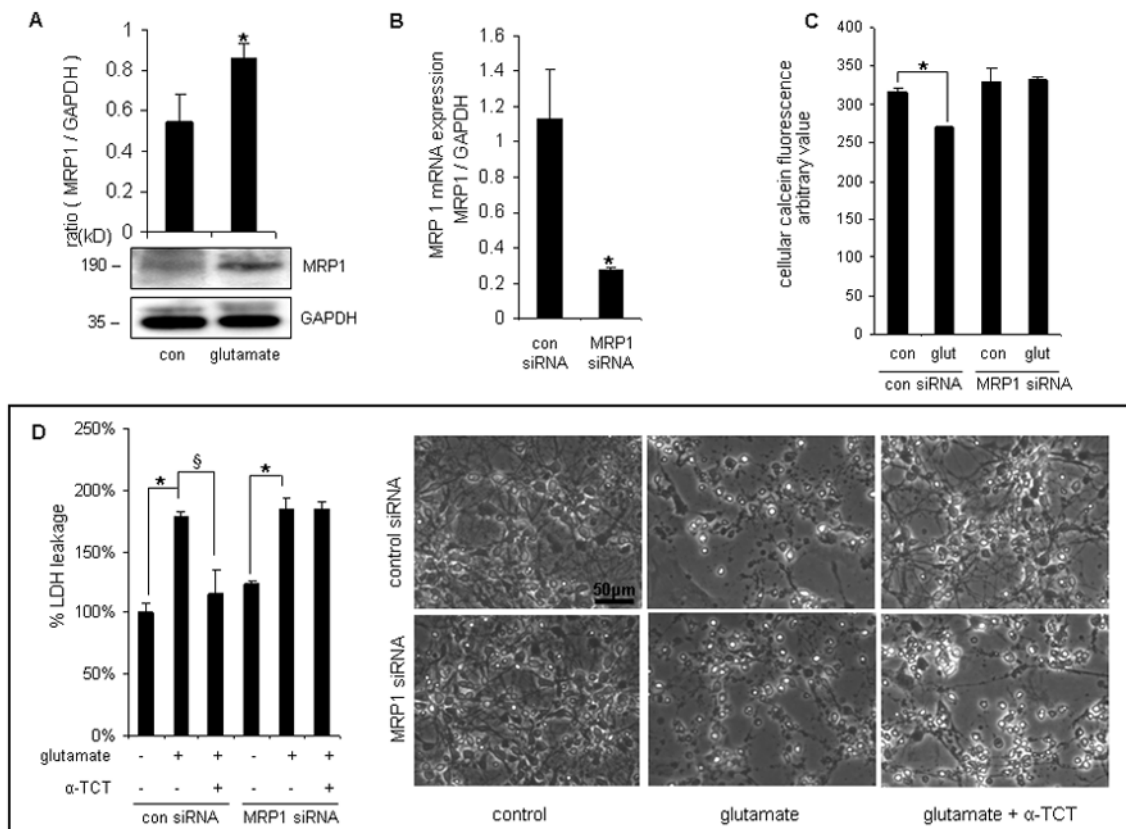


Figure 1. MRP1 knockdown attenuated the neuroprotection of α -tocotrienol in primary cortical neurons

After 24h of seeding, primary cortical neurons were treated with glutamate (5mmol/L, 24h). Glutamate challenge induced the expression of MRP1 protein (A). After MRP1 siRNA transfection, MRP1 mRNA expression was significantly down-regulated (B). C, Glutamate-challenged primary neurons with MRP1 knockdown exhibited loss of functional MRP1 by retaining more calcein compared to corresponding control cells. D, α -TCT (1 μ mol/L) was added into cell culture medium 6h before glutamate treatment. After glutamate (5mmol/L, 24h) challenge, LDH leakage was measured. Neuroprotection of α -TCT was compromised under conditions of MRP1 knockdown. Bar=50 μ m, n=3, * P <0.05 compared with control; § P < 0.05 compared with control siRNA-transfected, glutamate-treated primary neurons.

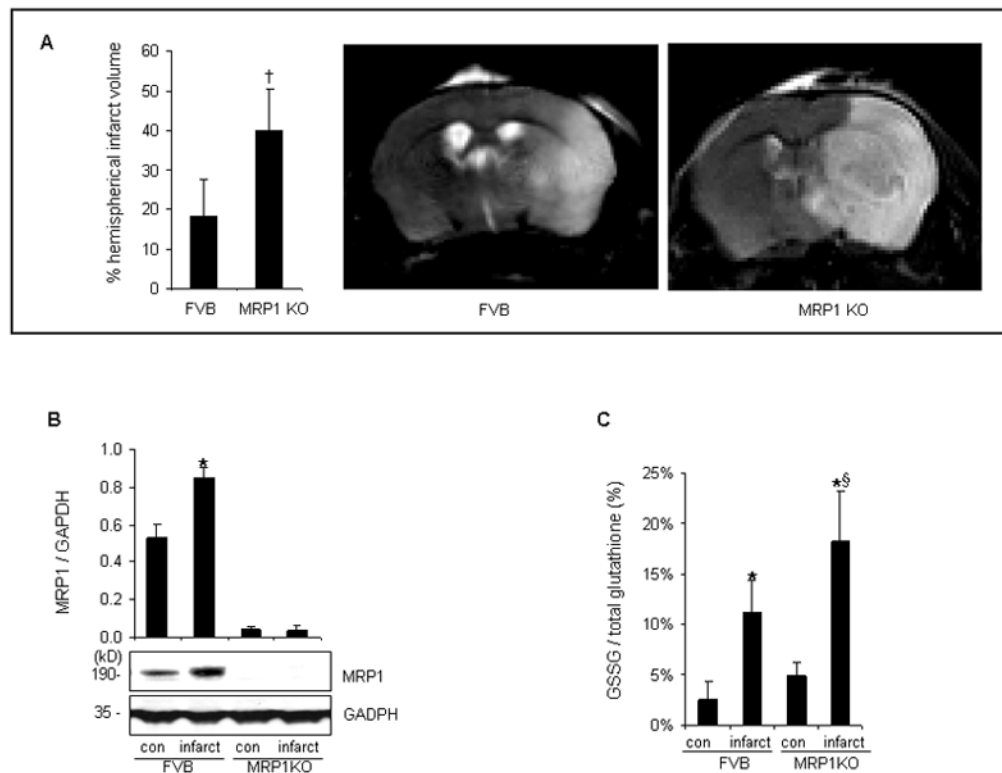


Figure 2. MCAO-induced brain injury was exacerbated in MRP1 deficient mice

Transient focal cerebral ischemia was induced in MRP1 deficient (n=20) or FVB mice (n=15) by MCAO. **A**, MRP1 KO mice presented larger stroke-induced lesion compared to FVB mice after 48h reperfusion (FVB, n=8; MRP1 KO, n=12). **B**, Protein expression of MRP1 was increased in infarct hemisphere of FVB mice (n=3). **C**, MRP1 deficient mice contained significantly higher level of GSSG in stroke-affected hemisphere compared to that of FVB mice (n=4). * $P < 0.05$ compared with corresponding contralateral hemisphere; [†] $P < 0.05$ compared with FVB; [§] $P < 0.05$ compared with infarct hemisphere of FVB.

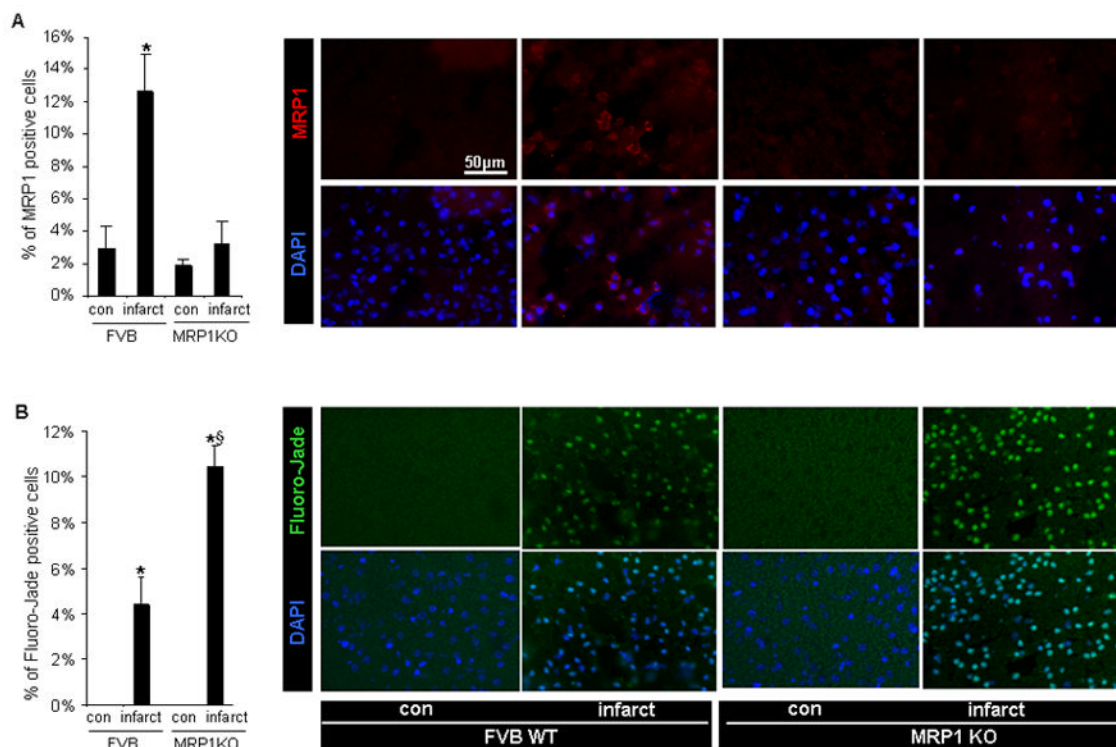


Figure 3. MCAO-induced neurodegeneration was accentuated in MRP1 deficient mice
A, MRP1 expression was upregulated at the stroke affected cortex of FVB mice (red-MRP1 protein; blue-DAPI stained nuclei) **B**, The infarct hemisphere of MRP1 deficient mice presented abundant Fluoro-Jade signals in cortical region (green-Fluoro-Jade positive neurons; blue-DAPI stained nuclei). Bar=50 μ m, n=3, * P <0.05 compared with corresponding contralateral hemisphere; § P <0.05 compared with infarct hemisphere of FVB.

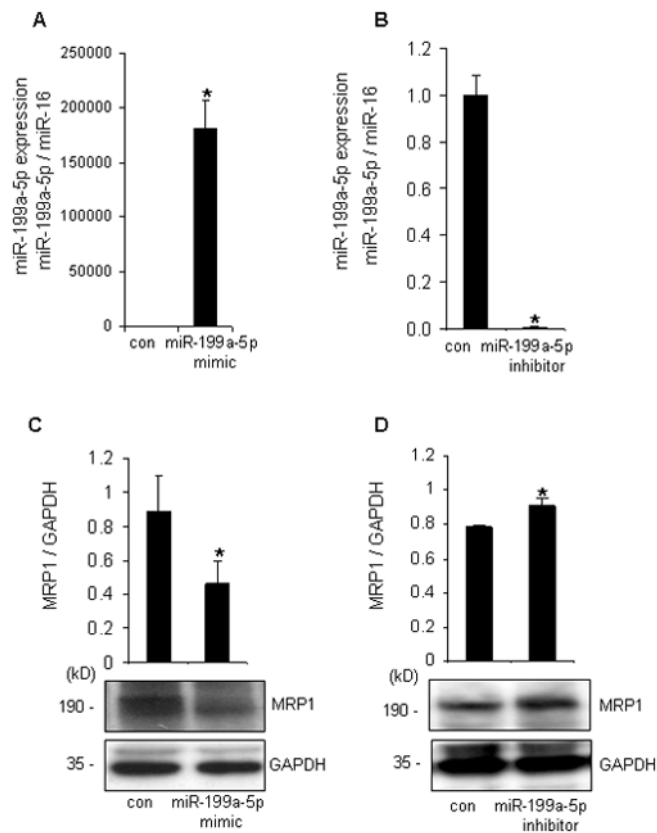


Figure 4. miR-199a-5p silences MRP1 in primary cortical neurons

After 72h of miR-199a-5p mimic delivery, miR-199a-5p expression was significantly upregulated (A) while miR-199a-5p hairpin inhibitor significantly downregulated miR-199a-5p (B). miR-199a-5p mimic/inhibitor negatively regulated protein (C-D) of MRP1 in primary cortical neurons. n=3, * P <0.05 compared with control.

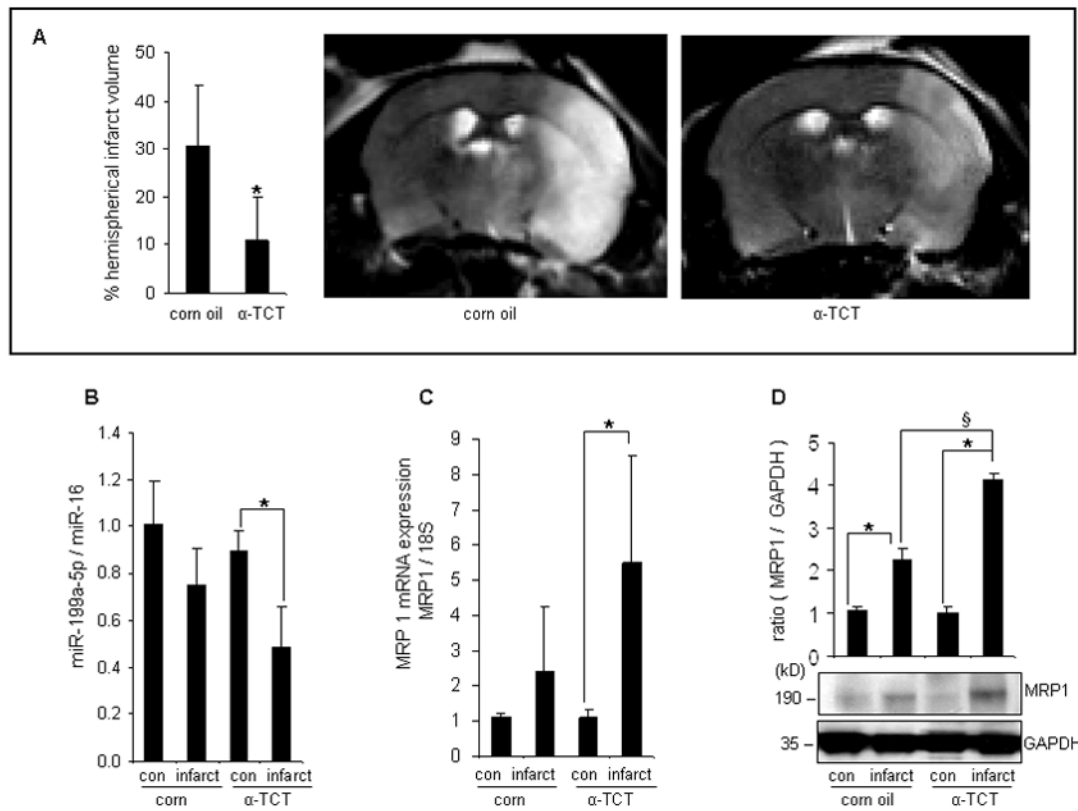


Figure 5. Orally supplemented α -tocotrienol protected against stroke via MRP1 upregulation
 C57BL/6 mice were orally gavaged with vitamin E-stripped corn oil (n=18) or 50mg α -TCT per kg body weight (n=23) for 13 weeks then MCAO was performed. **A**, α -TCT supplementation significantly reduced MCAO-induced brain injury (corn oil supplemented mice, n=9; α -TCT supplemented mice, n=10). miR-199a-5p expression was significantly down-regulated (**B**) while MRP1 mRNA (**C**) and protein (**D**) levels were significantly elevated in the stroke affected tissue of α -TCT fed mice (n=3). * P <0.05 compared with corresponding control; § P <0.05 compared with infarct hemisphere of corn oil fed mice.

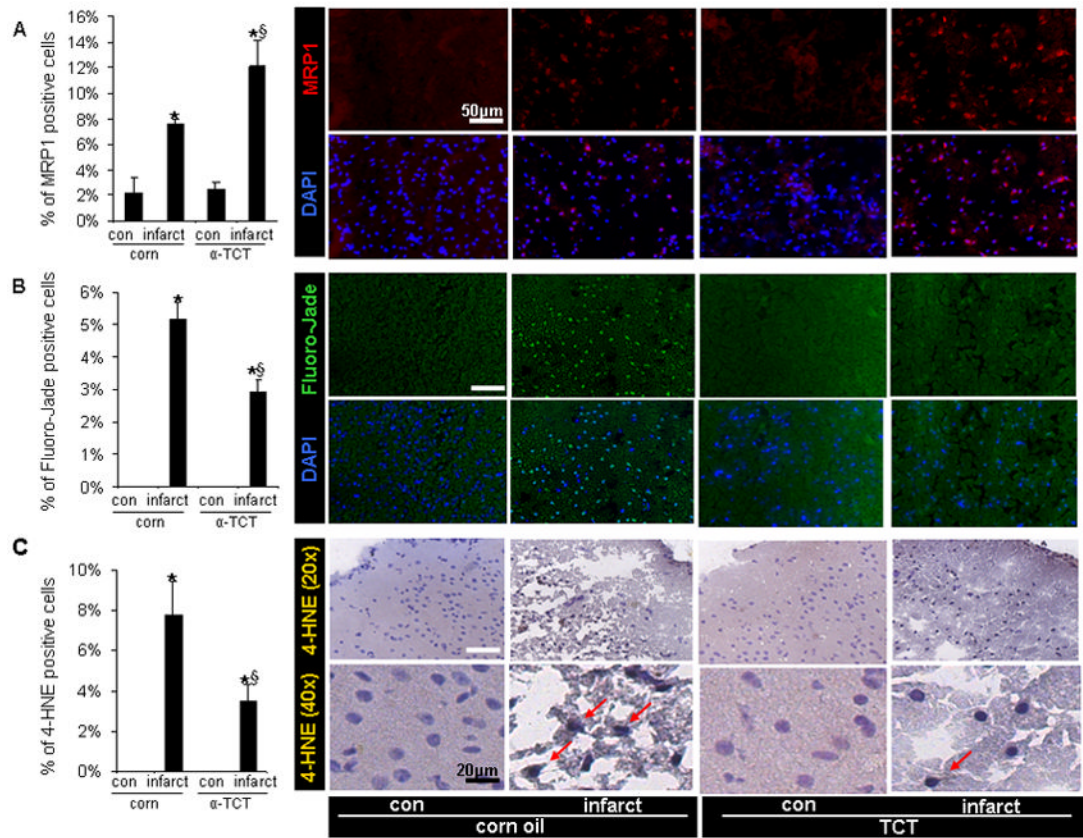


Figure 6. Orally supplemented α -tocotrienol attenuated MCAO-induced neurodegeneration and lipid peroxidation

A, The abundance of MRP1 positive cells were significantly upregulated in the stroke affected cortex of α -TCT supplemented mice (red-MRP1 protein; blue-DAPI stained nuclei). **B**, Fluoro-Jade positive neurons were fewer in the infarct hemisphere of α -TCT fed group compared to those at the infarct site of corn oil fed group (green-Fluoro-Jade; blue-DAPI stained nuclei). **C**, The abundance of 4-HNE-positive cells was lower in infarct hemisphere of α -TCT supplemented mice. Bar(white)=50 μ m, Bar(black)=20 μ m. n=3, * P <0.05 compared with corresponding contralateral hemisphere; § P <0.05 compared with infarct hemisphere of corn oil fed mice.