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# Natural Vitamin E $\alpha$ -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; Chandan K. Sen, PhD

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

**Methods**—Stroke-dependent brain tissue damage was studied in MRP1-deficient mice and  $\alpha$ -TCT-supplemented mice.

**Results**—Elevated MRP1 expression was observed in glutamate-challenged primary cortical neuronal cells and in stroke-affected brain tissue. MRP1-deficient mice displayed larger stroke-induced lesions, recognizing a protective role of MRP1. In vitro, protection against glutamate-induced neurotoxicity by  $\alpha$ -TCT was attenuated under conditions of MRP1 knockdown; this suggests the role of MRP1 in  $\alpha$ -TCT-dependent neuroprotection. In vivo studies demonstrated that oral supplementation of  $\alpha$ -TCT protected against murine stroke. MRP1 expression was elevated in the stroke-affected cortical tissue of  $\alpha$ -TCT-supplemented mice. Efforts to elucidate the underlying mechanism identified MRP1 as a target of microRNA (miR)-199a-5p. In  $\alpha$ -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 add a new dimension to the current understanding of the molecular bases of  $\alpha$ -TCT neuroprotection in 2 ways: by identifying MRP1 as a  $\alpha$ -TCT-sensitive target and by unveiling the general prospect that oral  $\alpha$ -TCT may regulate miR expression in stroke-affected brain tissue. (*Stroke*. 2011;42:2308-2314.)

**Key Words:** antioxidant ■ vitamin E ■ microRNA ■ glutathione

Vitamin E is a generic term for tocopherols (TCP) and tocotrienols (TCT). Although TCPs have been widely studied for decades, the significance of  $\alpha$ -TCT as the most potent neuroprotective form of natural vitamin E has been uncovered recently. We have reported that nanomolar concentrations of  $\alpha$ -TCT, not  $\alpha$ -TCP, prevent stroke-associated neurodegeneration.<sup>1-2</sup> Alpha-TCT protects neural cells by 2 major mechanisms, including inhibition of inducible c-Src and 12-lipoxygenase pathways.<sup>1,3</sup> Recently, we have reported the key role of intracellular oxidized glutathione (GSSG) in causing neural cell death.<sup>4</sup> Under normal physiological conditions, in most tissues GSSG represents 1% of 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 depleted

in the face of oxidant insult. Excessive cellular GSSG is toxic and therefore is pumped out at the expense of adenosine triphosphate. 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 overt.<sup>4</sup>

Multidrug resistance-associated protein 1 (MRP1) plays a key role in clearing intracellular GSSG.<sup>5</sup> 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  $\alpha$ -TCT for its ability to enhance MRP1 function in the context of stroke-induced brain injury.

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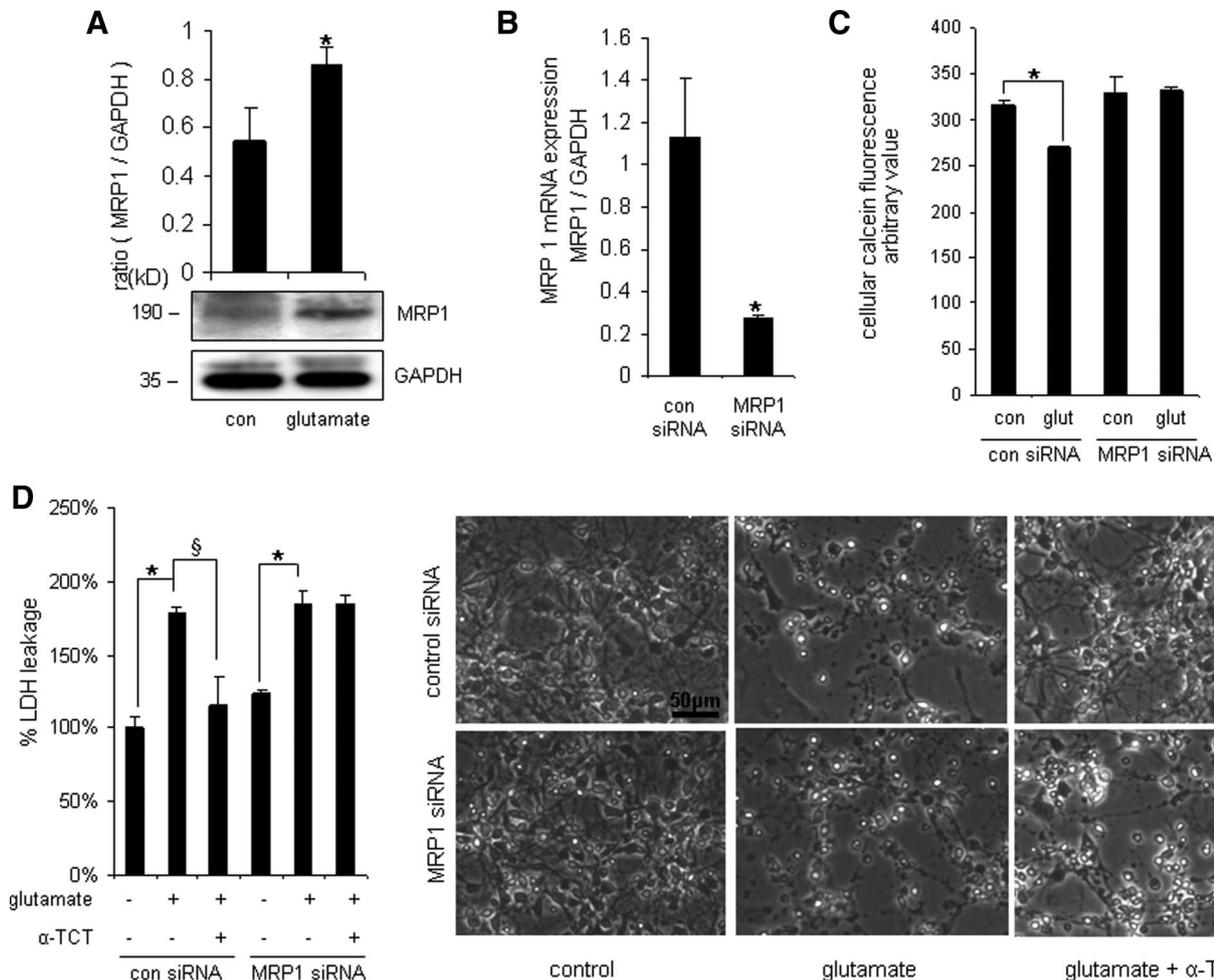
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**Figure 1.** MRP1 knockdown attenuated neuroprotection of  $\alpha$ -tocotrienol in primary cortical neurons. After 24 hours of seeding, primary cortical neurons were treated with glutamate (5mmol/L, 24 hours). Glutamate challenge induced the expression of MRP1 protein (A). After MRP1 siRNA transfection, MRP1 mRNA expression was significantly downregulated (B). C, Glutamate-challenged primary neurons with MRP1 knockdown exhibited loss of functional MRP1 by retaining more calcein compared with corresponding control cells. D,  $\alpha$ -TCT (1 $\mu$ mol/L) was added into cell culture medium 6 hours before glutamate treatment. After glutamate (5mmol/L, 24 hours) challenge, LDH leakage was measured. Neuroprotection of  $\alpha$ -TCT was compromised under conditions of MRP1 knockdown. Bar=50  $\mu$ m, n=3, \* $P$ <0.05 compared with control;  $\S P$ <0.05 compared with control siRNA-transfected, glutamate-treated primary neurons. siRNA indicates small interfering RNA; mRNA, messenger RNA; TCT, Tocotrienol; LDH, lactate dehydrogenase.

## Materials and Methods

For specific details, see online supplement at <http://aha.strokejournal.org>.

### Primary Cortical Neurons

Cells were isolated from the cerebral cortex of rat feti (Sprague-Dawley, day 17 of gestation; Harlan; see online supplement at <http://aha.strokejournal.org>).

### siRNA Delivery and Analysis of Genes

DharmaFECT 1 transfection reagent was used to transfect cells with 100nmol/L siRNA pool (Dharmacon RNA Technologies; see online supplement at <http://aha.strokejournal.org>).

### Delivery of miR Mimic and Inhibitor

DharmaFECT 1 transfection reagent was used to transfect cells with miRIDIAN rno-miR-199a-5p mimic or inhibitor (Dharmacon RNA Technologies; see online supplement at <http://aha.strokejournal.org>).

### pGL3-MRP1-3' Untranslated Region Luciferase Reporter Assay

See online supplement at <http://aha.strokejournal.org>

### Quantification of miR Expression

miR fraction was isolated using miRVana miRNA isolation kit (Ambion). miR-199a-5p levels were quantified using Taqman Universal Master Mix (Applied Biosystems; see online supplement at <http://aha.strokejournal.org>).

### Western Blot

Samples (40–50  $\mu$ g of protein/lane) were separated on a 4% to 12% sodium dodecyl sulfate polyacrylamide and probed with anti-MRP1 (1:20 dilution, Enzo Life Sciences; see online supplement at <http://aha.strokejournal.org>).

### Cell Viability Assay

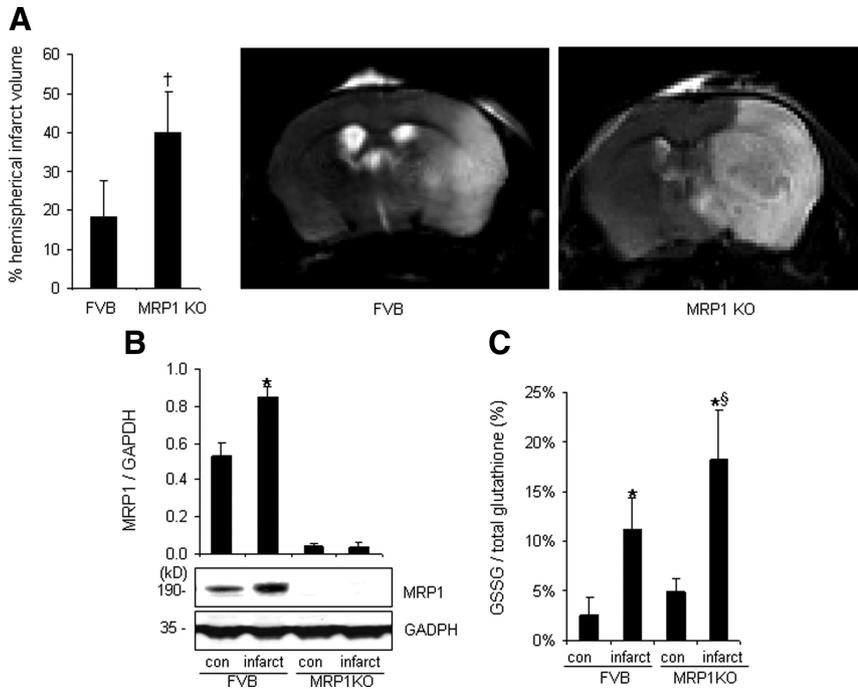
See online supplement at <http://aha.strokejournal.org>.

### Immunocytochemistry

See online supplement at <http://aha.strokejournal.org>.

### Calcein Clearance Assay

After MRP1 siRNA transfection, primary cortical neurons were treated with glutamate. Calcein-AM (5  $\mu$ mol/L, Invitrogen) was loaded to the cells for 30 minutes, and loss of cellular fluorescence was analyzed (see online supplement at <http://aha.strokejournal.org>).



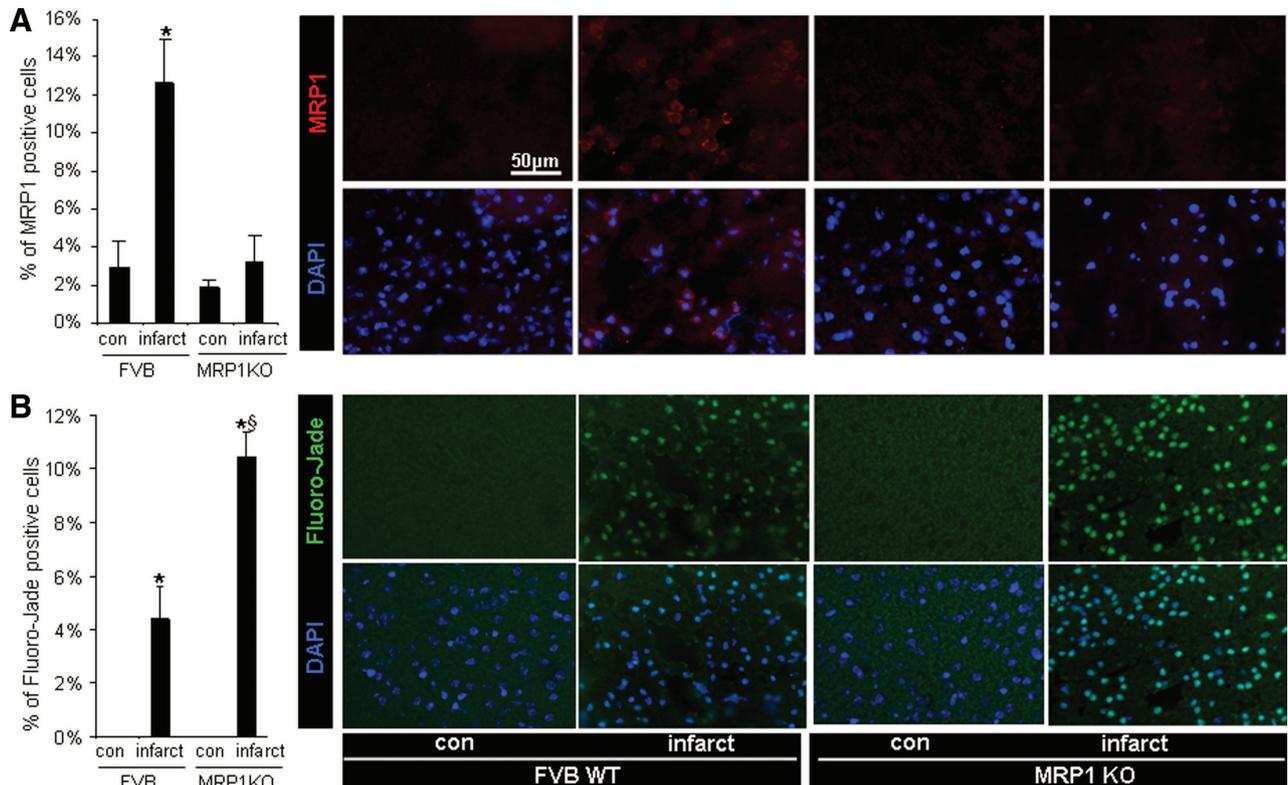
**Figure 2.** MCAO-induced brain injury was exacerbated in MRP1-deficient mice. Transient focal cerebral ischemia was induced in MRP1-deficient (n=20) or friend virus B-type (FVB) mice (n=15) by MCAO. **A**, MRP1 KO mice presented larger stroke-induced lesion compared with FVB mice after 48 hours reperfusion (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 with 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. MCAO indicates middle cerebral artery occlusion; KO, knockout; GSSG, intracellular oxidized glutathione.

**Mouse Stroke Model**

Transient focal cerebral ischemia was induced in 8-week-old, MRP1-deficient (n=20, male; Taconic) or background friend virus B-type (FVB) mice (n=15, male; Taconic) by middle cerebral artery occlusion (MCAO; see online supplement at <http://aha.strokejournal.org>).

**Magnetic Resonance Imaging**

T2-weighted imaging was performed on stroke-affected mice. Imaging experiments were carried out using an 11.7 T magnetic resonance imaging system comprised of a vertical bore magnet (Bruker Biospin; see online supplement at <http://aha.strokejournal.org>).



**Figure 3.** MCAO-induced neurodegeneration was accentuated in MRP1-deficient mice. **A**, MRP1 expression was upregulated at the stroke-affected cortex of friend virus B-type (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 μm, n=3, \**P*<0.05 compared with corresponding contralateral hemisphere; §*P*<0.05 compared with infarct hemisphere of FVB. MCAO indicates middle cerebral artery occlusion.

### $\alpha$ -Tocotrienol Supplementation

C57BL/6 (5-week old, male; Harlan) mice were randomly divided into 2 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  $\alpha$ -TCT supplement group. The test group was orally gavaged with  $\alpha$ -TCT (Carotech) in vitamin-E-stripped corn oil at a dosage of 50 mg/kg body weight for 13 weeks as reported previously.<sup>1</sup> MCAO was performed at 20 to 24 hours after the last supplementation. After 48 hours of MCAO, T2-weighted images were recorded. Mice suffering from surgical complications (eg, hemorrhage or death) during MCAO were excluded. Immediately after imaging, tissues from control and stroke-affected hemispheres of control (n=9) and  $\alpha$ -TCT-supplemented (n=10) mice were harvested. Mice were maintained under standard conditions at 22±2°C with 12:12 dark:light cycles. All animal protocols were approved by the Institutional Animal Care and Use Committee of Ohio State University.

### HPLC-Electrochemical Detection

See online supplement at <http://aha.strokejournal.org>.

### mRNA Expression Assay From Laser-Captured Microdissected Somatosensory Cortex of Brain Sample

Laser microdissection and pressure catapulting was performed on coronal slices of mouse brains using the microlaser system from PALM Microlaser Technologies AG. (See online supplement at <http://aha.strokejournal.org>).

### Histology

Coronal slices of cortical sections were stained with rabbit polyclonal antibody to MRP1 (1:200, Abbiotec), 0.0001% Fluoro-Jade C (Millipore), or 4-hydroxynonenal (1:1000; Enzo Life Sciences; see online supplement at <http://aha.strokejournal.org>).

### Statistics

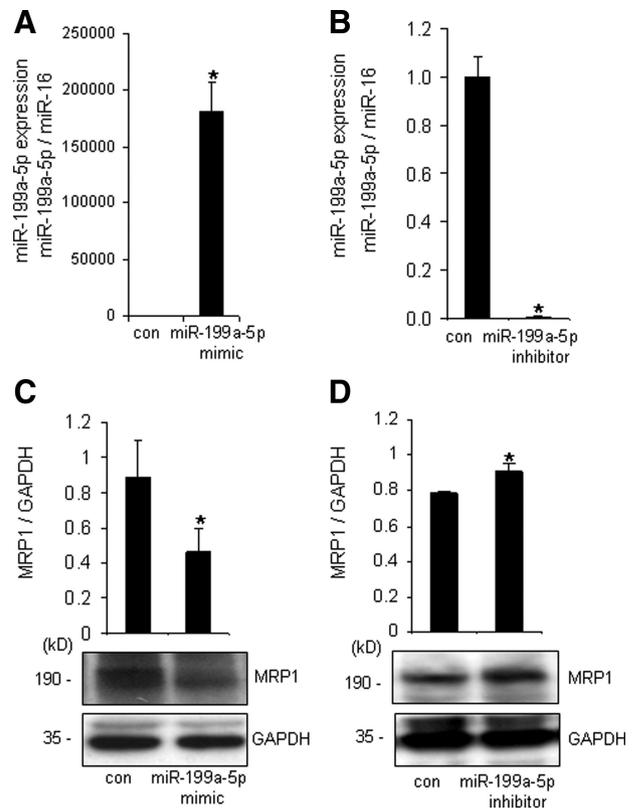
Data are reported as mean±SD. Differences between means were tested using Student *t* test or 1-way ANOVA with Tukey's test. *P*<0.05 was considered statistically significant.

## Results

### Neuroprotection of $\alpha$ -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 in HT4 neural cells (Figure S1A).

Previously we have reported that  $\alpha$ -TCT is potently neuroprotective against a number of insults, including GSSG-induced cell death.<sup>3–4,6</sup> To test whether such neuroprotective property of  $\alpha$ -TCT depends on MRP1 in neural cells, cellular GSSG clearance was studied utilizing a MRP1 knockdown approach (Figure 1B and Figure S1B-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

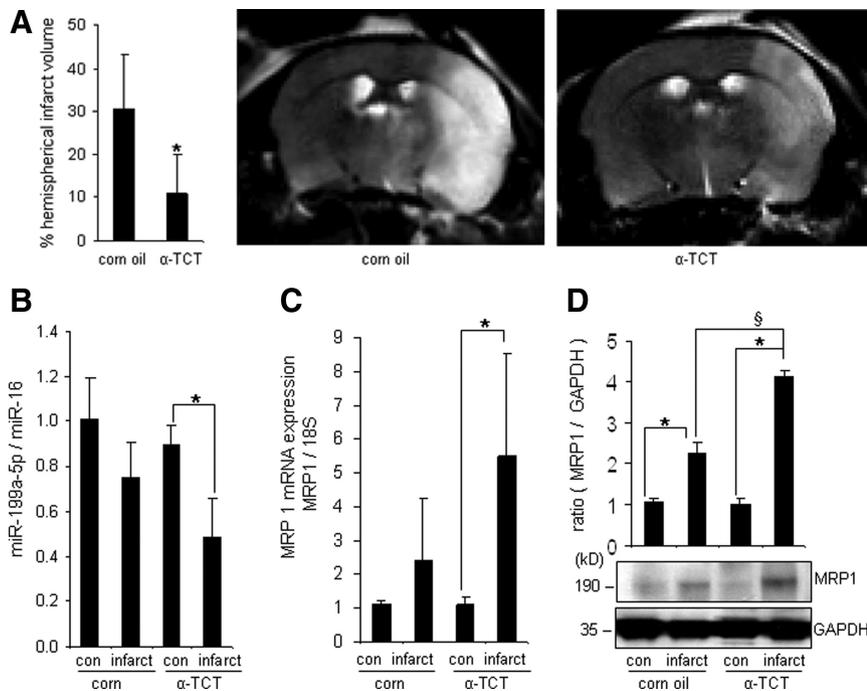


**Figure 4.** miR-199a-5p silences MRP1 in primary cortical neurons. After 72 hours of miR-199a-5p mimic delivery, miR-199a-5p expression was significantly upregulated (A) while miR-199a-5p hairpin inhibitor significantly downregulated miR-199a-5p (B). miR-199a-5p mimic/inhibitor negatively regulated protein (C-D) of MRP1 in primary cortical neurons. n=3, \**P*<0.05 compared with control.

ability of  $\alpha$ -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 middle cerebral artery–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 with 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, support-



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

ing 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 miR, we first performed in silico studies using databases such as Target Scan ([www.targetscan.org](http://www.targetscan.org)), Miranda ([www.microrna.org](http://www.microrna.org)), and PicTar ([www.pictar.org](http://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 of MRP1 transcript. Next, we turned toward 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 and Figure S2A). miR-199a-5p silenced MRP1 in both primary (Figure 4C-D) as well as in 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' untranslated region of MRP1 mRNA containing the putative miR-199a-5p binding sequence cloned into a firefly luciferase reporter construct was used. This construct was cotransfected 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; this finding recognizes MRP1 as a direct target of miR-199a-5p (Figure S2D).

### Oral $\alpha$ -Tocotrienol Protected Against Stroke via MRP1 Upregulation

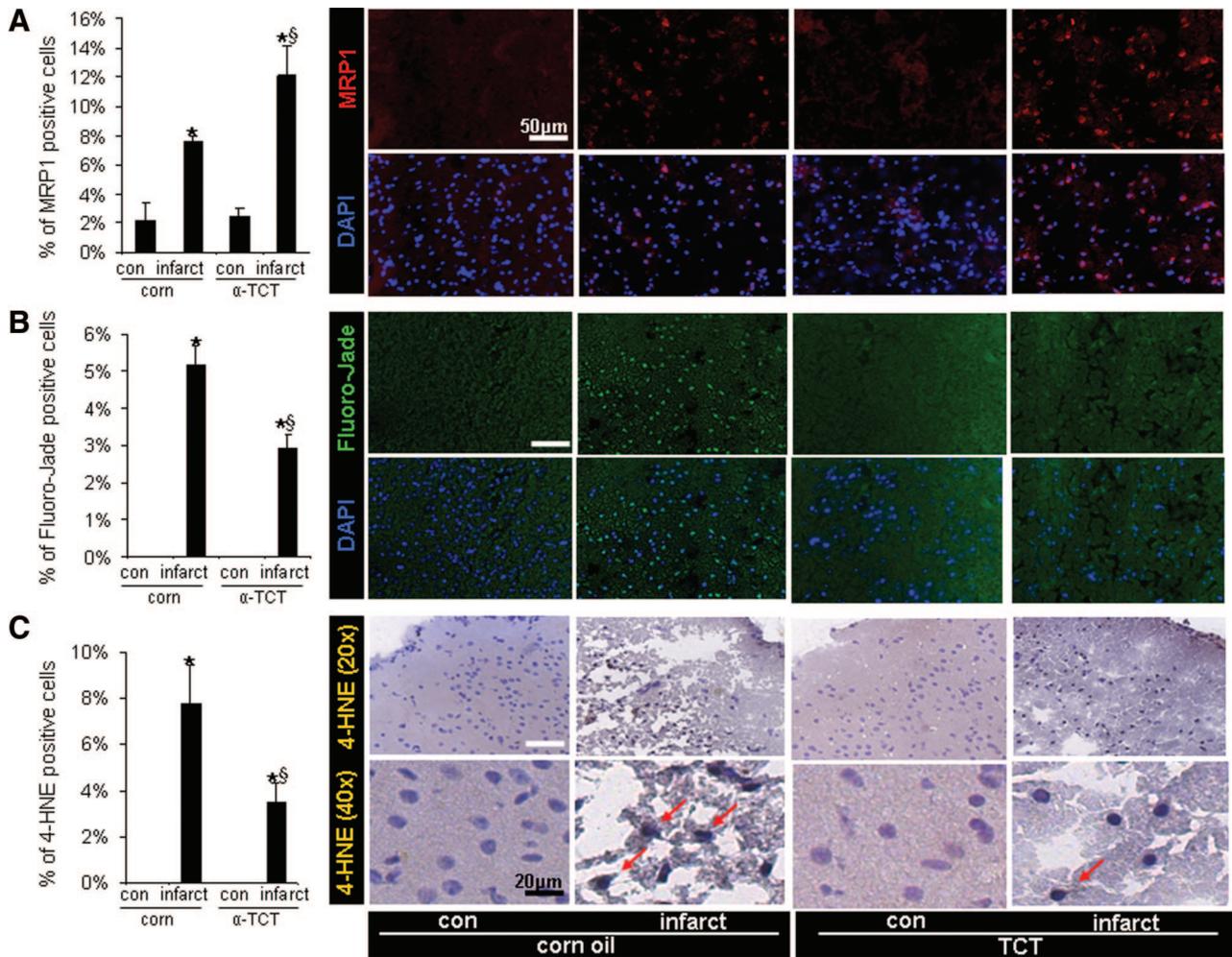
To test the neuroprotective effects of  $\alpha$ -TCT against stroke in vivo, randomly divided mice were gavaged with either vitamin-E-stripped corn oil or  $\alpha$ -TCT (50 mg/kg body

weight) for 13 weeks. MCAO was performed 1 day after the last supplementation. MCAO-affected brains were harvested 48 hours after reperfusion (Figure S3A). Alpha-TCT supplementation significantly increased brain  $\alpha$ -TCT level (Figure S3B) without changing  $\alpha$ -TCP levels (Figure S3C). MCAO limited blood flow in  $\alpha$ -TCT-supplemented, as well as in control groups, comparably (Figure S3D). However, MCAO-induced hemispherical infarct volume was significantly attenuated in  $\alpha$ -TCT-supplemented group (Figure 5A).

To test whether the neuroprotective properties of  $\alpha$ -TCT against stroke in vivo was dependent on the ability of  $\alpha$ -TCT to upregulate MRP1, cortical tissue elements were microdissected from infarct, as well as contralateral noninfarct, sites using a laser microdissection and pressure catapulting system (Figure S4). Notably,  $\alpha$ -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  $\alpha$ -TCT-supplemented mice as compared to corn oil supplemented mice (Figure 6A). Alpha-TCT supplementation attenuated stroke-induced neurodegeneration (Figure 6B). Stroke induced lipid peroxidation, as evidenced by elevated levels of 4-hydroxynonenal-positive cells, in the infarct tissue. Consistent with other observations indicating a beneficial influence of  $\alpha$ -TCT supplementation, 4-hydroxynonenal-positive cells were fewer in the infarct hemisphere of  $\alpha$ -TCT-supplemented mice (Figure 6C).

### Discussion

Following a number of failed clinical trials testing the conventional form of vitamin E,  $\alpha$ -TCP,<sup>7-8</sup> interest in naturally occurring forms of vitamin E such as  $\gamma$ -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  $\alpha$ -TCP,



**Figure 6.** Orally supplemented  $\alpha$ -tocotrienol attenuated MCAO-induced neurodegeneration and lipid peroxidation. **A**, The abundance of MRP1-positive cells were significantly upregulated in the stroke-affected cortex of  $\alpha$ -TCT-supplemented mice (red-MRP1 protein; blue-DAPI stained nuclei). **B**, Fluoro-Jade-positive neurons were fewer in the infarct hemisphere of  $\alpha$ -TCT-fed group compared with 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  $\alpha$ -TCT-supplemented mice. Bar (white)=50  $\mu$ m, Bar (black)=20  $\mu$ m. n=3, \* $P$ <0.05 compared with corresponding contralateral hemisphere; § $P$ <0.05 compared with infarct hemisphere of corn oil fed mice. MCAO indicates middle cerebral artery occlusion; MRP1, multidrug resistance-associated protein 1; TCT, tocotrienol; DAPI, 4',6-diamidino-2-phenylindole; HNE, hydroxynonenal.

the only form of vitamin E tested in such trials, represents only a fraction of the natural vitamin E family.<sup>7-8</sup> 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  $\alpha$ -TCT at nanomolar concentration represents the most potent functional property of the entire vitamin E family.<sup>6</sup> Mechanisms explaining such property include inhibition of inducible c-Src, as well as 12-lipoxygenase activity, in stroke-related neurodegenerative settings.<sup>1,9</sup>

Stroke-associated ischemic insult is known to compromise adenosine triphosphate production as well as deplete the reducing equivalent pool of the brain. This, in turn, compromises the function of adenosine-triphosphate-dependent ion pumps, as well as the ability of the brain to manage oxidant insult. As a result,  $K^+$  efflux and  $Ca^{2+}$  influx are increased in response to membrane depolarization. In contrast, excessive GSSG builds up within the cell. Both elevated intracellular  $Ca^{2+}$ , as well as GSSG, are directly implicated in cell death

signaling.<sup>10-11</sup> Our previous work highlights the critical significance of elevated and trapped cellular GSSG in executing neural cell death.<sup>4</sup>

MRP1 represents a major GSSG clearing system in neural cells.<sup>12</sup> 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 stroke.<sup>13</sup> In addition, ischemia-reperfusion depletes niocinamide dinucleotide phosphate, impairing all reductase functions dependent on this reducing equivalent.<sup>14</sup> Therefore, a sharp rise in brain tissue GSSG/GSH ratio occurs following stroke.<sup>4</sup> In the current article, we note that such elevation of intracellular GSSG is associated with 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 survival.<sup>12</sup> 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, ie, Alzheimer's disease and epilepsy, MRP1 expression is elevated in the brain.<sup>15–16</sup> 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 the first evidence that MRP1 is subject to post-transcriptional gene silencing by miRs. This finding provided a great segue into addressing the significance of miRs in stroke.<sup>17</sup> Antagomir miR-497 treatment has been recognized for its potential to attenuate injury related to acute ischemic stroke.<sup>18</sup> The miR-200 family and miR-182 have been found to be upregulated in ischemic preconditioning.<sup>19</sup> Although the significance of miR-199a-5p in stroke has not been studied, miR-199a-5p is recognized as a hypoxia-sensitive miR.<sup>20</sup> Thus, the hypoxia component of stroke-related ischemia may be responsible for downregulating miR-199a-5p following stroke.<sup>21</sup>

### Conclusions

The findings of this study add a new dimension to the current understanding of the molecular bases of  $\alpha$ -TCT neuroprotection in 2 ways: by identifying MRP1 as a  $\alpha$ -TCT-sensitive target and by unveiling the general prospect that oral  $\alpha$ -TCT may regulate microRNA expression in stroke-affected brain tissue. Neuroprotective, as well as hypocholesterolemic, properties of  $\alpha$ -TCT make it a good candidate for nutrition-based intervention in people at high risk for stroke. Transient ischemic attack, or mini-stroke, serves as a sentinel warning sign for high-risk stroke patients.<sup>22</sup> Prophylactic stroke therapy therefore provides an opportunity for intervention in patients experiencing transient ischemic attack before a major stroke event. Outcomes of the current study warrant clinical assessment of  $\alpha$ -TCT in transient ischemic attack patients. Furthermore,  $\alpha$ -TCT is a nutrient that is certified by the U.S. Food and Drug Administration to be Generally Recognized As Safe (GRN307) and is not a drug with potential side effects. Thus,  $\alpha$ -TCT may be considered as a preventive nutritional countermeasure for people at high risk for stroke.

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### Disclosures

None.

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## ONLINE SUPPLEMENT

### Supplemental Materials and Methods

#### Materials

The following materials were obtained from the source indicated: L-glutamic acid monosodium salt, and lactate dehydrogenase (LDH) cytotoxicity assay kit (Sigma-Aldrich, St. Louis, MO);  $\alpha$ -tocotrienol (Carotech Inc, Malaysia); DharmaFECT™1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO); Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA); *mirVana*™ miRNA isolation kit (Ambion, Austin, TX); PicoPure RNA Isolation kit (Arcturus, Sunnyvale, CA); dual-luciferase reporter assay system (Promega Corporation, Madison, WI); calcein acetoxymethyl ester, Lipofectamine™ LTX and PLUS™ reagent (Invitrogen Corporation, Carlsbad, CA); 4-hydroxynonenal (HNE) and monoclonal antibody to MRP1 (Enzo Life Sciences, Plymouth Meeting, PA); rabbit polyclonal MRP1 antibody (Abbotec, San Diego, CA); Fluoro-Jade® C (Millipore, Billerica, MA).

For cell culture, Dulbecco's modified Eagle medium, fetal calf serum, and penicillin and streptomycin were purchased from Invitrogen Corporation, Carlsbad, CA. Culture dishes were obtained from Nunc, Denmark.

#### Cell culture

Mouse hippocampal HT4 neural cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in humidified atmosphere of 95% air and 5% CO<sub>2</sub> as described previously<sup>1-3</sup>. *Glutamate treatment*. Immediately before experiments, the culture medium was replaced with fresh medium supplemented with serum and antibiotics. Glutamate (5 mmol/L for primary neurons and 10 mmol/L for HT4 neural cells) was added to the cell culture medium as an aqueous solution. No change in the medium pH was observed in response to the addition of glutamate<sup>2,4</sup>.  *$\alpha$ -Tocotrienol (TCT) treatment*. A stock solution of  $\alpha$ -TCT (Carotech Inc, Malaysia) was prepared in ethanol. Before experiments, culture medium was replaced with fresh medium supplemented with serum and antibiotic,  $\alpha$ -TCT was then added to the culture medium as described in the corresponding legends.

#### Primary cortical neurons

Cells were isolated from the cerebral cortex of rat feti (Sprague-Dawley, day 17 of gestation; Harlan, Indianapolis, IN) as described previously<sup>1,4</sup>. After isolation from the brain, cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 40  $\mu$ mol/L cystine, and antibiotics (100  $\mu$ g/ml streptomycin, 100 units/ml penicillin, and 0.25  $\mu$ g/ml amphotericin). Cultures were maintained at 37°C in 5% CO<sub>2</sub> and 95% air in a humidified incubator. All experiments were carried out 24 hours after plating.

#### siRNA delivery and analysis of genes

Primary cortical neurons ( $1.5-2.5 \times 10^6$  cells/ well in 12-well plate) or HT4 neural cells ( $0.1 \times 10^6$  cells / well in 12-well plate) were seeded in antibiotic free medium for 24h

prior to transfection. DharmaFECT™1 transfection reagent was used to transfect cells with 100 nmol/L siRNA pool (Dharmacon RNA Technologies, Lafayette, CO) for 72h as described previously<sup>5-7</sup>. For controls, siControl non-targeting siRNA pool (mixture of 4 siRNA, designed to have  $\geq 4$  mismatches with the corresponding gene) was used. HT4 cells were harvested and re-seeded for treatment with glutamate or  $\alpha$ -TCT as indicated in the respective figure legends. For the primary neurons, media were changed after 24h of seeding. Neurons were treated with glutamate or  $\alpha$ -TCT as indicated after 72h of transfection. For determination of mRNA expression after siRNA transfection, total RNA was isolated from cells using the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA). The abundance of mRNA for MRP1 was quantified using real time PCR using SYBR green-I (Applied Biosystems, Forster City, CA). The following primer sets were used: m\_MRP1\_F, 5'-GGT CCT GTT TCC CCC TCT ACT TCT T-3'; m\_MRP1\_R, 5'-GCA GTG TTG GGC TGA CCA GTA A-3'; m\_GAPDH\_F, 5'-ATG ACC ACA GTC CAT GCC ATC ACT-3'; m\_GAPDH\_R, 5'-TGT TGA AGT CGC AGG AGA CAA CCT-3'; r\_MRP1\_F, 5'-TGA ACC ATG AGT GTG CAG AAG GT-3'; r\_MRP1\_R, 5'-TCA CAC CAA GCC AGC ATC CTT-3'; r\_GAPDH\_F, 5'- TAT GAC TCT ACC CAC GGC AAG TTC A-3'; r\_GAPDH\_R, 5'- CAG TGG ATG CAG GGA TGA TGT TCT-3'.

#### **miRIDIAN microRNA (miR) mimic/ inhibitor delivery**

Primary cortical neurons ( $1.5-2.5 \times 10^6$  cells/ well in 12-well plate) or HT4 neural cells ( $0.1 \times 10^6$  cells / well in 12-well plate) were seeded in antibiotic free medium for 24h prior to transfection. DharmaFECT™ 1 transfection reagent was used to transfect cells with miRIDIAN rno-miR-199a-5p mimic / rno-miR-199a-5p inhibitor or mmu-miR-199a-5p mimic / mmu-miR-199a-5p hairpin inhibitor (Dharmacon RNA Technologies, Lafayette, CO) as per the manufacturer's instructions. miRIDIAN miR mimic or inhibitor negative controls (Dharmacon RNA Technologies, Lafayette, CO) were used for control transfections. Samples were collected after 72h of miR mimic/inhibitor delivery for quantification of miR, mRNA and protein expression as described<sup>8</sup>.

#### **pGL3-MRP1-3'UTR luciferase reporter assay**

miRIDIAN mmu-miR-199a-5p mimic or mmu-miR-199a-5p hairpin inhibitor was delivered to HT4 neural cells followed by transfection with pGL3-MRP1-3'UTR firefly luciferase expression construct (Signosis, Sunnyvale, CA) together with renilla luciferase pRL-cmv expression construct using Lipofectamine™ LTX PLUS™ reagent. Luciferase assay were performed using the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activity was normalized to renilla luciferase expression for each sample as described<sup>8</sup>.

#### **Quantification of microRNA expression**

Total RNA including miR fraction was isolated using miRVana™ miRNA isolation kit (Ambion, Austin, TX), according to the manufacturer's protocol. miR-199a-5p levels were quantified using Taqman Universal Master Mix (Applied Biosystems, Forster City, CA). miR levels were quantified with the  $2^{(-\Delta\Delta CT)}$  relative quantification method using miR-16 as the house keeping miR<sup>7-10</sup>.

#### **Western blot**

After protein extraction, the protein concentration was determined using BCA protein reagents. The samples (40-50  $\mu$ g of protein / lane) were separated on a 4-12% SDS-polyacrylamide gel electrophoresis as described<sup>2,11-12</sup> and probed with anti-MRP1 (1:20 dilution, Enzo Life Sciences, Plymouth Meeting, PA). To evaluate the loading efficiency, membranes were probed with anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO).

### **Cell viability**

The viability of primary cortical neurons or HT4 neural cells in culture was assessed by measuring leakage of lactate dehydrogenase (LDH) from cells into media using an *in vitro* toxicology assay kit from Sigma-Aldrich (St. Louis, MO, USA). The protocol has been described in detail in a previous report<sup>5-6,11,13</sup>. In brief, LDH leakage was determined using the following equation: % total LDH leaked = (LDH activity in the cell culture medium / total LDH activity)  $\times$  100. Total LDH activity represents the sum of LDH activities in the cell monolayer, detached cells, and the cell culture medium.

### **Immunocytochemistry**

HT4 neural cells ( $0.5 \times 10^6$  cells / well) were seeded in 35mm plates for 24h then treated with or without 10 mmol/L glutamate. Cells were washed with PBS three times and fixed in 10% buffered formalin for 20 min, then underwent permeabilization using 0.1% Triton X-100/PBS for 15 min. The cells were washed and incubated with 10% goat serum (Vector Laboratories, Burlingame, CA) for 1h at room temperature, and incubated with MRP1 antibody (1:50, Abcam, Cambridge, MA) overnight at 4°C. After incubation with primary antibody, cells were washed with PBS three times and incubated with an Alexa-fluor 488 (1:200 dilution) for 1h at room temperature. After three washes and incubation with 4',6'-diamino-2-phenylindole (1:10,000 dilution) for 2 min, cells were mounted in gelmount (aqueous mount, Vector Laboratories, Burlingame, CA) for microscopic imaging as described previously<sup>5,11</sup>.

### **Calcein clearance assay**

Calcein clearance assay was used to measure MRP1 activity in both primary cortical neurons and HT4 neural cells against glutamate insult. To improve poor specificity of pharmacological inhibitors, RNA interference approach was applied to measure specific MRP1 activity. After 72h of MRP1 siRNA transfection as described above, HT4 cells were re-seeded and treated with glutamate as described in relevant legends. After glutamate challenge, calcein-AM (25 nmol/L) was loaded to the cells for 15 min at 37°C. Cells were washed with PBS, collected and analyzed using the Accuri C6<sup>TM</sup> (Accuri Cytometers, Ann Arbor, MI) flowcytometer. MRP1 activity was measured on the basis of intracellular calcein retention<sup>14</sup>. For primary neurons, media were changed after 72h of transfection, and the neurons were treated with 5 mmol/L glutamate for 1h. After glutamate challenge, calcein-AM (5  $\mu$ mol/L) was loaded to the cells for 30 min at 37°C. Then, fluorescence was measured by using the Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) with the excitation wavelength of 485nm and the emission wavelength of 528nm.

### **Mouse stroke model**

Transient focal cerebral ischemia was induced in 8 weeks old MRP1 deficient (n=20, male, Taconic, Hudson, NY) or background FVB mice (n=15, male, Taconic, Hudson, NY) by middle cerebral artery occlusion (MCAO) as previously described<sup>1,11,15-16</sup>. Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique. Briefly, mice were anesthetized by inhaling halothan, and 6-0 nylon monofilament was inserted into the internal carotid artery, *via* the external carotid artery. Then the filament tip was positioned for occlusion at a distance of 6mm beyond the internal carotid artery-ptyergopalatine artery bifurcation. We observed that this approach results in a  $70 \pm 10\%$  drop in cerebral blood flow as measured by laser Doppler (DRT4, Moor Instruments). Once the filament was secured, the incision was sutured and the animal was allowed to recover from anesthesia in its home cage. After 90min of occlusion, the animal was briefly re-anesthetized, and reperfusion was initiated via withdrawal of the filament from MCA. This surgical protocol typically results in a core infarct limited to the parietal cerebral cortex and caudate putamen of the right hemisphere. After 48h of reperfusion, T2-weighted image was taken to measure infarct volume. 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 MRP1 (n=12) and FVB (n=8) were harvested.

## **MRI**

T2-weighted imaging was performed on stroke-affected mice. Imaging experiments were carried out using a 11.7T (500 MHz) MR system comprised of a vertical bore magnet (Bruker Biospin, Ettlingen, Germany) as described previously by our group<sup>11,17-18</sup>. Briefly, animals were placed inside a 30 mm radio frequency coil (resonator) and finally the whole arrangement was placed inside the vertical magnet. Shimming was performed to adjust field inhomogeneities on the subject. After several localizer scans were completed for three different orientations (saggital, coronal and axial), a T2-weighted spin echo rapid acquisition with relaxation enhancement (RARE) sequence was optimized for some key parameters such as: field of view (FOV) = 30×30 mm, acquisition matrix 256×256, repetition time (TR) = 3000 ms, echo time (TE) = 30 ms, flip angle (FA) = 180 degrees, images in acquisition = 15, resolution = 8.533 pixels/mm, and number of averages 4 was used to acquire T2-weighted MR images from the mouse head on the 11.7-T MRI system to generate 15 images corresponding to 15 short axis slices. For stroke-volume calculations, raw MRI images were converted to digital imaging and communications in medicine (DICOM) format and read into ImageJ software (NIH).

## **$\alpha$ -Tocotrienol supplementation**

C57BL/6 (5 weeks, male, Harlan, Indianapolis, IN) mice were randomly divided into two groups, control (n=18) and supplemented (n=23) group. 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  $\alpha$ -TCT supplement solution was prepared in vitamin E-stripped corn oil. The test group was orally gavaged with  $\alpha$ -TCT (Carotech Inc, Malaysia) in oil (same as placebo) at a dosage of 50 mg/kg body weight for 13 weeks. Incorporation of orally supplemented vitamin E to the brain is a slow process. Longer supplementation period improves bioavailability of vitamin E to the

brain. Stroke was performed at 20 to 24h after the last supplementation of  $\alpha$ -TCT or corn oil. After 48h of MCAO, T2-weighted image was taken to measure infarct volume. 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  $\alpha$ -TCT fed (n=10) mice were harvested. Mice were maintained under standard conditions at 22 $\pm$ 2°C with 12:12 dark: light cycles. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University, Columbus, Ohio.

### **HPLC-electrochemical detection**

Vitamin E analyses of TCT or corn oil fed mice brains were performed using a HPLC-coulometric electrode array detector (Coularray Detector - model 5600 with 12 channels; ESA Inc., Chelmsford, MA). This system enables the simultaneous detection of TCTs and TCPs in the same run as described by us previously<sup>2,19</sup>. Glutathione measurement was performed using an HPLC system coupled with a electrochemical coulometric detector as described<sup>1,4,20</sup>. The CoulArray detector employs multiple channels set at specific redox potentials. Data were collected using channels set at 600, 700, and 800mV. 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 were as described previously<sup>2,20</sup>.

### **mRNA expression assay from laser-captured microdissected somatosensory cortex of brain tissue**

Laser microdissection and pressure catapulting (LMPC) was performed using the microlaser system from PALM Microlaser Technologies AG (Bernreid, Germany) as described<sup>8,21-23</sup>. Briefly, mice were euthanized immediately after MRI imaging and coronal slices of brain tissue were collected using a mouse brain matrix. OCT-embedded in slices were subsequently cut in 12 $\mu$ m thick sections on a Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany). Settings used for laser cutting were UV-Energy of 70-80 and UV-Focus of 70. Matched area ( $2 \times 10^6 \mu\text{m}^2$ ) of contralateral or stroke-affected somatosensory cortex was captured into 25 $\mu$ l of RNA extraction buffer. The total RNA was isolated using PicoPure RNA Isolation kit (Arcturus, Sunnyvale, CA) to measure MRP1 mRNA expression. To determine miR-199a-5p expression in MCAO-induced mouse brain, miR fraction was isolated using *miRVana*<sup>TM</sup> miR isolation kit (Ambion, Austin, TX) as described above.

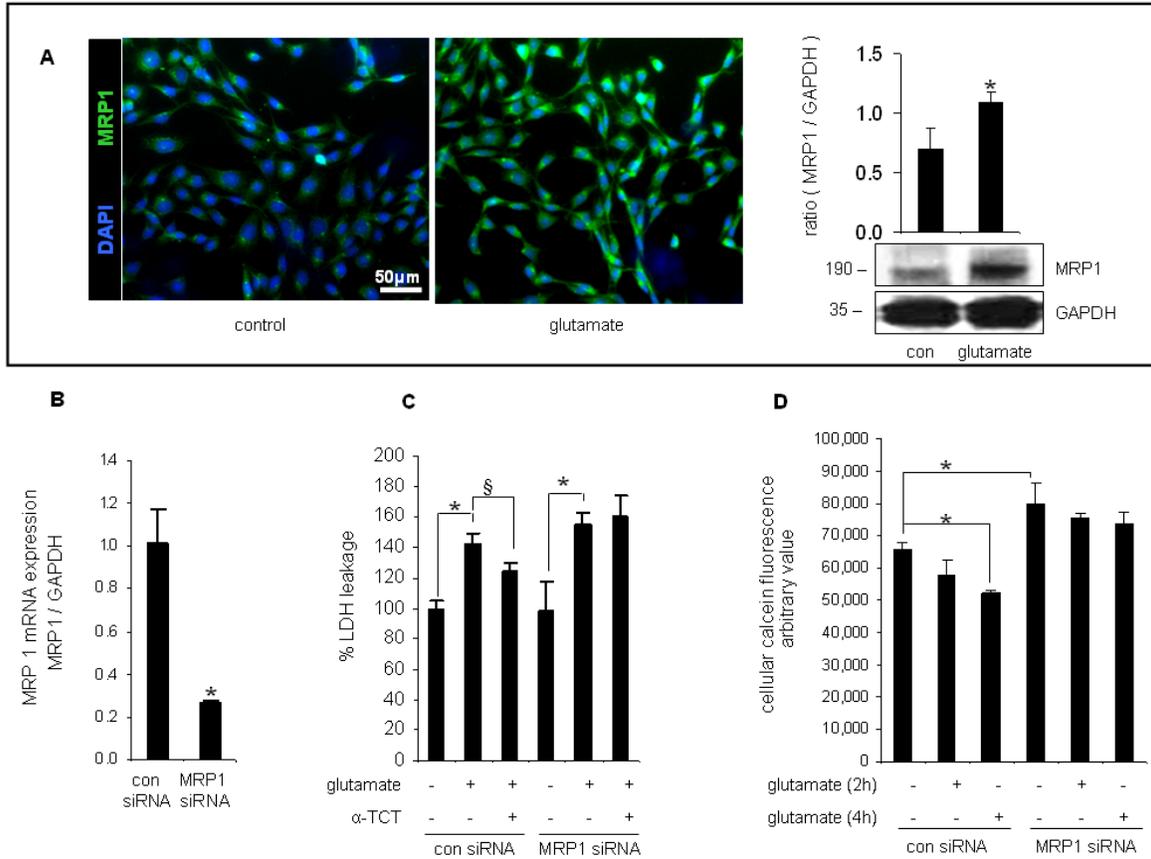
### **Histology**

OCT-embedded frozen brain was sectioned (12 $\mu$ m) and mounted onto slides. brain sections were stained with rabbit polyclonal antibody to MRP1 (1: 200, Abbiotec, San Diego, CA), 0.0001% Fluoro-Jade® C (Millipore, Billerica, MA) or 4-HNE (1:1000, Enzo Life Sciences, Plymouth Meeting, PA). Coronal slices of cortical sections were analyzed by fluorescence microscopy (Axiovert 200M, Zeiss, Göttingen, Germany) and images were captured using Axiovert v4.8 software (Zeiss)<sup>6,24</sup>.

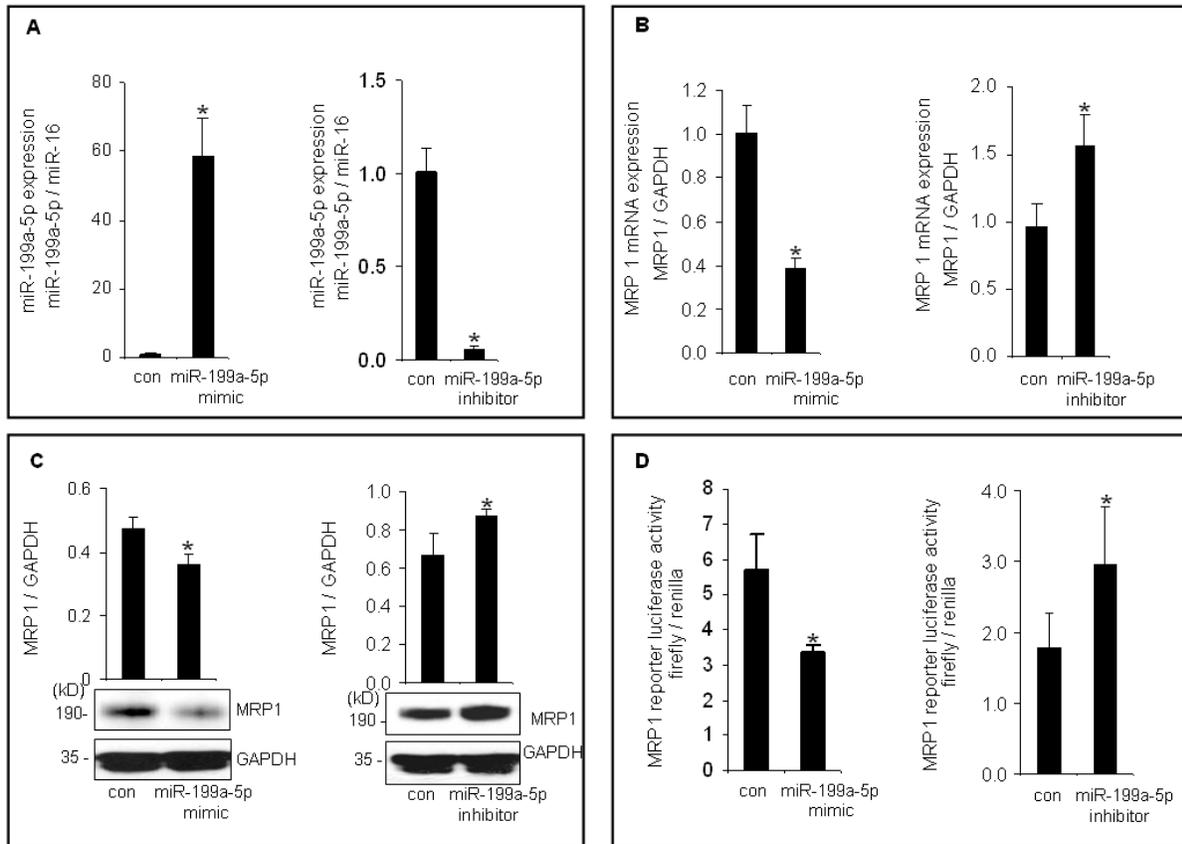
### **Statistics**

Data are reported as mean  $\pm$  SD of at least three independent experiments. Difference in means was tested using Student's *t*-test or one-way ANOVA with Tukey's test. *P*<0.05 was considered statistically significant.

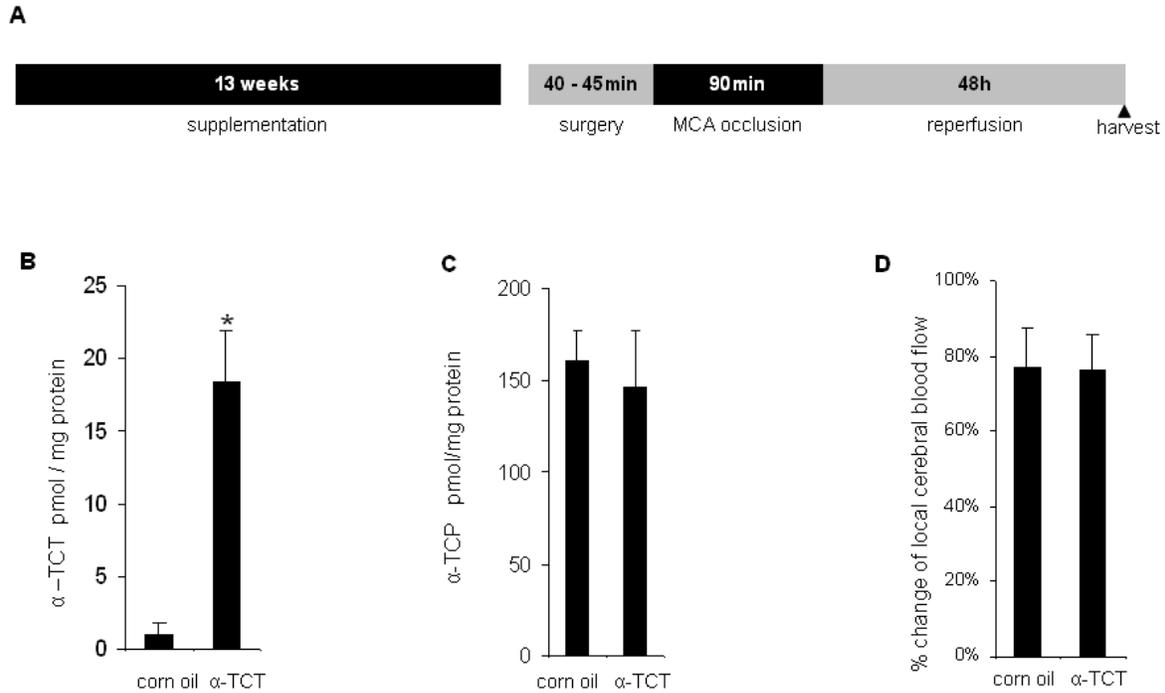
Supplemental Figures and Figure Legends



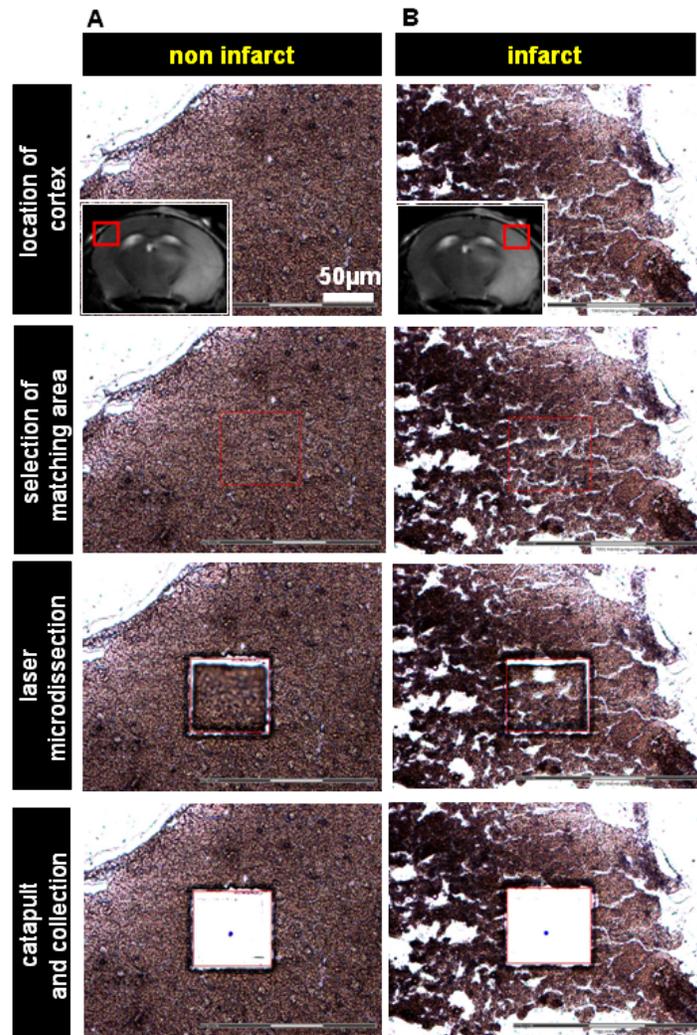
**Figure S1. MRP1 knockdown attenuated the neuroprotection of  $\alpha$ -tocotrienol. A,** Glutamate (10mmol/L, 12h) challenge induced the expression of MRP1 in HT4 neural cells (blue-DAPI stained nuclei; green-MRP1 protein). After MRP1 siRNA transfection, MRP1 mRNA expression was significantly down-regulated (**B**). **C,** Cells were re-split after transfection.  $\alpha$ -TCT (1 $\mu$ mol/L) was added into cell culture medium 6h before glutamate treatment. After glutamate (10mmol/L, 12h) challenge, LDH leakage was measured. Neuroprotection of  $\alpha$ -TCT was compromised under conditions of MRP1 knockdown. **D,** Glutamate-challenged cells with MRP1 knockdown exhibited loss of functional MRP1 by retaining more calcein compared to corresponding control cells. Bar=50 $\mu$ m. n=3, \* $P$ <0.05 compared with control;  $^{\S}$  $P$ <0.05 compared with control siRNA-transfected, glutamate-treated HT4 cells.



**Figure S2. miR-199a-5p targets MRP1 expression.** After 72h of miR-199a-5p mimic delivery, miR-199a-5p expression was significantly upregulated while miR-199a-5p hairpin inhibitor significantly downregulated miR-199a-5p in HT4 neural cells (A). miR-199a-5p mimic/inhibitor negatively regulated mRNA (B), and protein (C) of MRP1. To test whether MRP1 is a direct target of miR-199a-5p, HT4 cells were transfected with a pGL3-MRP1-3'UTR firefly luciferase expression construct and co-transfected with control renilla luciferase reporter construct along with miR-199a-5p mimic or inhibitor. miR-199a-5p mimic delivered cells showed lower luciferase activity while miR-199a-5p hairpin inhibitor delivered cells showed higher luciferase activity (D). n=3, \*P<0.05 compared with control.



**Figure S3. Orally supplemented  $\alpha$ -tocotrienol did not change brain  $\alpha$ -tocopherol levels or cerebral blood flow.** **A**, C57BL/6 mice were randomly divided into two groups, and orally gavaged with vitamin E-stripped corn oil (n=18) or 50mg  $\alpha$ -TCT per kg body weight (n=23) for 13 weeks. After 24h of last supplementation, MCAO was performed for 90min. T2 weighted MRI imaging was performed 48h after reperfusion, then mice were sacrificed and brain were harvested. **B-C**, Oral  $\alpha$ -TCT supplementation significantly increased  $\alpha$ -TCT level in the brain without affecting brain tissue  $\alpha$ -TCP level (n=5). **D**, Reduction in the MCA-area blood flow in corn oil fed and  $\alpha$ -TCT fed mice during occlusion was found to be comparable (corn oil, n=9;  $\alpha$ -TCT, n=10). \* $P$ <0.05 compared with corresponding control.



**Figure S4. Collection of somatosensory cortical tissue elements using a laser microdissection and pressure catapulting (LMPC) system.** MCAO-challenged brains were embedded in OCT. OCT-embedded slices were cut in 12 $\mu$ m thick coronal section. Matched area ( $2 \times 10^6 \mu\text{m}^2$ ) of contralateral or stroke-affected cortex was captured using a LMPC system (**A**, control hemisphere; **B**, infarct hemisphere). Bar=50 $\mu$ m.

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