Ferulic Acid Suppresses Glutamate Release Through Inhibition of Voltage-Dependent Calcium Entry in Rat Cerebrocortical Nerve Terminals

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ABSTRACT This study investigated the effects and possible mechanism of ferulic acid, a naturally occurring phenolic compound, on endogenous glutamate release in the nerve terminals of the cerebral cortex in rats. Results show that ferulic acid inhibited the release of glutamate evoked by the K⁺ channel blocker 4-aminopyridine (4-AP). The effect of ferulic acid on the evoked glutamate release was prevented by chelating the extracellular Ca²⁺ ions, but was insensitive to the glutamate transporter inhibitor DL-threo-beta-benzyl-oxyaspartate. Ferulic acid suppressed the depolarization-induced increase in a cytosolic-free Ca²⁺ concentration, but did not alter 4-AP-mediated depolarization. Furthermore, the effect of ferulic acid on evoked glutamate release was abolished by blocking the Ca_v2.2 (N-type) and Ca_v2.1 (P/Q-type) channels, but not by blocking ryanodine receptors or mitochondrial Na⁺/Ca²⁺ exchange. These results show that ferulic acid inhibits glutamate release from cortical synaptosomes in rats through the suppression of presynaptic voltage-dependent Ca²⁺ entry.

KEY WORDS: • antioxidants • brain • calcium • cerebrocortical nerve terminals • ferulic acid • glutamate release • neuroprotection • phenolic compounds • voltage-dependent Ca²⁺ channels

INTRODUCTION

FERULIC ACID IS A PHENOLIC COMPOUND commonly found in fruits, vegetables, and grains. Several biological activities of ferulic acid (4-hydroxy-3-methoxy cinnamic acid, Fig. 1) have emerged, for example, antioxidant, anti-inflammatory, anticancer, antiapoptotic, antidiabetic, and hepatoprotective properties.¹ In addition to these properties, ferulic acid attenuates oxidative stress, glutamate, or amyloid-induced neurotoxicity,^{2–8} protects against cerebral ischemia-induced brain damage,⁹ and ameliorates β -amyloid-induced memory impairment.¹⁰ These findings suggest a neuroprotective role for ferulic acid; however, the mechanism whereby ferulic acid exerts this capability is unclear.

In the brain, glutamate is a major excitatory neurotransmitter and plays an important role in functions, such as synaptic plasticity, learning, and memory.^{11,12} Excessive release of glutamate induces an increase in intracellular Ca²⁺ levels. This, in turn, triggers a cascade of cellular responses, including an enhanced oxygen-free radical

production, disturbed mitochondrial function, and protease activation, ultimately leading to neuronal cell death.^{13,14} The neuronal damage induced by over-excitation is likely involved in a number of neuropathological conditions, ranging from acute insults, such as stroke, epileptic seizures, traumatic brain, and spinal cord injury, to chronic neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.^{15–17} Consequently, if a compound can attenuate glutamate release from nerve terminals, it may have a neuroprotective effect against the pathological conditions related to excessive glutamate release. Some neuroprotective agents have been revealed to decrease glutamate release in human and rat brain tissues.^{18–20} Likewise, ferulic acid has a neuroprotective-like effect and whether ferulic acid has an effect on endogenous glutamate release should be evaluated.

To our knowledge, there are no studies addressing whether ferulic acid directly affects glutamate release at the presynaptic level. Thus, the current study used isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex as a model to determine the effects of ferulic acid on glutamate release. In contrast to brain slices, synaptosomes do not suffer from any postsynaptic interactions and are, therefore, extensively used to evaluate presynaptic phenomena.²¹ In addition, the possible underlying mechanism was also investigated.

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FIG. 1. Chemical structure of ferulic acid.

MATERIALS AND METHODS

Materials

3',3',3'-Dipropylthiadicarbocyanine iodide [DiSC₃(5)] and Fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA, USA). Dantrolene, DL-threo- β -benzyloxyaspartate (DL-TBOA), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), and ω -conotoxin MVIIC (ω -CgTX MVIIC) were obtained from Tocris Cookson (Bristol, United Kingdom). Ethylene glycol bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), ferulic acid, and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Animals

Adult male Sprague-Dawley rats (100–200 g) were used in these studies. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Fu Jen Catholic University Animal Care and Utilization Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Synaptosomal preparation and glutamate release assay

Synaptosomes were prepared as described previously²² from the cerebral cortices of rats.

Synaptosomes (0.5 mg) were resuspended in 2 mL of the HEPES-buffered incubation medium (HBM: 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂·6H₂O, 1.2 mM Na₂HPO₄, 10 mM glucose, and 10 mM HEPES, pH 7.4) that contained 16 μ M bovine serum albumin, and incubated in a cuvette maintained at 37°C with stirring in a Perkin-Elmer LS-50B spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Glutamate release was assayed in the presence of NADP⁺ (2 mM), glutamate dehydrogenase (50 U/mL), and CaCl₂ (1 mM) by on-line fluorimetry, as described previously.²³ Release was stimulated as indicated with 4-aminopyridine (4-AP, 1 mM), or KCl (15 mM), 10 min after the start of incubation and monitored at 2-sec intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate was expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively released after 5 min of depolarization.

Synaptosomal plasma membrane potential

The synaptosomal membrane potential can be monitored by positively charged, membrane potential-sensitive carbocyanine dyes, such as DiSC₃(5), which is a positively charged carbocyanine that accumulates in polarized synaptosomes that are negatively charged on the inside. At high concentrations, the dye molecules accumulate and the fluorescence is quenched. Upon depolarization, the dye moves out and, hence, the fluorescence increases.²⁴ Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3-min incubation, 5 μ M DiSC₃(5) was added and allowed to equilibrate before the addition of CaCl₂ (1 mM) after 4-min incubation. Then, 4-AP (1 mM) was added to depolarize the synaptosomes at 10 min, and DiSC₃(5) fluorescence was monitored at excitation and emission wave lengths of 646 and 674 nm, respectively.

Cytosolic-free Ca²⁺ concentration

The Ca²⁺ concentration ([Ca²⁺]_C) was measured using the Ca²⁺ indicator Fura-2-AM. Synaptosomes (0.5 mg/mL) were preincubated in HBM with 16 μ M BSA in the presence of 5 μ M Fura-2-AM and 0.1 mM CaCl₂ for 30 min at 37°C in a stirred test tube. After Fura-2-AM loading, synaptosomes were centrifuged in a microcentrifuge for 30 sec at 3000 g (5000 rpm). The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated cuvette in a Perkin-Elmer LS-50B spectrofluorometer. CaCl₂ (1 mM) was added after 3 min, and further additions were made after an additional 10 min. Fluorescence data were accumulated at excitation wave lengths of 340 and 380 nm (emission wavelength of 505 nm) at 7.5-sec intervals. Calibration procedures were performed as described previously,²⁵ using 0.1% SDS to obtain the maximal fluorescence with Fura-2 saturation with Ca²⁺, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca²⁺ complex. [Ca²⁺]_C was calculated using equations described previously.²⁶

Data analysis

Data were analyzed using Lotus 1-2-3, Microcal Excel, and Microcal Origin. Data are expressed as mean $\pm$ SEM. To test the significance of the effect of a drug versus control, an unpaired Student's *t*-test was used. When an additional comparison was required (such as whether a second treatment influenced the action of ferulic acid), a one-way analysis of variance was used followed by a *post hoc* LSD comparison. Analysis was completed via software SPSS (17.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

To examine the effect of ferulic acid on glutamate release, nerve terminals were depolarized with the K⁺ channel blocker 4-AP, which, by increasing the excitability of the synaptosomal plasma membrane. This increases the opening

of voltage-dependent Ca^{2+} channels (VDCCs) to elevate cytoplasmic-free $[\text{Ca}^{2+}]_c$ and, thus, induces the release of glutamate.²⁷ In synaptosomes incubated in the presence of 1 mM CaCl_2 , 4-AP (1 mM) evoked a glutamate release of 7.4 ± 0.04 nmol/mg/5 min. Application of ferulic acid (50 μM) produced an inhibition of 4-AP-evoked glutamate release to 3.6 ± 0.1 nmol/mg/5 min ($n=6$; $P<.001$), without altering the basal release of glutamate ($P>.05$; Fig. 2A). A maximal inhibition of 66% occurred with 100 μM ferulic acid. The IC_{50} value for ferulic acid inhibition of 4-AP-evoked glutamate release, derived from a concentration-response curve, was 31 μM (Fig. 2B). In addition, glutamate release evoked by 4-AP (1 mM) in the absence of extracellular Ca^{2+} was unaffected by ferulic acid (50 μM ; $n=6$; Fig. 2C). Furthermore, ferulic acid (50 μM) still caused a significant inhibition on 4-AP-induced release of glutamate in the presence of DL-TBOA (10 μM), a nonselective inhibitor of all excitatory amino acid transporter subtypes ($P<.05$; $n=5$; Fig. 2D). DL-TBOA by itself increased control 4-AP-evoked glutamate release (because of inhibition of reuptake of released glutamate; $P<.001$; $n=5$). These results demonstrate that the inhibition of 4-AP-evoked glutamate release by ferulic acid is mediated by a reduction in the Ca^{2+} -dependent exocytotic component of glutamate release.

To further understand the mechanism responsible for the ferulic acid-mediated inhibition of glutamate release, we used a membrane-potential-sensitive dye, DiSC₃(5), to determine the effect of ferulic acid on the synaptosomal plasma membrane potential. Fig. 3A shows that 4-AP (1 mM) caused an increase in DiSC₃(5) fluorescence by 15.8 ± 0.6 fluorescence units/5 min. Application of ferulic acid (50 μM) for 10 min before 4-AP addition did not alter the resting membrane potential, and produced no significant change in the 4-AP-mediated increase in DiSC₃(5) fluorescence (15.5 ± 0.9 fluorescence units/5 min; $n=5$). In addition, we confirmed the ferulic acid-mediated inhibition of glutamate release using an alternative secretagogue, high external $[\text{K}^+]$ (Fig. 3B). Elevated extracellular KCl depolarizes the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of voltage-dependent ion channels. Whereas Na^+ channels are inactivated under these conditions, VDCCs are activated nonetheless to mediate Ca^{2+} entry, which supports neurotransmitter release.²⁸ Addition of 15 mM KCl evoked controlled glutamate release of 9.4 ± 0.2 nmol/mg over 5 min, which was decreased to 4.9 ± 0.3 nmol/mg over 5 min in the presence of ferulic acid (50 μM ; $n=6$; $P<.01$; Fig. 3B). This indicates that the observed inhibition of evoked glutamate release by ferulic acid is unlikely to have been caused by a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential, or attenuation of the depolarization produced by 4-AP.

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a lowering of intraterminal Ca^{2+} levels. To investigate whether a decrease in $[\text{Ca}^{2+}]_c$ was responsible for the ferulic acid-mediated inhibition of release, we used a Ca^{2+}

indicator Fura-2 to monitor intraterminal Ca^{2+} levels directly. In Figure 4, after addition of 4-AP (1 mM), $[\text{Ca}^{2+}]_c$ in synaptosomes was elevated to a plateau level of 219.5 ± 6.8 nM. This 4-AP-evoked rise in $[\text{Ca}^{2+}]_c$ was decreased by application of ferulic acid (50 μM) to 172.4 ± 6.9 nM ($n=6$; $P<.001$).

In the adult rat cerebrocortical nerve terminal preparation, the release of glutamate evoked by depolarization is reported to be caused by Ca^{2+} influx through $\text{Ca}_v2.2$ (N-type) and $\text{Ca}_v2.1$ (P/Q-type) channels and Ca^{2+} release from internal stores, such as endoplasmic reticulum (ER) and mitochondria.^{29–33} To assess the role of $\text{Ca}_v2.2$ and $\text{Ca}_v2.1$ channels, synaptosomes were preincubated with 2 μM ω -conotoxin MVIIC (ω -CgTX MVIIC), a wide-spectrum blocker of $\text{Ca}_v2.2$ and $\text{Ca}_v2.1$ channels. As shown in Figure 5A, glutamate release evoked by 1 mM 4-AP under control conditions was significantly decreased in the presence of 2 μM ω -CgTX MVIIC alone or 50 μM ferulic acid alone ($P<.001$). When ω -CgTX MVIIC (2 μM) and ferulic acid (50 μM) were applied simultaneously, the inhibition of glutamate release following 4-AP depolarization was not significantly different from the effect of ω -CgTX MVIIC alone ($n=6$; Fig. 5A, B). On the other hand, a potential role of intracellular Ca^{2+} release in ferulic acid-mediated inhibition of glutamate release was tested in the presence of dantrolene, an inhibitor of intracellular Ca^{2+} release from ER, and CGP37157, a membrane-permeant blocker of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange. Dantrolene (100 μM) reduced control 4-AP-evoked release ($P<.01$; $n=5$). In the presence of dantrolene, however, ferulic acid (50 μM) still effectively inhibited 4-AP-evoked glutamate release ($P<.05$; Fig. 5B). Similarly to dantrolene, CGP37157 (100 μM) decreased the release of glutamate evoked by 4-AP (1 mM; $P<.01$), but it had no effect on the ferulic acid-mediated inhibition of 4-AP-evoked glutamate release ($n=5$; Fig. 5B).

DISCUSSION

In this work, isolated cerebrocortical nerve terminals (synaptosomes) retaining the morphological and functional characteristics of *in vivo* nerve terminals²¹ were used to obtain clear evidence that ferulic acid inhibits the depolarization-evoked release of glutamate. To the best of our knowledge, this study presents the first examination of the effect of ferulic acid on endogenous glutamate release at the presynaptic level. Several possible mechanisms for this effect are discussed as follows.

Neurotransmitter release is a complex phenomenon and can be modulated at several putative sites in the nerve terminal, including Na^+ channels, K^+ channels, Ca^{2+} channels, and the release process itself.^{12,34,35} Therefore, when addressing the mechanism responsible for the ferulic acid-mediated inhibition of Ca^{2+} -dependent glutamate release, this study considered two scenarios that might be involved: (1) alteration of the synaptosomal plasma membrane potential and downstream modulation of Ca^{2+} influx into the terminal, and (2) direct regulation of VDCCs affecting Ca^{2+}

FIG. 2. Ferulic acid inhibits 4-aminopyridine (4-AP)-induced glutamate release from rat cerebrocortical nerve terminals; this effect is due to a decrease in vesicular exocytosis. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/mL and incubated for 3 min before the addition of 1 mM CaCl_2 . 4-AP (1 mM) was added after a further 10 min to effect depolarization (arrow). Total glutamate release ($+\text{Ca}^{2+}$; **A**) and Ca^{2+} -independent glutamate release ($-\text{Ca}^{2+}$; **C**) was measured under control conditions or in the presence of 50 μM ferulic acid added 10 min before the addition of 4-AP. (**B**) Concentration-response curve for ferulic acid inhibition of 4-AP (1 mM)-evoked glutamate release. (**D**) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 50 μM ferulic acid and absence and presence of 10 μM DL-threo- β -benzyloxyaspartate (DL-TBOA). DL-TBOA was added 20 min before depolarization, while ferulic acid was added 10 min before depolarization. Results are mean $\pm$ SEM of four to five independent experiments. * $P < .05$, one-way analysis of variance (ANOVA). *** $P < .001$, unpaired Student's t -test. EGTA, ethylene glycol bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid.

FIG. 3. Ferulic acid does not alter the synaptosomal membrane potential. (A) Synaptosomal membrane potential monitored with 3',3'-dipropylthiadicarbocyanine iodide [DiSC₃(5)] in the absence (control) and in the presence of 50 μ M ferulic acid, added 10 min before depolarization with 1 mM 4-AP. (B) Glutamate release was induced by 15 mM KCl in the absence (control) or presence of 50 μ M ferulic acid, added 10 min before depolarization. Result are means $\pm$ SEM of five to six independent experiments. *** P < .001, unpaired Student's t -test.

FIG. 4. Ferulic acid reduces the 4-AP-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). Cytosolic-free $[Ca^{2+}]_c$ (nM) was monitored using Fura-2. Synaptosomes were stimulated with 1 mM 4-AP in the absence (control) and in the presence of 50 μ M ferulic acid, added 10 min before depolarization. Results are mean $\pm$ SEM of six independent experiments. *** P < .001, unpaired Student's t -test.

entry. The first possibility is unlikely for three reasons. First, 4-AP- versus KCl-evoked glutamate release is significantly inhibited by ferulic acid. 4-AP-mediated depolarization involves upstream K^+ and Na^+ channel activity, 15 mM external KCl affects experimental clamping of the K^+ electrochemical potential, and hence, the membrane potential.³⁶ The latter type of depolarization bypasses regulation at the level of K^+ channels and also effectively removes voltage-dependent Na^+ involvement, in that, these channels are desensitized in response to strong and continued depolarization.²⁸ Based on the mechanistic differences between the 4-AP- and KCl-mediated depolarization discussed above, it is unlikely that Na^+ channels are involved in the ferulic acid modulation of glutamate release. Second, no significant effect of ferulic acid on synaptosomal plasma membrane potential appears either in the resting condition or upon depolarization with 4-AP (indicating a lack of effect on K^+ conductance). Third, ferulic acid did not affect the 4-AP-evoked Ca^{2+} -independent glutamate release, which depends only on the membrane potential.³⁷ This indicates that ferulic acid does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. The glutamate transporter inhibitor DL-TBOA failed to affect the ferulic acid inhibition of 4-AP-evoked glutamate release, supporting this suggestion. Accordingly, these results clearly suggest that the ferulic acid-mediated inhibition of 4-AP-evoked glutamate release is mediated by a decrease in the Ca^{2+} -dependent exocytotic component of glutamate release. Moreover, this phenomenon is not because of a decrease in synaptosomal excitability caused by the modulation of Na^+ or K^+ ion channels.

If it is not the modulation of synaptosomal excitability, then the locus of action of ferulic acid must lie further downstream in the stimulus-exocytosis coupling cascade. Using Fura-2, this study demonstrated that ferulic

acid significantly decreases the 4-AP-evoked increase in $[Ca^{2+}]_c$. Furthermore, the inhibition of glutamate release by ferulic acid is highly sensitive to blockade of the $Ca_v2.2$ and $Ca_v2.1$ channels, which are known to directly participate in triggering glutamate release from synaptosomes.^{30–33} In

addition to VDCCs, however, intracellular stores of Ca^{2+} released through the ER ryanodine receptors and mitochondria may be responsible for $[Ca^{2+}]_c$ increase following depolarization.²⁹ This possibility was eliminated by the observation that the inhibitory effect of ferulic acid on 4-AP-evoked glutamate release was not affected by the ER ryanodine receptor inhibitor dantrolene, and by the mitochondrial Na^+/Ca^{2+} exchange inhibitor CGP37157. Although there is no direct evidence that ferulic acid acts on presynaptic Ca^{2+} channels, these results imply that the inhibition of glutamate release by ferulic acid occurs primarily through the suppression of Ca^{2+} influx through $Ca_v2.2$ and $Ca_v2.1$ channels. However, the blockade of $Ca_v2.2$ and $Ca_v2.1$ channel activity did not completely eliminate the inhibitory effect of ferulic acid on the 4-AP-evoked glutamate release (~6% of the activity remained), raising the possibility that the $Ca_v2.2$ - and $Ca_v2.1$ -resistant Ca^{2+} channel types are also involved in the action of ferulic acid.

Ferulic acid is widely found in fruits, vegetables, cereals, and coffee. Consumption of these foods may result in ~150–250 mg/day of FA intake.³⁸ Ferulic acid can cross the blood–brain barrier and enter the brain by rapid kinetics.³⁹ Studies have shown that ferulic acid improves memory deficits and brain damage induced by a variety of toxins, including glutamate and $A\beta1-42$.^{7,10,40} Although the exact mechanisms responsible for the neuroprotective effect of ferulic acid remain to be elucidated, the possible involvement of scavenging free radicals or antioxidant properties has been reported.^{3,41,42} In this work, the ability of ferulic acid to depress glutamate release is of particular significance and presents an additional explanation for the neuroprotective effect of ferulic acid in several neuronal injuries, especially in glutamate-induced neurotoxicity; this is because excessive glutamate release has been proposed to be involved in the pathophysiology of several neurological states, including ischemic brain damage and neurodegenerative diseases.^{43,44} On the other hand, the concentration of ferulic acid used to inhibit glutamate release in our study *in vitro* (50 μ M) is high, but the action of ferulic acid is specific. The observation supporting this statement revealed the following: (1) the effect of ferulic acid on the evoked glutamate release was prevented by chelating the extracellular Ca^{2+} ions, but was insensitive to the glutamate transporter inhibitor; (2) ferulic acid decreased the

FIG. 5. Ferulic acid-mediated inhibition of 4-AP-evoked glutamate release is abolished by $Ca_v2.2$ and $Ca_v2.1$ channels blockade. **(A)** Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 50 μ M ferulic acid, 2 μ M ω -conotoxin MVIIC (ω -CgTX MVIIC), 2 μ M ω -CgTX MVIIC, and 50 μ M ferulic acid. **(B)** Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence or presence of 50 μ M ferulic acid, and absence and presence of 2 μ M ω -CgTX MVIIC, 100 μ M dantrolene, or 100 μ M 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157). Ferulic acid was added 10 min before depolarization, whereas the other drugs were added 30 min before depolarization. Results are mean $\pm$ SEM of five to six independent experiments. * $P < .05$, one-way ANOVA. *** $P < .001$, unpaired Student's *t*-test.

depolarization-induced increase in $[Ca^{2+}]_C$, whereas it did not alter 4-AP-mediated depolarization; and (3) ferulic acid-mediated inhibition of glutamate release was abolished by the N-, P-, and Q-type Ca^{2+} channel blocker, but not by the ryanodine receptor blocker, or the mitochondrial Na^+/Ca^{2+} exchanger blocker.

In conclusion, the results described in this report demonstrate that ferulic acid inhibits glutamate release from cerebrocortical synaptosomes via a suppression of voltage-dependent Ca^{2+} entry, supporting the hypothesis that ferulic acid may have efficacy as a brain therapeutic agent against excitotoxicity, but the relevance of our finding to *in vivo* clinical situations remains to be determined. However, this finding may provide further understanding of the mode of ferulic acid action in the brain, thereby emphasizing the therapeutic potential of this compound in treating neurodegenerative disease.

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AUTHOR DISCLOSURE STATEMENT

The authors affirm that no competing financial interests exist.

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