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European Journal of Pharmacology 449 (2002) 261–267

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Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice

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Received 14 March 2002; received in revised form 24 June 2002; accepted 26 June 2002

Abstract

We previously showed that rosmarinic acid from the leaves of *Perilla frutescens* Britton var. *acuta* Kudo (Perillae Herba) has antidepressive-like activity. The aim of the present study was to examine (i) whether caffeic acid, a major metabolite of rosmarinic acid, also has antidepressive-like activity, and (ii) whether these substances inhibit either the uptake of monoamines to synaptosomes or mitochondrial monoamine oxidase activity. Rosmarinic acid (2 mg/kg, i.p.) and caffeic acid (4 mg/kg, i.p.) each significantly reduced the duration of immobility in the forced swimming test in mice. In contrast, neither substance, at doses that produced a significant reduction in the immobile response in the forced swimming test, affected spontaneous motor activity. These results indicate that, like rosmarinic acid, caffeic acid also possesses antidepressive-like activity. In neuropharmacological studies, neither rosmarinic acid (10^{-9}–10^{-3} M) nor caffeic acid (10^{-9}–10^{-3} M) affected either the uptake of monoamines to synaptosomes or mitochondrial monoamine oxidase activity in the mouse brain. These results suggest that both caffeic acid and rosmarinic acid may produce antidepressive-like activity via some mechanism(s) other than the inhibition of monoamine transporters and monoamine oxidase.
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Keywords: Rosmarinic acid; Caffeic acid; Forced swimming test; Antidepressant; (Mouse)

1. Introduction

Several traditional oriental herbal medicines, such as Hange-kouboku-to and Saiboku-to, have been used for separation of the depression and/or anxiety-related disorders, such as anxiety neurosis and anxiety hysteria (Kubota, 1996; Fukushima, 1997; Luo et al., 2000). Various findings in recent preclinical studies have supported the therapeutic value of herbal medicines in a clinical setting. For example, Saiboku-to has been shown to have an anxiolytic effect in studies using the elevated plus-maze test (Kuribara et al., 1996) and light–dark tests (Yuzurihara et al., 2000). Hange-kouboku-to has also recently been shown to have an antidepressive-like effect using the tail suspension and forced swimming tests (Luo et al., 2000).

The leaves of *Perilla frutescens* Britton var. *acuta* Kudo (Perillae Herba) are commonly found in traditional oriental herbal medicines, including Hange-kouboku-to and Saiboku-to, which are primarily used to treat depression and anxiety-related disorders (Kubota, 1996; Fukushima, 1997). Previously, we identified rosmarinic acid as a novel antidepressive-like substance from components within Perillae Herba (Takeda et al., 2002) based on several findings. First, some extracts from Perillae Herba produced antidepressive-like activity in the forced swimming test. Second, three-dimensional high-performance liquid chromatography and fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) spectral analysis clearly showed extracts from Perillae Herba, which produced an antidepressive-like effect, contained abundant rosmarinic acid. Third, extracts from another species of Perillae Herba, which contains only low levels of rosmarinic acid, did not have an anti-immobility effect. Finally, rosmarinic acid itself also has antidepressive-like activity.

Previous pharmacokinetic studies have clearly demonstrated that rosmarinic acid as well as Perillae Herba is

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PII: S0014-2999(02)02037-X

metabolized to caffeic acid in vivo (Nakazawa and Ohsawa, 1998, 2000). Therefore, the present study was designed to examine whether caffeic acid also produces antidepressive-like activity in the forced swimming test. In addition, the effects of these substances on monoamine uptake into synaptosomes and mitochondrial monoamine oxidase activity were also examined, since the inhibition of either monoamine transporters or monoamine-degrading enzymes may underlie the therapeutic value of existing clinically effective antidepressants (Vetulani and Nalepa, 2000).

2. Materials and methods

The present studies were conducted in accordance with Guide for Care and Use of Laboratory Animals as adopted by the Committee on Care and Use of Laboratory Animals of Tokyo Medical University and the Japanese Pharmacological Society.

2.1. Animals

Male ICR mice (Charles River, Japan) weighing 30–35 g were housed at a room temperature of 23 ± 1 °C with a 12-h light–dark cycle (lights on from 6:00 am to 6:00 pm). Food and water were available ad libitum.

2.2. Forced swimming test

The forced swimming test was performed over 2 days, i.e. a day for the pre-swimming session and a day for the test session, following the procedure described by Porsolt et al. (1977, 1978) with a minor modification (Takeda et al., 2002). Briefly, in the pre-swimming session, mice were individually placed for 15 min in cylinders (19 cm in diameter and 25 cm high) filled with water (24 ± 1 °C) up to 15 cm deep. Twenty-four hours later, mice underwent the test session. In the test session, mice were again placed in cylinders filled with water, and the duration of immobility was recorded for 5 min by an activity-monitoring system (SUPER-MEX, Muromachi Kikai, Japan). Rosmarinic acid (1–4 mg/kg, i.p.), caffeic acid (1–4 mg/kg, i.p.) or the vehicle (10 ml/kg, i.p.) was administered 30 min prior to the start of the test session.

2.3. Measurement of spontaneous motor activity

The spontaneous motor activity of mice was measured by an activity-monitoring system (SUPER-MEX, Muromachi Kikai). Briefly, mice were placed in cylinders (19 cm in diameter and 25 cm high) and total motor activity counts in each 10-min segment were automatically recorded for 90 min prior to the injections and for 180 min following the administration of rosmarinic acid (2 mg/kg, i.p.), caffeic acid (4 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.).

2.4. Monoamine uptake assay

[^{3}H]Norepinephrine, [^{3}H]dopamine and [^{3}H]5-hydroxytryptamine (5-HT) uptake were determined in crude synaptosomes from mouse brain, as previously described (Inazu et al., 2001). To obtain tissues, mice were sacrificed by decapitation, their brains were quickly removed and the cerebral cortex, striatum and hippocampus were dissected on ice. The cerebral cortex, striatum and hippocampus were used for [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]5-HT uptake assays, respectively. Tissues were homogenized in 20 volumes of 0.32 M sucrose with a Teflon pestle in a fitted glass tube. Homogenates were centrifuged at 1000 × g for 10 min, with retention of the supernatant followed by centrifugation at 27,000 × g for 20 min. The resulting pellet was resuspended in 0.32 sucrose, and then ice-cold Krebs Henseleit buffer (121 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.4 nM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.13 mM EDTA·2Na, 0.2% ascorbic acid and 0.1 mM pargyline) bubbled with 95% O_2 and 5% CO_2 was added. Protein concentration was determined by the Coomassie Plus Protein Assay Reagent (Pierce Chemical, IL, USA). Five and eight mg of tissues (original wet weight) per ml were used for dopamine and norepinephrine or 5-HT uptake assays, respectively. Uptake assays were performed in tubes containing synaptosomes, various concentrations of

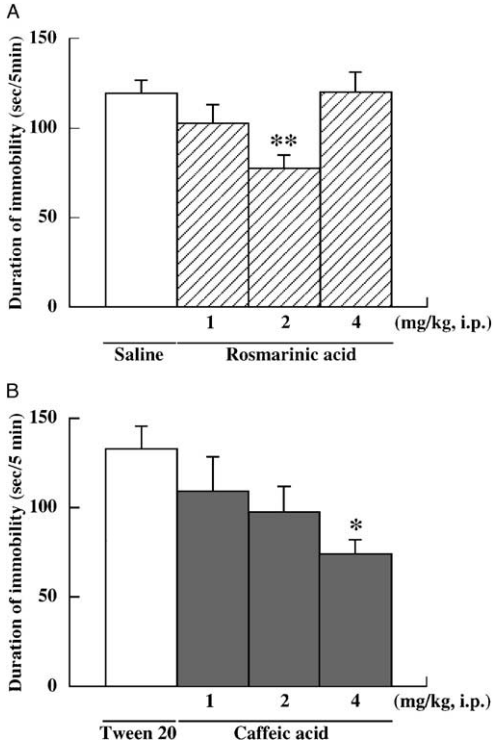

Fig. 1. Effects of rosmarinic acid (A) and caffeic acid (B) on forced swimming-induced immobility in mice. Drugs or vehicle (saline or 1% Tween 20) were administered intraperitoneally 30 min before the second measurement of immobility. Each column represents the mean with S.E.M. of eight mice. * $P<0.05$, ** $P<0.01$ vs. vehicle-treated group.

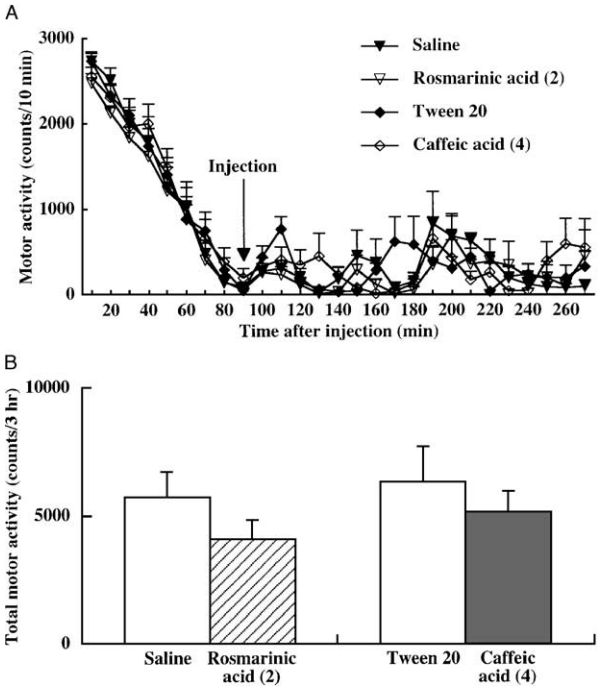

Fig. 2. Effects of rosmarinic acid and caffeic acid on mouse spontaneous motor activity. Motor activity counts in each 10-min segment were recorded for 90 min prior to the injections and for 180 min following the administration of drugs or vehicle (saline or 1% Tween 20). Time-course of changes in motor activity and total motor activity for 180 min after vehicle or drug administration are shown in panels (A) and (B), respectively. Each point and column represents the mean with S.E.M. of eight mice.

test compounds and [^{3}H]norepinephrine, [^{3}H]dopamine or [^{3}H]5-HT in assay buffer with a final volume of 1 ml. Synaptosomes were preincubated with the test compounds for 10 min at 37 °C and then [^{3}H]norepinephrine, [^{3}H]dopamine or [^{3}H]5-HT (final concentration = 100 nM) was added. Uptake was terminated after 5 min by rapid filtration through a Brandel cell harvester (M-24R, Neuroscience) and the mixture was rinsed with 3 × 5 ml of ice-cold saline through Whatman GF/B glass-fiber filters that had been presoaked in saline for at least 1 h. Filter mats were allowed to air dry and then placed in scintillation vials containing 4 ml of scintillation fluid. Radioactivity was determined by liquid scintillation spectrometry. Non-specific uptake was defined as that present at 2 °C. Specific uptake (temperature-dependent uptake) was determined by subtracting the accumulation at 2 °C (non-specific uptake) from that at 37 °C (total uptake). Results are expressed as percent specific uptake.

2.5. Monoamine oxidase assay

Monoamine oxidase activity was measured using [^{14}C]5-HT and [^{14}C]β-phenylethylamine as previously described (Egashira and Yamanaka, 1993; Egashira et al., 1996, 1999). Mice were sacrificed by decapitation and then their brains were quickly removed. The brains were homogenized in 10 volume 0.32 M sucrose solution adjusted to pH 7.4 with 0.5 M $NaHCO_3$. Mitochondrial fractions were prepared by differential centrifugation (Egashira and Yamanaka, 1993). The mitochondria were washed twice by resuspension in 0.32 M sucrose solution and used as enzyme preparations. The enzyme preparation was preincubated for 20 min at 20 °C with drugs at concentrations of 1 μM to 1 mM before adding the substrates. Simultaneously, the enzyme preparation served as a blank was processed by heat treatment at 100 °C for 5 min. The remaining monoamine oxidase activity was measured after the substrates were added. The reaction was initiated by adding 25 μl of substrate including labeled 5-HT (100 μM) or β-phenylethylamine (10 μM), and the mixture was incubated for 30 min at 37 °C. The

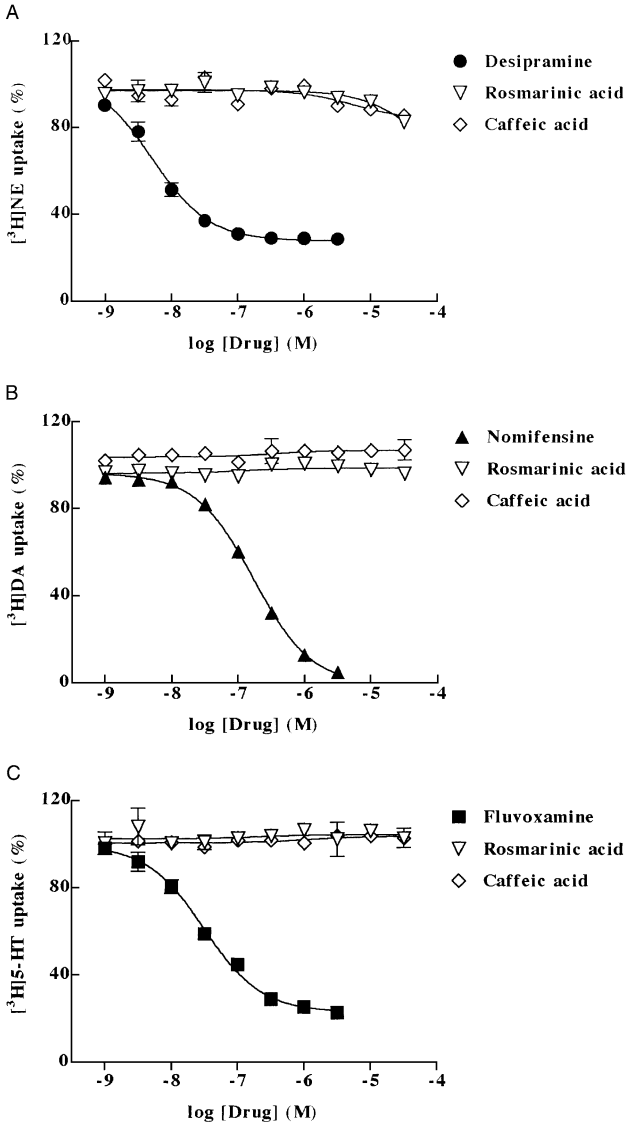

Fig. 3. Effects of rosmarinic acid and caffeic acid on [^{3}H]norepinephrine (A), [^{3}H]dopamine (B) and [^{3}H]5-HT (C) uptake into mouse synaptosomes. Synaptosomes were preincubated with rosmarinic acid or caffeic acid for 10 min, and the uptake of 100 nM [^{3}H]norepinephrine, [^{3}H]dopamine or [^{3}H]5-HT was then measured for 5 min. Each point represents the mean with S.E.M. of three experiments.

reaction was then stopped by adding hydrochloric acid (2 N). The reaction products were extracted with 2 ml of benzene-ethyl acetate (1:1, volume/volume) for separate the radiolabelled metabolites from the radiolabelled parent compound. Triton X-100-toluene scintillation liquid (10 ml) was added to 1-ml samples of the extract, and radioactivity was measured by liquid scintillation spectrometry. Enzyme activity was expressed as nanomoles per minute per milligram of protein. The protein concentrations of the enzyme preparations were measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The protein concentration of the enzyme preparations was adjusted to 1 mg/ml.

2.6. Drugs

Rosmarinic acid was provided by Tsumura (Ibaraki, Japan). Caffeic acid and desipramine were purchased from Sigma (St. Louis, MO, USA). Nomifensine was purchased from Research Biochemicals Int. (Natick, MA, USA). Fluvoxamine was provided by Meiji Seika Kaisya (Kanagawa, Japan). Levo-[Ring-2,5,6-^{3}H]-norepinephrine (specific activity: 1.48–2.96 TBq/mmol), 3,4-[Ring-2,5,6-^{3}H]-dopamine (1.11–2.22 GBq/mmol), 5-[1,2-^{3}H]-HT (0.55–1.11 TBq/mmol), 5-[2-^{14}C]-HT (1.48–2.22 GBq/mmol) and β-[ethyl-1-^{14}C]-phenylethylamine (1.48–2.22 GBq/mmol) were purchased from NEN Life Science products (Boston, MA, USA). For in vivo studies, doses of test compounds were calculated as the free base and were administered as a solution with saline or as a suspension with 1% Tween 20. For in vitro studies, compounds were dissolved in assay buffer or 0.1% dimethyl sulfoxide (DMSO).

2.7. Statistical analysis

All data are presented as the mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for the statistical evaluation ($P<0.05$ and 0.01).

3. Results

3.1. Effects of rosmarinic acid and caffeic acid on the duration of immobility in the forced swimming test

The effects of rosmarinic acid and caffeic acid on the duration of immobility of mice in the forced swimming test are shown in Fig. 1. Significance was shown by the ANOVA using four groups from Fig. 1A ($F(3,28)=4.658$, $P<0.01$) and Fig. 1B ($F(3,28)=2.994$, $P<0.05$). Rosmarinic acid had a significant effect at a dose of 2 mg/kg ($P<0.01$), however, this effect disappeared at 4 mg/kg. In contrast, caffeic acid (1–4 mg/kg, i.p.) dose-dependently reduced the duration of immobility, and a significant effect was observed at 4 mg/kg ($P<0.05$) (Fig. 1B).

3.2. Effects of rosmarinic acid and caffeic acid on spontaneous motor activity in mice

The effects of rosmarinic acid and caffeic acid on spontaneous motor activity in mice are shown in Fig. 2. Neither rosmarinic acid (2 mg/kg, i.p.) nor caffeic acid (4 mg/kg, i.p.), at doses that significantly reduced the immobile response in the forced swimming test, affected spontaneous motor activity.

3.3. Effects of rosmarinic acid and caffeic acid on [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]5-HT uptake into synaptosomes from mouse brain regions

The effects of rosmarinic acid and caffeic acid on [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]5-HT uptake into synaptosomes from mouse brain regions are shown in Fig. 3. [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]5-HT uptake was potently inhibited by the monoamine reuptake inhibitors desipramine, nomifensine and fluvoxamine, respectively. Under these experimental conditions, however, neither rosmarinic acid nor caffeic acid altered the synaptosomal uptake for any of the monoamines ($IC_{50}>300$ μM).

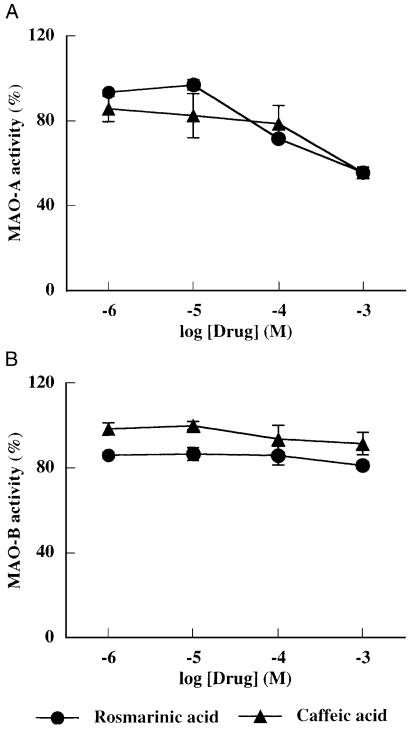

Fig. 4. Effects of rosmarinic acid and caffeic acid on monoamine oxidase activity in mitochondria of mouse brain. After incubation for 20 min with rosmarinic acid or caffeic acid, monoamine oxidase-A (A) and monoamine oxidase-B (B) activities were determined with 200 μM 5-HT and 50 μM β-phenylethylamine as substrates, respectively, for 30 min. Each point represents the mean with S.E.M. of three experiments.

3.4. Effects of rosmarinic acid and caffeic acid on monoamine oxidase activity in mitochondria

The effects of rosmarinic acid and caffeic acid on monoamine oxidase activity in mouse mitochondria are shown in Fig. 4. As shown in Fig. 4A, rosmarinic acid and caffeic acid slightly inhibited monoamine oxidase-A activity by 40.4% and 35.5%, respectively, with an IC_{50}>1 mM. In contrast, monoamine oxidase-B activity was not affected at all by treatment with either test compound (IC_{50}>1 mM) (Fig. 4B).

4. Discussion

We previously identified rosmarinic acid from Perillae Herba as a novel antidepressive-like substance (Takeda et al., 2002). In the present study, we found that, in addition to rosmarinic acid, its major metabolite caffeic acid also significantly reduced the duration of immobility of mice in the forced swimming test, a well-accepted stress model of depression. Although drugs that alter general motor activity may give false positive/negative results in the forced swimming test, the present study demonstrated that both rosmarinic acid and caffeic acid at doses that produced an antidepressive-like effect did not significantly change motor activity in naive mice. Therefore, it is unlikely that the antidepressive-like effects of these substances observed in the forced swimming test are based on the stimulation of general motor activity. Thus, caffeic acid, like rosmarinic acid, may possess antidepressive-like properties.

Rosmarinic acid produced a significant antidepressive-like effect at only one dose. This limited effect of rosmarinic acid would throw doubt whether rosmarinic acid has real antidepressive potential. However, our recent study using the conditioned fear stress paradigm, an animal model of anxiety and/or depression, also demonstrated that rosmarinic acid produced U-shaped reduction in the freezing behavior in the same way as the observation in the present study, and significant effects were observed at two doses (unpublished observation). The effectiveness of rosmarinic acid in both studies using two different types of stress models suggest that rosmarinic acid may have an ability to inhibit emotional changes produced by stress, whereas, there may be a critical dose range for producing the efficacy.

The reason for lack of dose-dependent effect of rosmarinic acid is unknown, however, under the present circumstances, we provide one possibility that its metabolites might be involved. Previous pharmacokinetic study has defined a number of metabolites of rosmarinic acid besides caffeic acid, i.e. coumaric acid, ferulic acid and hydroxyphenylpropionic acid (Nakazawa and Ohsawa, 1998). Although the psychopharmacological effects of these metabolites have not been examined, any of them might possibly interfere with the antidepressive-like effect of rosmarinic acid.

The action of antidepressants, which represent a large and increasing share of the public health expenditure, cannot yet be explained by a definite mechanism (Sandler, 1998). The initial hypothesis regarding the action of these drugs was based mainly on the primary effect of tricyclic and monoamine oxidase inhibitors on monoamine transporters and monoamine oxidase, respectively, i.e. the so-called monoamine hypothesis (Bunney and Davis, 1965; Schildkraut, 1965). This hypothesis stated that the increased availability of monoamine neurotransmitters at synaptic sites and the consequent receptor changes would compensate for a deficit in monoaminergic transmission and cause a therapeutic effect. However, this hypothesis was soon revealed to be too simplistic, mainly due to the temporal discrepancy between the timing of the primary biochemical effect of the drugs (minutes to hours) and the onset of therapeutic action (weeks). Another reason was the lack of a clear correlation between the depressive state and a depletion of monoamines (Gumnick and Nemeroff, 2000; Popoli et al., 2000). Furthermore, while there is considerable evidence to support the effectiveness of pharmacotherapy in the treatment of depression using these drugs, 30–40% of all patients do not respond sufficiently to the initial treatment, and the therapeutic action of these drugs has a delayed onset (Doris et al., 1999; Gumnick and Nemeroff, 2000). Under the present circumstances, the appearance of a substance with a novel antidepressive mechanism may provide an opportunity to solve these problems. Interestingly, we found in the present study that neither rosmarinic acid nor caffeic acid affected the uptake of monoamines to synaptosomes or mitochondrial monoamine oxidase activity in the mouse brain. These results suggest that both caffeic acid and rosmarinic acid may produce antidepressive-like activity via some mechanism(s) other than the inhibition of monoamine transporters and monoamine oxidase. Further studies on the mechanisms involved in the antidepressive-like properties of both substances could help to explain the pathophysiology underlying depression, and pave the way for new therapeutic strategies.

The detailed mechanisms involved in the antidepressive-like properties of rosmarinic acid and caffeic acids are not yet clear. However, previous pharmacological studies have revealed that rosmarinic acid inhibits the histamine release from mast cells (Rimando et al., 1987), and that caffeic acid can activate the α_1-adrenoreceptor system (Cheng and Liu, 2000) and inhibit the production and release of nitric oxide (NO) (Soliman and Mazzio, 1998; Yokozawa and Chen, 2000). It has been suggested that these systems in the brain may contribute to stress and depression. Forced swimming increased the brain content of histamine and histamine turnover (Noguchi et al., 1992), and H_1 and H_3 receptor antagonists reduced the duration of immobility in the forced swimming test (Noguchi et al., 1992; Pérez-García et al., 1999). Some antidepressants such as doxepin, mianserin and amitriptyline are potent competitive H_1 receptor antagonists, as determined in several different assay systems (Richelson,

1992). Furthermore, previous studies using the forced swimming test have suggested that either the activation of α_1-adrenoreceptors or the inhibition of NO production may also be involved in the expression of antidepressive-like effects (Martin et al., 1986; Jefferys and Funder, 1996; Song et al., 1996; Harkin et al., 1999; Yildiz et al., 2000). Therefore, these brain systems should be examined in future studies to elucidate the detailed mechanisms involved in the antidepressive effects of rosmarinic acid and caffeic acid. Further, it is also of interest to discover whether both substances modify monoamine transmission by a direct action at monoamine receptors, in addition to α_1-adrenoreceptors. These neurochemical and receptor binding profiles should also be clarified in future studies.

It is also possible that their effects may involve the direct modulation of a second messenger system. It has recently been reported that caffeic acid inhibits both protein kinase A and protein kinase C activity in vitro (Nardini et al., 2000). Increased evidence suggests that the therapeutic effects of existing antidepressants are associated with adaptive changes in post-receptor signaling, rather than with their primary action (Popoli et al., 2000). For example, chronic administration of various types of monoamine reuptake inhibitors that exhibited therapeutic activity clinically decreased the activity of protein kinase C or protein kinase A (Moyer et al., 1986; Nestler et al., 1989; Mann et al., 1995). Although it is not known whether the previously reported in vitro inhibition of the activity of protein kinase C and protein kinase A by caffeic acid could play a significant role in the modulation of cell functions in vivo, these inhibitory effects of caffeic acid might be involved in the antidepressive-like activity found in the present study.

In conclusion, the present study clearly demonstrated that both caffeic acid and rosmarinic acid reduced the duration of immobility in the forced swimming test in mice. However, neither substance affected the uptake of monoamines or monoamine oxidase activity. These results suggest that rosmarinic acid and caffeic acid may have antidepressive mechanisms different from those of either monoamine transporter or monoamine oxidase inhibitors currently used clinically.

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