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Inhibitory Effects of Paeonol on Morphine-Induced Locomotor Sensitization and Conditioned Place Preference in Mice

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The inhibitory effects of paeonol, a major compound of *Paeoniae radix*, on the development of locomotor sensitization, conditioned place preference (CPP) and dopamine receptor supersensitivity induced by the repeated administration of morphine were investigated through behavioral experiments. A single administration of morphine produces hyperlocomotion. Repeated administration of morphine develops sensitization (reverse tolerance), a progressive enhancement of locomotion, which is used as a model for studying the drug-induced drug-seeking behaviors, and CPP, which is used as a model for studying drug reinforcement. Paeonol inhibited morphine-induced hyperlocomotion, sensitization and CPP. In addition, paeonol inhibited the development of postsynaptic dopamine receptors supersensitivity, which may be an underlying common mechanism that mediates the morphine-induced dopaminergic behaviors such as sensitization and CPP. Apomorphine (a dopamine agonist)-induced climbing behaviors also were inhibited by a single direct administration of paeonol. These results provide evidence that paeonol exerts anti-dopaminergic activity, and it is suggested that paeonol may be useful for the prevention and therapy of these adverse actions of morphine.

Key words: Paeonol, Morphine, Hyperlocomotion, Sensitization, Conditioned place preference, Dopamine receptor supersensitivity, Climbing behavior

INTRODUCTION

A single administration of morphine in rodents induces hyperlocomotion and stereotyped behaviors (Narita *et al.*, 1993; Hunada *et al.*, 1994). Dopaminergic and noradrenergic neurons in the central nervous system play important roles in the behavioral effects of abuse drugs (Rethy *et al.*, 1971; Kuschenski and Hornykiewicz, 1974; Iwamoto, 1981). Repeated exposure to morphine produces progressive enhancement in behavior, a phenomenon known as behavioral sensitization or reverse tolerance (Babbini and Davis, 1972; Shuster *et al.*, 1975; Kaliva and Duffy, 1987; Kuribara and Tadokoro, 1989). Sensitization provides a useful animal model for drug-seeking behaviors (Robinson and Berridge, 1993). In addition, the repeated intermittent administration of morphine to animals induces reinforcing properties. The paradigm known as conditioned place preference (CPP) has been widely used in animal research to investigate the drug reinforcement (Mucha *et al.*, 1982; Bardo *et al.*, 1984).

Repeated use of addictive drugs produces multiple changes in the brain that lead to sensitization and dependence. Both sensitization and CPP commonly share dopaminergic neurons projecting to the ventral tegmental area of the midbrain and to the nucleus accumbens and other forebrain structures (Kalivas and Duffy, 1987; Kalivas and Stewart, 1991; Wood and Alter, 1998). The research, so far, demonstrates that the central dopaminergic systems play important roles in sensitization and CPP to morphine. In addition, the chronic administration of morphine leads to postsynaptic receptor changes such as increased sensitivity to dopamine receptor stimulation. In other word, both postsynaptic dopamine receptor supersensitivity and behavioral sensitization result simultaneously from chronic administration of morphine (Shuster *et al.*, 1975; Titzman *et al.*, 1979; Kalivas and Stewart, 1991). Several anti-dopaminergic drugs have been investigated to determine

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their usefulness in the treatment of adverse effects such as drug-seeking and reinforcement of abuse drugs (Jeziorski and White, 1995; Li *et al.*, 2004; Li *et al.*, 2004). Recently, natural products for the treatment of adverse effects of abuse drugs have been of more interest in because of their own lower toxicity (Kim *et al.*, 1996; Huong *et al.*, 1997; Kim *et al.*, 1998; Kim *et al.*, 1999).

Paeonol, a major component of *Paeoniae radix*, has antioxidant and anti-inflammatory activities (Chou, 2003). Paeonol also has sedative, antileptic, analgesic and anxiolytic-like effects (Mi *et al.*, 2005). On the other hand, a folk medicine composed of seven herbal drugs, including *Paeoniae radix and Panax ginseng*, has been traditionally used as an antidote in the treatment of morphine-tolerant/dependent patients in Asian countries. Previously, we reported that ginseng inhibited hyperlocomotion, sensitization and CPP induced by morphine.

Here, we investigated whether or not paeonol derived from *Paeoniae Radix* might be beneficial in the treatment of adverse effects induced by morphine, one of the abuse drugs. We also evaluated the inhibitory effects of paeonol on morphine-induced sensitization and CPP and assessed whether or not these effects result in anti-dopaminergic activity.

MATERIALS AND METHODS

Animals and drugs

Group of 10-15 ICR male mice weighing 22-27 g, were used in all experiments. They were housed, 10-15 animals per cage with water and food available *ad libitum* under an artificial 12:12 (h) light/dark cycle (light at 07:00) and constant temperature (22±2°C). The drugs used were morphine hydrochloride (Dae-Won Pharm. Co., Seoul, Korea), apomorphine hydrochloride (Sigma, St. Louis, MO, U.S.A.). Apomorphine was dissolved immediately before use in physiological saline containing 0.1% ascorbic acid. All other drugs were dissolved in physiological saline.

Isolation of paeonol

The sample (2 kg) of *Paeoniae Radix* (root) purchased from the market was cut into the small size and extracted with methanol in water bath. The methanol extract was evaporated to be dry. The residue (500 g) was suspended with water and partitioned with hexane. The hexane solution was evaporated to dry. The hexane fraction (67 g) was fractionated on silica gel (60-230 mesh) column chromatography using a gradient of hexane-acetone (100:1 → 1:1) afforded three fractions (Fr. 1~3). The Fr. 1 afforded crude crystal and it was re-crystallized in ethyl acetate to give white crystal (21 g).

Measurement of hyperlocomotion induced by morphine

The locomotor activity of mice was measured using a tilting-type ambulometer (AMB-10, O'Hara, Tokyo, Japan). Each mouse was placed in the activity cage (20 cm in diameter, 18 cm in height). Drugs were administered after an adaptation period of 10 min. Paeonol (25, 50 and 100 mg/kg) was administered orally (p.o.) 1 h prior to the subcutaneous (s.c.) injection of morphine (10 mg/kg, Dae-Won Pharm. Co., Korea). Drugs were administered after an adaptation period of 10 min. Paeonol (25, 50 and 100 mg/kg) was administered orally (p.o.) 1 h prior to the subsutaneous (s.c.) injection of morphine (10 mg/kg). In the preliminary experiments, the combined effects of morphine and paeonol were investigated at various time intervals. The maximal inhibitory effects of paeonol on morphine-induced locomotor activity were observed when paeonol was administered 1 h prior to the morphine injection. In addition, the preliminary experiments indicated that the locomotor activity induced by morphine for 1 h was consistent and reliable. Therefore, the locomotor activity was measured for 1 h after the morphine injection.

Measurement of locomotor sensitization induced by morphine

Morphine (10 mg/kg. s.c.) was administered once a day for 7 days according to our previous methods to induce locomotor sensitization (Kim *et al.*, 1995; Kim *et al.*, 2002). Paeonol (25, 50 and 100 mg/kg, p.o.) was administered once a day 1 h before the injection of morphine injection for 7 days. The morphine-induced locomotor activity was also measured for 1 h by using a tilting-type ambulometer. The mice were first allowed to perambulate for 10 min in the activity cages followed by a 1-h test period immediately after the morphine injection. The development of sensitization day after day was evidenced by an increased locomotor activity response to morphine as compared with that of at day 1. The inhibition of reverse tolerance by paeonol was evidenced by a lesser locomotor activity produced by morphine.

Measurement of CPP induced by morphine

Apparatus

The CPP apparatus was made by a modification of the method of Mucha *et al.* (1982). It consisted of two square-based Plaxiglas compartment (15×15×15 cm), one with a white and the other with a black box joined by a gray tunnel (3×3×7.5 cm) that could be closed by guillotine doors. To provide tactile differences between the floors of the compartments, the white compartment had wire mesh and the black compartment had a metal grid. Removal of the guillotine doors during the pre-testing and the final testing phase allowed animals freely access to both com-

partments. Movement was recorded for 15 min via a photo-beam detector connected via electrical interface to an IBM-compatible PC computer.

Procedure

The control mice received saline immediately before the exposure to the white or black compartment. Morphine (5 mg/kg, s.c.) was given immediately before the mice were placed in the white compartment. To test the effect of paeonol alone or in combination with morphine, paeonol (25, 50, 100 mg/kg, p.o.) was administered 1 h prior to the morphine or saline injection, respectively. Phase 1 (pre-testing phase): on day 1, the mice were pre-exposed to the test apparatus for 15 min. The guillotine doors were raised and each animal was allowed to move freely between the two compartments. On day 2, baseline preference was determined for the non-preferred side vs. the preferred side for 15 min. Phase II (conditioning phase): on days 3, 5, 7 and 9, mice were injected with the drug before being confined in the white compartment, the non-preferred side, for 60 min. On days 4, 6, 8 and 10, the mice were injected with saline before confinement in the black compartment, the preferred side, for 1 h. Phase III (testing phase): on day 11, the guillotine doors were raised. The mice were placed in the central tunnel and the time spent by the mice in the two compartments was recorded for 15 min. The scores were calculated from the changes of the testing phase and the pre-testing phase in the white compartment.

Measurement of dopamine receptor supersensitivity induced by morphine

Additional groups of mice that had received the same morphine dose (10 mg/kg, s.c.) as in the reverse tolerance experiment were used to determine the inhibitory effect of paeonol (25, 50 and 100 mg/kg, p.o.) on the development of behavioral sensitization to apomorphine (2 mg/kg, s.c.), a dopamine receptor agonist. Morphine was administered once a day for 7 days according to the paradigm of the reverse tolerance test. On day 8, 24 h after the final injection of morphine, dopamine receptor supersensitivity was evidenced by an increased locomotor activity induced by apomorphine. The apparatus and procedure used were the same as described in previous reports (Bhargava, 1980; Kim *et al.*, 1995). The locomotor activity induced by apomorphine was measured for 10 min. The inhibitory effect of paeonol on the development of morphine-induced behavioral sensitization to apomorphine was evidenced by lesser locomotor activity in response to apomorphine.

Measurement of apomorphine-induced climbing behavior

Climbing behavior in mice was measured using the three point rating scale of Protais *et al.* (1976). The apparatus and procedure used were the same as described in our previous report (Kim *et al.*, 1995). Immediately, after a subcutaneous injection of apomorphine (2 mg/kg), the mice were put into cylindrical individual cages (12 cm in diameter and 14 cm in height) with the floor and wall consisting of vertical metal bars (2 mm in diameter and I cm apart). After a 5-min period of exploratory activity, climbing behavior was measured by all-or-none scores at 10, 20 and 30 min after the administration of apomorphine, and the three scores were averaged. The scores of this behavior were evaluated as follows: four paws on the floor (0 point), fore feet holding the wall (1 point), and four paws holding the wall (2 points). Paeonol (25, 50 and 100 mg/kg, p.o.) was administered to mice 1 h before the apomorphine injection.

Statistics

The data were expressed as mean±SEM. The significance of the hyperactivity, reverse tolerance, CPP and dopamine receptor supersensitivity results was assessed by an analysis of variance (ANOVA) followed by Dunnett's test.

RESULTS

Inhibitory effects of paeonol on morphine induced hyperlocomotion

The morphine-treated group showed a marked increase in locomotor activity by 616% (1,8757 counts, $P<0.005$) when compared with that of saline group (301 counts). Meanwhile, paeonol (25, 50 and 100 mg/kg, p.o.) administered 1 h prior to the morphine injection, inhibited about 9.2% (1,686 counts), 23.3% (1,425 counts) and 41.4% (1,089 counts, $P<0.05$), respectively, compared with that of morphine control group (Fig. 1).

Inhibitory effects of paeonol on morphine induced sensitization

Morphine (10 mg/kg, s.c.)-induced locomotor activity was progressively enhanced by the repeated administration of morphine once a day for 7 days when compared with that of saline group, suggesting the development of sensitization to hyperlocomotion induced by morphine. Meanwhile, paeonol (25, 50 and 100 mg/kg, p.o.) administered 1 h before the morphine injection inhibited about 24.6% (2,547 counts, $P<0.05$), 26.4% (2,608 counts, $P<0.05$), and 32.8% (2,398 counts, $P<0.01$), respectively, compared with that of morphine group at day 6 (Fig. 2).

Inhibitory effects of paeonol on morphine induced CPP

Groups treated only with paeonol (100 mg/kg) did not

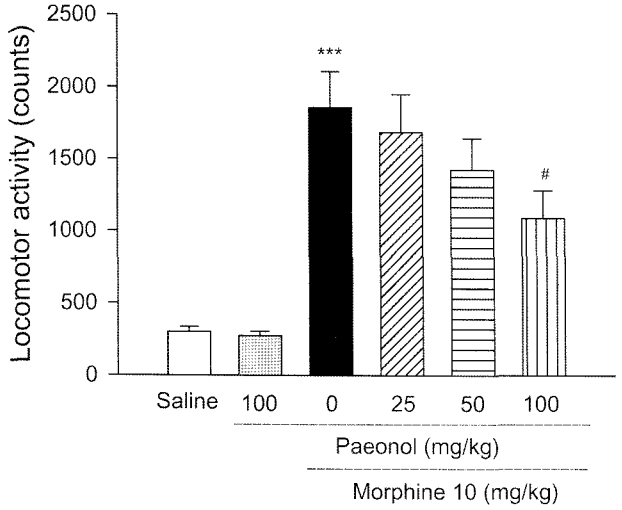

Fig. 1. Inhibitory effect of paeonol on the hyperlocomotion induced by morphine. Paeonol (25, 50 and 100 mg/kg) was administered orally to the mice 1 h before the morphine (10 mg/kg, s.c.) injection. The locomotor activity was measured for 1 h. Each value is the mean ± SEM of at least 10 mice. ***P<0.005, compared with that of the saline group. #P<0.05, compared with that of morphine group.

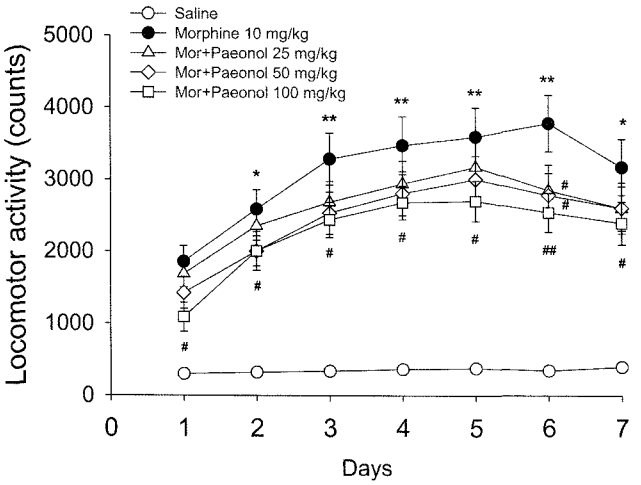

Fig. 2. Inhibitory effect of paeonol on the development of sensitization induced by morphine. Morphine (10 mg/kg, s.c.) was administered to the mice once a day for 7 days. Paeonol (25, 50 and 100 mg/kg) was administered orally to the same mice 1 h before the morphine injection. The locomotor activity was measured for 1 h after the morphine injection. Each value is the mean ± SEM of at least 10 mice. *P<0.05, **P<0.01, compared with that of the morphine control group at day 1. #P<0.05, ##P<0.01, compared with that of morphine control group.

show CPP, compared with that of the saline group. The group treated with morphine (5 mg/kg) showed significant effect of CPP with 107 sec, 102 sec less than the -5 sec of the saline group (P<0.01). The group pretreated with paeonol (100 mg/kg) showed a mark inhibition of morphine-induced CPP, yielding -80 sec 187 sec less than the 107 sec of the morphine control group (P<0.01). In addition, the group pretreated with paeonol (25 and 50 mg/kg) also

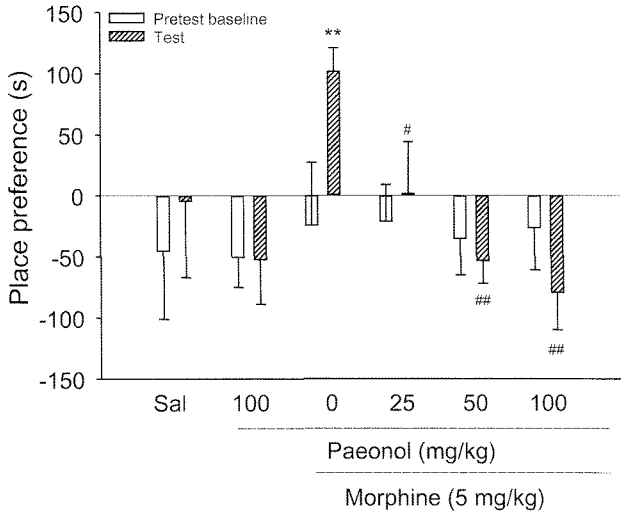

Fig. 3. Inhibitory effect of paeonol on morphine-induced CPP. In the conditioning phase, mice were injected with morphine according to the CPP test paradigm. Paeonol (25, 50 and 100 mg/kg) was administered 1 h before the morphine (5 mg/kg) injection. The scores were expressed as the differences in time spent by the mice between the testing phase and the pre-testing phase in the drug-paired compartment. Each value is the mean ± SEM of at least 10 mice. **P<0.01, compared with that of the vehicle group. #P<0.05, ##P<0.01, compared with that of morphine group.

showed significant inhibition of morphine-induced CPP, respectively (P<0.05 and P<0.01) (Fig. 3).

Inhibitory effects of paeonol on dopamine receptor supersensitivity

The mice which received the same chronic administration of morphine (10 mg/kg) as in the locomotor sensitization test produced an enhanced locomotor activity to apomorphine (2 mg/kg), showing 98.9 counts (P<0.005), when compared with that saline group (31.2 counts), suggesting the development of postsynaptic DA receptor supersensitivity in morphine-induced locomotor sensitized mice. However, paeonol (25, 50 and 100 mg/kg) administration 1 h before the morphine injection inhibited about 42.7% (56.7 counts, P<0.05), 50.6% (48.9 counts, P<0.05) and 67.8% (31.8 counts, P<0.01), respectively, compared with that of morphine control group (Fig. 4). These results suggest that paeonol inhibits the development of postsynaptic dopamine receptor supersensitivity in morphine-induced locomotor sensitized mice.

Inhibitory effects of paeonol on apomorphine induced climbing behaviors

The maximum response of climbing behaviors by apomorphine (2 mg/kg) was observed in this experiment (P<0.005). The groups treated only with saline and paeonol themselves did not show any climbing behavior. Pretreatment with paeonol (50 and 100 mg/kg) produced a signi-

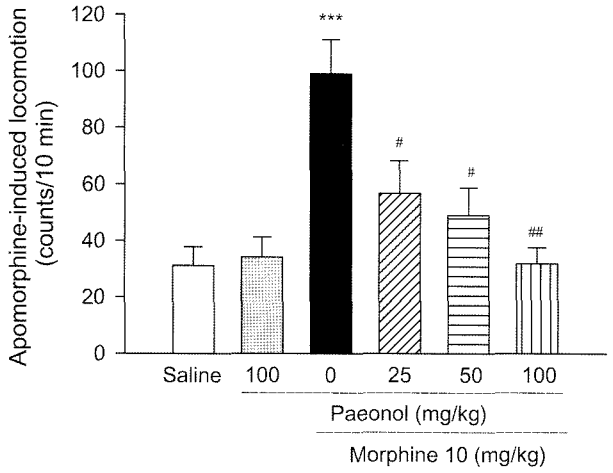

Fig. 4. Inhibitory effect of paeonol on dopamine receptor supersensitivity to apomorphine in morphine-induced sensitized mice. Additional groups of the mice that received the same chronic morphine as in reverse tolerance test were used to investigate the effect of paeonol on the development of behavioral sensitization to apomorphine in morphine-induced behavioral sensitization. Morphine-induced behavioral sensitization was evidenced by measuring the enhanced locomotor activity to apomorphine. Each value is the mean ± SEM of at least 10 mice. ***P<0.005, compared with that of the saline group. #P<0.05, ##P<0.01, compared with that of morphine group.

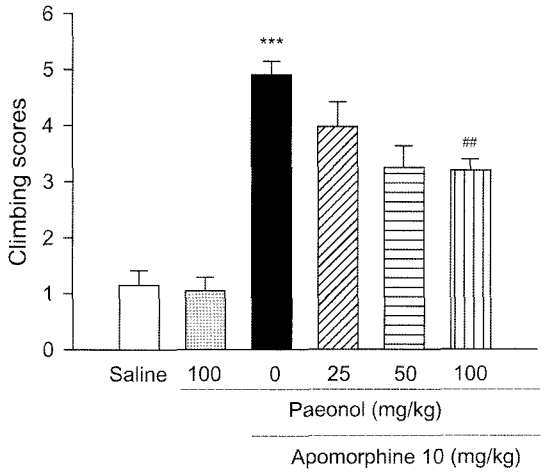

Fig. 5. Inhibitory effect of apomorphine-induced climbing behavior. Paeonol (25, 50 and 100 mg/kg) was administered 1 h before the apomorphine (2 mg/kg) injection. Immediately, after the apomorphine injection, the mice were put into cylindrical individual cages. After a 5-min period of exploratory activity, climbing behavior was measured by all or non score at 10, 20 and 30 min after the administration of apomorphine, and the three scores were added and averaged. Each value is the mean ± SEM of at least 10 mice. ***P<0.005, compared with that of the saline group. #P<0.05, ##P<0.01, compared with that of morphine group.

ficant inhibition of apomorphine-induced climbing behavior resulting in less score than the apomorphine-induced climbing behavior score, yielding 3.24 and 3.19 scores, respectively (P<0.05 and P<0.01, Fig. 5). These results show that a single administration of paeonol inhibits apomorphine-induced climbing behavior, demonstrating that paeonol has the anti-dopaminergic activity.

DISCUSSION

The primary goals of addiction treatment are the facilitation of abstinence and the prevention of relapse. Pharmacological treatment often is used to reduce withdrawal symptoms, but thus far has not been effective in preventing relapse. Still, there has been considerable interest in identifying drugs that reduce and/or inhibit the adverse actions such as tolerance to and dependence on abuse drugs. The effects of naltrexone, a long-acting, orally administered, pure opioid antagonist, have been extensively studied, and narcotic antagonists may be useful in treating addictions to other psychostimulants (Chiu *et al.*, 2005). However, the clinical use of naltrexone is limited because it is associated with low rates of compliance, which have been well documented. On the other hand, the use of clonidine to aid in opioid withdrawal, has important treatment implications. Medications for withdrawal control can be helpful in the short run for discouraging relapse.

Reverse tolerance is a behavioral phenomenon associated with repeated administration of abuse drugs. Current theories suggest that sensitization is important in drug abuse, and may be responsible for the development of drug-seeking behaviors (Robinson and Berridge, 1993). The drug seeking behaviors that arise from repeated administration of psychomotor-stimulants such as morphine, cocaine and methamphetamine is thought to result from sensitization. The motor effects of morphine largely depend on the dopaminergic system, since dopaminergic antagonists block morphine-induced hyperactivity (Funada *et al.*, 1994; Manzanedo *et al.*, 1999). It has been observed that dopamine receptor antagonists effectively inhibit morphine-induced sensitization to hyperactivity (Leone and Di Chihara, 1990; Li *et al.*, 2004). Changes in dopaminergic transmission could be involved in the behavioral sensitization to the hyperactivity induced by morphine (Seerano *et al.*, 2002).

Drug-seeking behaviors with reinforcement do not enable the patients to maintain abstinence. Accordingly, we are much more interested in drugs that inhibit sensitization and/or CPP, because it is believed that the inhibition of sensitization and CPP may reduce drug-seeking behaviors of addictive patients. Morphine and heroin are among the most commonly abused opiates because of their effects on the brain reward circuitry. Their reinforcing effects and the intense craving they produce after long-term use drive abuse of these drugs. In addition, the reinforcing effects of abuse drugs are subject to sensitization. Sensitization

resulting from repeated administration requires persistent activation of the mesolimbic dopaminergic systems. The activation of this system is also implicated in drug reinforcement.

Therefore, a pivotal role for dopaminergic mechanisms in opioid-induced sensitization and CPP has been proposed (Van Ree *et al.*, 1999). For example, the CPP produced by morphine is abolished by pretreatment with dopamine receptor antagonists (Beninger and Miller, 1998). However, recently, natural products for the treatment of adverse effects have been of interest because of their own lower toxicity (Kim *et al.*, 1996, 1998, 1999; Huong *et al.*, 1997). Ginseng's beneficial effects on the adverse actions of psychotropic agents were initially reported by Kim *et al.* (1995), who observed that *Panax ginseng* inhibited the analgesic tolerance and physical dependence induced by morphine (Kim *et al.*, 1990). They additionally reported that *Panax ginseng* also attenuated hyperactivity, sensitization, and CPP induced by psychotropic agents, such as morphine, cocaine, and methamphetamine, showing anti-dopaminergic activity (Kim *et al.*, 1990; Kim *et al.*, 1995; Kim *et al.*, 1996).

Although a folk medicine composed of seven herbal drugs, including *Paeoniae radix*, has been used as an antidote in the treatment of morphine-tolerant/dependent patients, there is no report on the therapeutic and/or preventive effects of paeonol isolated *Paeoniae radix* on the adverse actions of morphine to our knowledge. The results of the current experiments indicate that paeonol, a major compound *Paeoniae radix* inhibited hyperactivity, reverse tolerance and CPP in mice. Moreover, we found that a single administration of paeonol inhibited apomorphine-induced climbing behavior. From these behavioral experiments, we could suggested that inhibitory effects of paeonol also might be mediated by dopaminergic transmission in brain because paeonol inhibited dopamine receptor supersensitivity and apomorphine-induced climbing behavior.

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