Potentiation of Adenosine A₁ Receptor Agonist CPA-Induced Antinociception by Paeoniflorin in Mice

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The effect of paeoniflorin (PF), a major constituent isolated from Paeony radix, on N⁶-Cyclopentyladenosine (CPA), a selective adenosine A₁ receptor (A₁ receptor) agonist, induced antinociception was examined in mice. In the tail-pressure test, CPA (0.05, 0.1, 0.2 mg/kg, s.c.) could induce antinociception in a dose-dependent manner. PF (5, 10, 20 mg/kg, s.c.) alone failed to exhibit any antinociceptive effect in mice; however, pretreatment of PF (20 mg/kg, s.c.) could significantly enhance CPA-induced antinociception. Additionally, pretreatment of 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.25 mg/kg, s.c.), a selective A₁ receptor antagonist, could antagonize the antinociceptive effect of combining CPA with PF. Furthermore, in the competitive binding experiments, PF did not displace the binding of [³H]-6-Cyclopentyl-1,3-dipropylxanthine ([³H]-DPCPX) but displaced that of [³H]-2-Chloro-N⁶-cyclopentyladenosine ([³H]-CCPA, a selective A₁ receptor agonist) to the membrane preparation of rat cerebral cortex. These results suggested that PF might selectively increase the binding and antinociceptive effect of CPA by binding with A₁ receptor.

Key words paeoniflorin; antinociception; adenosine; adenosine A₁ receptor

Paeony radix has long been used as a component of traditional Japanese and Chinese prescriptions to treat certain types of nociceptive diseases, such as muscle pain and menstrual pain.1—3) Paeoniflorin (PF) (Fig. 1), a major constituent isolated, was attributed to the analgesic property of Paeony radix.4) It was previously reported that PF could alleviate nociceptive response in formalin test and that it exerted its antinociceptive effect mainly through the activation of κ-opioid receptors in mice.5)

Recent evidences revealed that some pharmacological profiles of PF could be antagonized by the selective A₁ receptor antagonist DPCPX.6—8) This result was further confirmed in PF-induced neuroprotection in cerebral ischemia.9) However, observation that PF failed to displace the binding of [³H]-DPCPX to membrane preparation of the rat cerebral cortex suggested that PF might influence adenosine A₁ receptor function in a manner different from the classical A₁ receptor agonists.9,10) Adenosine plays an important role among the modulators implicated in antinociceptive responses. In particular, activation of A₁ receptors has been shown to induce antinociceptive effects.11—13) However, formalin test is an inflammatory pain mode,14) in which endogenous adenosine is released and regulates pain transmission primarily by activating A₁ receptor.12—14) It may debase the veracity of the nociceptive evaluation system, in which A₁ receptor is involved. Therefore, it is interesting to examine the antinociceptive effect of PF in a non-inflammatory experiment.

In the present study, in order to remove the interference of endogenous adenosine, the tail-pressure test, a nociceptive experiment without any apparent inflammation in the periphery,15) is introduced to investigate the potential antinociceptive effect of PF on its own, as well as the possible relationship between PF and A₁ receptor system.

MATERIALS AND METHODS

Animals Kun-Ming male mice (Shanghai Experimental Animal Center, Chinese Academy of Sciences), weighing 18—22 g at the beginning of the experiment were used. Ten or more animals were used for one group in this study. Five animals were housed per cage under a 12 h light and dark cycle for at least 7 d before the start of the experiment. Sprague-Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences), weighing 250—300 g were used for membrane preparation. The animals had free access to solid food and water ad libitum in the clean room maintained at 22±2°C. The study was performed in compliance with National Institutes of Health (NIH) guidelines and was approved by Animal Care and Use Committee, Shanghai Institute of Materia Medica, CAS.

Drugs The preparative separation and purification of PF were described as Chen et al.16) and the purity is above 98% determined by high-performance liquid chromatographic (HPLC) assay. The following drugs were obtained from commercial sources: N⁶-Cyclopentyladenosine (CPA) and 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) (Sigma Chemical Co., St. Louis, MO, U.S.A.); [³H]-8-Cyclopentyl-1,3-dipropylxanthine ([³H]-DPCPX) and [³H]-2-Chloro-N⁶-cyclopentyladenosine ([³H]-CCPA) (New England Nuclear, Stevenage, U.K.).

Treatment Schedule The graded doses (5, 10, 20 mg/kg) of PF or (0.05, 0.1, 0.2 mg/kg) of CPA were chosen in the tail-pressure test. The drugs were dissolved in saline and were injected s.c. 10 ml/kg body weight. The control animals received an equivalent volume of the vehicle at the same time when the mice received PF or CPA.
The moderate dose of CPA (0.1 mg/kg, s.c.) was used to study the potential enhancing effect of PF on CPA-induced antinociception. Briefly, PF (20 mg/kg, s.c.) were pretreated 30 min before the administration of CPA (0.1 mg/kg, s.c.). In addition, a selective A1 receptor antagonist DPCPX (0.25 mg/kg, s.c.) was pretreated 5 min before the injection of vehicle or PF (20 mg/kg, s.c.), followed by the administration of CPA (0.1 mg/kg, s.c.).

Tail-Pressure Test Tail-pressure test was performed using a published method,15 with minor modification. Briefly, pressure was applied to the tail 1 cm distant from its root using a pressure antinociception meter (XZC-A, Shandong Academy of Medical Sciences, China) with a wedge-shaped piston at a loading rate of 16 g/s. The weight (g) at which animals struggled or withdrew was considered the nociceptive threshold. To avoid tissue damage, pressure was limited to a maximum of 250 g unless the animals started to bite or vocalize before this weight was reached. Only mice with a baseline between 80 and 120 g were selected. Antinociceptive effects of drugs were measured 0.5, 1, 1.5, 2 and 3 h after administration. Antinociceptive response was expressed as a percentage of the nociceptive threshold (% NT): % NT = [(post-drug reaction pressure)/(pre-drug reaction pressure)]×100. The area under the curve (AUC), depicting % NT vs. time, was calculated by the trapezoidal rule in order to express the overall magnitude and duration of effect for tail-pressure.

Cortex Membrane Preparation Sprague-Dawley rats were killed by cervical dislocation and membrane prepared as described.16 In brief, brains were removed and immediately placed in ice-cold saline, before dissection of the cortex. Tissues were homogenized in 15 volumes (vol) of 0.32 mol/l sucrose using a glass/Teflon homogenizer, the homogenate was centrifuged at 1000×g for 10 min, and the resulting supernatant was centrifuged at 40000×g for 20 min. The synaptosomal/mitochondrial P2 pellet was lysed with 30 vol of ice-cold water for 30 min; then centrifuged at 48000×g for 10 min. The membrane pellet was resuspended in 30 vol of 50 mmol/l Tris–HCl buffer (pH 7.4), centrifuged at 48000×g for 10 min, resuspended in 5 vol of 50 mmol/l Tris–HCl buffer (pH 7.4), and stored at −80 °C. The protein concentration of the suspension was measured according to Bradford with bovine albumin as standard.17

\[ ^{[3}H\]-DPCPX and \[^{[3}H\]-CCPA Competitive Binding Assay \[^{[3}H\]-DPCPX (98.1 Ci/mmol) binding was performed for 3 h at 25 °C in the presence of 0.1 mmol/l \[^{[3}H\]-DPCPX, 2–3 μg cortical membrane suspension, and indicated concentrations of PF in 50 mmol/l Tris–HCl (pH 7.4), containing 2.5 U/ml adenosine deaminase. Then the binding was terminated by filtration onto the filter plate, followed by three washes with 50 mmol/l Tris–HCl (pH 7.4). Subsequently, the filter plate was dried at 40 °C for 1 h and 5 ml of MicroScint 20 (Packard bioscience) was added. After that, the filter plate was covered with TopSeal (Packard bioscience) then the radioactivity was determined in the TopCount (Micro β, PerkinElmer). For \[^{[3}H\]-CCPA (48.60 Ci/mmol) binding, the procedure was carried out as described for \[^{[3}H\]-DPCPX binding, with the following modifications. The final assay concentration of \[^{[3}H\]-CCPA was 1 mmol/l; and the amount of cortical membrane suspension was 4–6 μg.

Statistical Analysis Data were presented as the mean±S.E.M. Statistical differences were determined by Un-paired Student’s t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s test. For all cases, significance of differences were accepted at \(p<0.05\).

RESULTS

Effect of PF and CPA in Tail-Pressure Tests PF (5, 10, 20 mg/kg, s.c.) alone failed to exhibit any antinociceptive effect in the tail-pressure test in mice (Fig. 2). CPA (0.05, 0.1, 0.2 mg/kg, s.c.) produced a time- and dose-dependent antinociceptive effect in tail-pressure test. The maximum effect was obtained at 0.5 h after s.c. administration of CPA (Fig. 2). Compared with the saline-treated group, in the CPA treated groups (0.05, 0.1, 0.2 mg/kg, s.c.), the % NT and AUC were dose-dependently increased (Fig. 2). Additionally, the antinociceptive effect of CPA (0.1 mg/kg, s.c.) could be totally antagonized by pretreatment of A1 receptor antagonist DPCPX (0.25 mg/kg, s.c.) (Fig. 3). Furthermore, the antinociceptive effect of combining CPA

![Fig. 2. Effect of PF and CPA in the Tail-Pressure Test](image)

(a) Time-course of the effect of PF (5, 10, 20 mg/kg, s.c.) and CPA (0.05, 0.1, 0.2 mg/kg, s.c.) on the % NT in mice. Each point and vertical bar represents the mean±S.E.M. of 10—11 mice. (b) AUC of indicated each group. Each column and vertical bar represents the mean±S.E.M. of 10—11 mice. ∗ \(p<0.05\), ∗∗ \(p<0.01\), ∗∗∗ \(p<0.001\) vs. saline-treated animals (One-way ANOVA followed by Dunnett’s test).
with PF could be antagonized by pretreatment with DPCPX (0.25 mg/kg, s.c.). As for DPCPX-pretreated groups, there were no significant differences between the groups with and without PF (20 mg/kg, s.c.) administration (Fig 3). It indicated that the enhancing effect of PF on CPA-induced antinociception could be antagonized completely by the pretreatment of DPCPX.

**Effect of PF on the Binding of [3H]-DPCPX and [3H]-CCPA.** As shown in Fig. 4, PF did not displace the binding of [3H]-DPCPX, even at the highest concentration (100 μmol/l); but displaced the binding of [3H]-CCPA to membrane preparation of the rat cerebral cortex with IC_{50} of 10.3 nmol/l.

**DISCUSSION**

In the present study, we demonstrated that PF (20 mg/kg, s.c.) could potentiate the antinociceptive effect of CPA, a selective A1 receptor agonist in the tail-pressure test. PF on its own failed to exhibit any antinociceptive effect, however, when PF (20 mg/kg, s.c.) was pretreated 30 min before the administration of CPA, the antinociceptive effect of CPA was enhanced significantly (Figs. 2, 3). In addition, pretreatment of DPCPX (0.25 mg/kg, s.c.), a selective A1 receptor antagonist, could completely antagonize the antinociceptive effect of combining CPA with PF or CPA alone (Fig. 3).

Previous report suggested that PF could alleviate nociceptive response in formalin test and that the central antinociceptive effect of PF might be mediated by the activation of κ-opioid receptor. However, the formalin test is an inflammatory pain model, which resembles clinical postoperative pain. Following tissue inflammation, a large number of endogenous substances, including adenosine, are released, and these can excite or sensitize nociceptive afferents. Adenosine, an important inhibitory neuromodulator in the central nervous system (CNS) and peripheral sites, adenosine A1 receptor activation could produce pain suppression or antinociceptive action. In order to exclude the interference of endogenous adenosine, the tail-pressure test, a nociceptive experiment without any apparent inflammation in the periphery, was adopted in the present study to investigate the potential antinociceptive effect of PF on its own, as well as the possible relationship between PF and A1 receptor system. The reason why our *in vivo* results are different from the literature can be considered that although the basic level of adenosine release is insufficient to depress the formalin-induced licking paw, the antinociceptive effect of released adenosine will be largely enhanced in the present of PF. Another possible reason of the difference existing might be due to the difference in the animal species used. While Kunming strain mice were used in the present studies, ICR mice were used in the previous studies. In addition, a number of studies have shown that there is a link between adenosine systems and opioids. Systemic administration of adenosine and adenosine analogs enhances antinociception of morphine. Interactions of opioids with endogenous systems appear to be multifaceted and contribute to a number of aspects of opioid pharmacology. The previous report demonstrated that PF failed to displace the binding of [3H]-DPCPX, but displaced the binding of [3H]-NECA, an adenosine A1/A2 receptor agonist, to the membrane preparation of the rat cerebral cortex. In the present study, we also showed that PF did not displace the binding of [3H]-DPCPX to membrane preparation of the rat cerebral cortex, which were consistent with the findings reported previously. However, PF displaced the binding of [3H]-CCPA, a selective A1 receptor agonist, to membrane preparation of the rat cerebral cortex (Fig. 4). These results suggested that PF was able to bind with A1 receptor and PF might bind specifically with binding sites of A1 receptor only for CCPA, but
not for DPCPX. In addition, we found that PF significantly enhanced CPA-induced antinociception but failed to exhibit any antinociceptive effect by itself, suggesting that the binding of PF may result in conformational changes of A1 receptor that might selectively increase the binding and function of CPA. Furthermore, the antinociceptive effect of combining CPA with PF can be completely reversed by DPCPX. Combining with the previous study, in which PF was demonstrated to bind with A1 receptor in a manner different from the classical A1 receptor agonists,9 the results in the present study suggested that the enhancing effect of PF on CPA-induced antinociception is mediated by A1 receptor activation.

In conclusion, the present study demonstrated that PF had no antinociceptive effect on its own; but it did potentiate CPA-induced antinociception in tail-pressure test. Furthermore, the antinociceptive effect of combining PF with CPA could be completely reversed by DPCPX, an A1 receptor antagonist. Notably, PF might bind specifically with binding sites of A1 receptor only for agonist CCPA, but not for antagonist DPCPX. Thus we speculated that PF might be able to bind with A1 receptor that may selectively increase the binding and function of agonist CPA.

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REFERENCES