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Neuroprotective effect of paeoniflorin on cerebral ischemic rat by activating adenosine A_1 receptor in a manner different from its classical agonists

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1 The effects of paeoniflorin (PF), a compound isolated from *Paeony radix*, on neurological impairment and histologically measured infarction volume following transient and permanent focal ischemia were examined in Sprague–Dawley rats.

2 In transient ischemia model, rats were subjected to a 1.5-h occlusion of the middle cerebral artery (MCA). The administration of PF (2.5 and 5 mg kg^{-1}, s.c.) produced a dose-dependent decrease in both neurological impairment and the histologically measured infarction volume. Similar results were also obtained when PF (2.5, 5, and 10 mg kg^{-1}, s.c.) was given in permanent ischemia model.

3 The neuroprotective effect of PF (10 mg kg^{-1}, s.c.) was abolished by pretreatment of DPCPX (0.25 mg kg^{-1}, s.c.), a selective adenosine A_1 receptor (A_1R) antagonist.

4 PF (10, 40, and 160 mg kg^{-1}, i.v.) had no effect on mean arterial pressure (MAP) and heart rates (HR) in the conscious rat. Additionally, PF (10^{-3} mol l^{-1}) had no effect on noradrenaline- (NA-) or high K^+ concentration-induced contractions of isolated rabbit primary artery.

5 In competitive binding experiments, PF did not compete with the binding of [^{3}H]DPCPX, but displaced the binding of [^{3}H]NECA to the membrane preparation of rat cerebral cortex. This binding manner was distinguished from the classical A_1R agonists.

6 The results demonstrated that activation of A_1R might be involved in PF-induced neuroprotection in cerebral ischemia in rat. However, PF had no 'well-known' cardiovascular side effects of classical A_1R agonists. The results suggest that PF might have the potential therapeutic value as an anti-stroke drug.

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Abbreviations: A_1R, adenosine A_1 receptor; A_{2A}R, adenosine A_{2A} receptor; BBB, blood–brain barrier; CBF, cerebral blood flow; CNS, central nervous system; CPA, N^6-cyclopentyladenosine; CV-1808, 2-phenylaminoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HPLC, high-performance liquid chromatographic; HR, heart rates; MAP, mean arterial pressure; MCA, middle cerebral artery; NA, noradrenaline; NECA, *N*-ethylcarboxamidoadenosine; PF, paeoniflorin; TER, terazosin; TTC, 2,3,5-triphenyl tetrazolium chloride

Introduction

Cerebral ischemia begins as an imbalance between reduced energy supply and the high energy demands. The consequence of this energy imbalance is the depletion of ATP, which not only increases the susceptibility of brain tissues to oxidative stress but also triggers the onset of numerous ischemic cascades, leading to neuronal death (Small & Buchan, 1996; Ames, 2000). Thus, there is a choice of strategy to protect the brain against ischemic damage by reducing the energy demands of the neuronal tissue (Ames *et al*., 1995; Maynard *et al*., 1998; 1999; Ayoub *et al*., 1999). Adenosine is an important inhibitory neuromodulator in the CNS (Snyder, 1985; Greene & Haas 1991). Adenosine depresses cellular activity in all regions of the CNS tested, perhaps partly due to inhibition of the release of the major excitatory neurotransmitter glutamate, an action mediated by adenosine A_1 receptor (A_1R) (Dolphin & Prestwich, 1985; Heron *et al*., 1993). A_1R activation results in an inhibition of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in many cell lines (Kasai & Aosaki, 1989; Mogul *et al*., 1993) and NMDA receptor operated channels in the hippocampus (Schubert *et al*., 1994). In addition, hyperpolarization occurs to depress the excitability of neurons, *via* activation of outward K^+ channel currents or inward Cl^- currents (Greene & Haas, 1991; Rudolphi *et al*., 1992). Together, these effects account for the inhibitory property of adenosine and make A_1R a neuroprotective receptor in general.

Several lines of evidence indicate that activation of A_1R in the brain can reduce ischemic injury *in vivo* and hypoxic damage and death *in vitro* (Goldberg *et al*., 1988; Mori *et al*.,

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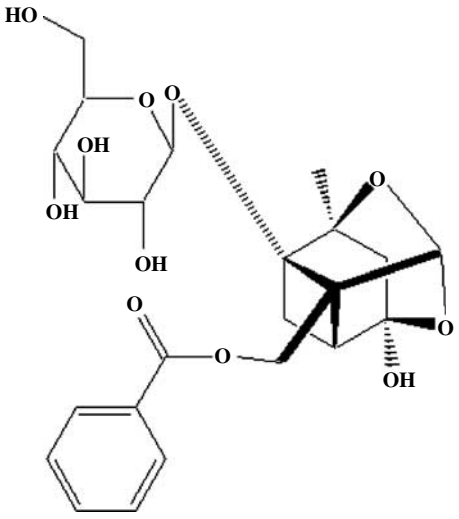

Figure 1 Chemical structure of PF.

1992; Heron *et al*., 1994; Von Lubitz *et al*., 1996). However, the possible therapeutic value of A_1R agonists in treating cerebral ischemia was dubious, due to their several severe cardiovascular side effects, likely mediated by A_1R, such as bradycardia and hypotension (Daval *et al*., 1991; Collis & Hourani, 1993). So, the preferred strategy in treating cerebral ischemia might be located in the search of A_1R agonists with less or no cardiovascular side effects (Sweeney, 1997).

Paeony radix, one of the Chinese traditional crude drugs, has been widely used as a component of traditional Chinese prescriptions to treat certain types of dementia, traumatic injuries and inflammation. Recently, it was reported that total paeony glycoside had protective effects on ischemia-like injury in cultured primary cortex neurons *in vitro* and local cerebral ischemia and acute complete cerebral ischemia *in vivo* (He *et al*., 2000; Liu *et al*., 2001; Wu & Zhu, 2001). Paeoniflorin (PF) (structure shown in Figure 1) is a characteristic monoterpene glucoside isolated from the root of *P. radix*. It was reported that PF could activate A_1R (Lai *et al*., 1998; Cheng *et al*., 1999; Tang *et al*., 2003), lower the injury induced by calcium overloading in cultured primary cortex neurons (Yang *et al*., 2001), but had no effect on the isolated rat artery (Tsai *et al*., 1999). Though the evidence is preliminary, these findings give a clue that PF might have a neuroprotective effect in the treatment of cerebral ischemia *via* activation of A_1R, with less or no cardiovascular side effects. With this clue in mind, the effects of PF on infarction volume as well as neurological impairment were examined following transient and permanent focal ischemia in rats. In addition, the possible mechanisms underlying its action were studied.

Methods

Chemicals and animals

The dried and powdered roots of *Paeony lactiflora,* one species in *Paeony,* were extracted with 70% ethanol under reflux. The concentrated extract was dissolved in water and tandem passed through a macroporous resin column. Wash the column with water first until no Molish reaction, and then with 40% ethanol. Concentration of the 40% eluate under reduced pressure gave the total paeony glycoside. The yellow powder was subjected to silica gel column chromatograph and then eluated with EtOAc/MeOH (20/1). The pure compound was yielded after the concentration of the collected eluate containing only PF (structure shown in Figure 1). The purity of PF is above 98% determined by high-performance liquid chromatographic (HPLC) assay. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N^6-cyclopentyladenosine (CPA), *N*-ethylcarboxamidoadenosine (NECA), 2-phenylaminoadenosine (CV-1808), noradrenaline (NA), and terazosin (TER) 2,3,5-triphenyl tetrazolium chloride (TTC) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. and [^{3}H]DPCPX and [^{3}H]NECA were from New England Nuclear, Stevenage, U.K.

Male Sprague–Dawley rats, weighing between 200 and 240 g, were used. The animals had free access to solid food and water *ad libitum* under standard conditions of temperature, humidity, and light. The study was performed in compliance with National Institutes of Health (NIH) guidelines and was approved by the Animal Care and Use Committee, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Animal preparation and middle cerebral artery (MCA) occlusion

Rats ($n = 8$–10 for each group) were anesthetized using 10% choral-hydrates (350 mg kg^{-1}, i.p.), then placed supine on a heated operating mat. A rectal thermometer inserted and the temperature was monitored and adjusted with heating lamps and a heated operating mat if the temperature fluctuated beyond predetermined limits of $37 \pm 1^{\circ}$C throughout the intraoperative period, and the values in the representative experiments were recorded at 0, 30, and 60 min after occlusion in permanent ischemia (Table 1).

Transient MCA occlusion was performed using a method described previously (Belayev *et al*., 1996; Takano *et al*., 1997), with minor modification. Briefly, the bifurcation of the left common carotid artery was exposed. The right MCA was occluded by insertion of a silicon-coated nylon suture (USS-DG, DERMALON, U.S.A.) through the common carotid artery as described previously (Lee *et al*., 2002). After closure of the operative sites, the animals were temporarily transferred to a cage with a heating lamp and the suture was gently removed at 1.5 h of MCA occlusion. In the case of permanent MCA occlusion, the procedures detailed above were followed, but the filament was left in place.

To allow for better postoperative recovery, we chose not to monitor physiological parameters in the present study because additional surgical procedures and blood losing are inevitable for this monitoring. Nevertheless, we performed a separate experiment to investigate the effects of PF (10 mg kg^{-1}, s.c.) with or without DPCPX (0.25 mg kg^{-1}, s.c.) on major physiological variables in ischemic rats ($n = 8$ for each group). Blood pH, blood gases (pO_2 and pCO_2), hemoglobin (Hb), hematocrit (Hct), oxygen saturation (SO_2%), or blood glucose (Gluc) were measured before ischemia, during the occlusion and 30 min after drugs administration.

Table 1 The rectal temperature (°C) of rats treated with saline or PF

Time (min)	*Saline* (n = 8)	*PF (10 mg kg^{-1})* (n = 8)	*DPCPX + PF (10 mg kg^{-1})* (n = 10)
0	37.45 ± 0.08	37.49 ± 0.07	37.50 ± 0.08
30	37.51 ± 0.08	37.52 ± 0.10	37.55 ± 0.10
60	37.60 ± 0.09	37.51 ± 0.08	37.44 ± 0.08

Results show mean ± s.e.m. of 8–10 animals per group. There were no statistical differences within or between the groups at any time point.

Administration of reagent following transient or permanent MCA occlusion

Rats were first injected with saline (2 ml kg^{-1}, s.c.) or PF (2.5, 5 and 10 mg kg^{-1}, s.c.) 15 min after transient or permanent MCA occlusion and then with the same dose of saline or PF 6 h after occlusion. In order to study the role of A_1R in the neuroprotection of PF, a selective A_1R antagonist DPCPX (0.25 mg kg^{-1}, s.c.) was administered 5 min before occlusion, followed by two times injection of PF (10 mg kg^{-1}, s.c.) as described previously.

Assessment of neurological impairment

Neurological impairment in the stroked animals was examined 22–24 h after MCA occlusion, according to the method described previously (Sydserff *et al*., 2002). Briefly, forelimb flexion, spontaneous rotation, and absence of response to contralateral whisker stimulation were scored on a 0–2 scale (0 = normal behaviour, 2 = severely impaired). In addition, torsion of the body towards the contralateral side was assessed on a 0–2 scale (0 = extensive torsion, 2 = succinct torsion). Thus, the impairment score was in the domain of 0–8.

Histological measurement of neuronal damage

Killing was performed 24 h after transient or permanent MCA occlusion by decapitation under halothane anesthesia. The brain was rapidly removed and cut into 2 mm coronal sections, and stained according to the standard TTC method (Bederson *et al*., 1986). The image of each slice was captured by using digital camera (NIKON, COOLPIX 4300), followed by analysis by the image system (Adobe ImageReady 7.0). The calculated infarction areas were then compiled to obtain the infarction volume per brain (in cubic micrometers). Infarction volume was corrected by using an 'indirect method' (area of intact contralateral (right) hemisphere minus area of intact region of the ipsilateral (left) hemisphere) to compensate for edema formation in the ipsilateral hemisphere (Lee *et al*., 2002).

Determination of MAP and HR

An indirect tail-cuff method (Cheng *et al*., 1999) was applied to determine the MAP and HR of conscious rats by an autodetector (RBP-1B, Sino–Japan Friendship Institute of Clinical Medicine). A phototransistor was used to detect pressure pulses through a cuff sensor at 28 ± 2°C. Rats ($n = 8$–9 for each group) were randomly injected with saline (2 ml kg^{-1}, intravenously (i.v.)), PF (10, 40, and 160 mg kg^{-1}, i.v.), and CPA (0.25 mg kg^{-1}, i.v.), a selective A_1R agonist as a positive control (Mathot *et al*., 1994; Van Schaick *et al*., 1997). MAP and HR were recorded by the autodetector at 0, 5, 10, 20, 40, 60, 90, and 120 min after the injection, respectively.

Primary artery preparations

The primary artery of rabbits was excised, dissected in Petri dishes containing the modified Krebs solution of the following composition (in mmol l^{-1}): NaCl 119, KCl 4.7, $CaCl_2$ 2.55, KH_2PO_4 1.6, $MgSO_4$ 1.18, $NaHCO_3$ 25, and glucose 11. The preparations were suspended in a tissue chamber containing 10 ml modified Krebs solution, kept at 37°C and gassed continuously with 95% O_2 and 5% CO_2. One end of the tissue was anchored to a stationary glass holder and the other to the force displacement transducer. The preparations were then subjected to a resting tension of 0.5 g by means of a micrometric device and contraction (area under the curve) was recorded with the aid of Medlab-U/4CS (MedEase Co., Ltd).

To study the effects of PF on the contraction induced by NA or high K^+ concentration, the preparations ($n = 8$ for each experiments) were allowed to recover from handling, then exposed to NA (10^{-6} mol l^{-1}) or K^+ (1.8×10^{-2} mol l^{-1}) for 10 min. When an even contractile response achieved, absolute values of contraction were recorded and considered as internal initial controls. Then PF (10^{-3} mol l^{-1}) was added for another 10 min, followed by TER (10^{-6} mol l^{-1}) or high K^+ washout, respectively.

Cortex membrane preparation

Sprague–Dawley rats were killed by cervical dislocation and membrane prepared as described (Finlayson *et al*., 1997). 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^{-1} sucrose using a glass/Teflon homogenizer, the homogenate was centrifuged at $1000 \times g$ for 10 min, and the resulting supernatant was centrifuged at $40{,}000 \times g$ for 20 min. The synaptosomal/mitochondrial P_2 pellet was lysed with 30 vol of ice-cold water for 30 min; then centrifuged at $48{,}000 \times g$ for 10 min. The membrane pellet was resuspended in 30 vol of 50 mmol l^{-1} Tris-HCl buffer (pH 7.4), centrifuged at $48{,}000 \times g$ for 10 min, resuspended in 5 vol of 50 mmol l^{-1} Tris-HCl buffer (pH 7.4), and stored at −80°C. The protein concentration of the suspension was measured according to Bradford (1976), with bovine albumin as standard.

[^{3}H]DPCPX, and [^{3}H]NECA competitive binding assay

[^{3}H]DPCPX (98.1 Ci mmol^{-1}) binding was performed for 3 h at 25°C in the presence of 0.1 nmol l^{-1} [^{3}H]DPCPX, 2–3 μg cortical membrane suspension, and indicated concentrations of PF or CPA in 50 mmol l^{-1} Tris-HCl (pH 7.4), containing 2.5 U ml^{-1} adenosine deaminase. Then the binding was terminated by filtration onto the filter plate, followed by three

washes with 50 mmol l^{-1} 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, Perkin-Elmer).

For [^{3}H]NECA (30 Ci mmol^{-1}) binding to A_1R, the cortical membrane was preincubated for 30 min at 37°C with 10 mmol l^{-1} CV-1808, a selective adenosine A_{2A} receptor (A_{2A}R) agonist, to abolish A_{2A}R binding. The followed procedure was carried out as described for [^{3}H]DPCPX binding, with the following modifications. The final assay concentration of [^{3}H]NECA was 25 nmol l^{-1}, the amount of cortical membrane suspension was 4–6 μg and the incubation period was 2 h.

Statistical analysis

Data were presented as the mean ± s.e.m. Statistical differences were determined by Paired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparison. For all cases, significance of differences were accepted at $P<0.05$.

Results

Effect of PF in transient and permanent focal ischemia

Transient occlusion of MCA for 1.5 h resulted in both neurological impairment and tissue damage, encompassing the subcortex nucleus and cortex, 24 h after occlusion. Administration of PF (2.5 mg kg^{-1}, s.c., twice) failed to produce a significant decrease in neurological impairment and damage, but injection of PF (5 mg kg^{-1}, s.c., twice) produced a substantial decrease in both neurological score (Figure 2a) and subcortex nucleus, cortex, and total infarction volume (Figure 2b).

In permanent model, PF (2.5, 5, 10 mg kg^{-1}, s.c., twice) produced a dose-dependent protection in infarction volume (Figure 3). Notably, the protective effect of PF (10 mg kg^{-1}, s.c., twice) could be abolished by pretreatment with DPCPX (0.25 mg kg^{-1}, s.c.), a selective A_1R antagonist (Figure 3).

At 24 h after permanent occlusion of left MCA, ischemic damage was more serious than the transient model. No significantly protective effects of PF (2.5 mg kg^{-1}, s.c., twice) were observed. However, PF (5 and 10 mg kg^{-1}, s.c., twice) significantly reduced the subcortex, cortex, and total infarction volume, except in the 5 mg kg^{-1} group, where no statistical differences were found in the subcortex.

Effect of PF on MAP and HR

The time profiles of MAP and HR after i.v. administration of saline, PF, or CPA to conscious rats are shown in Figure 4a and b. Fifteen minutes after i.v. injection of CPA at a dose of 0.25 mg kg^{-1} resulted in a 68% decrease of MAP from 109 to 35.8 mmHg, and a 43% decrease of HR from 370 to 200 beats per minute (bpm), approximately. However, no influences of PF were detected on MAP and HR, even in the highest dose (160 mg kg^{-1}, i.v.) group.

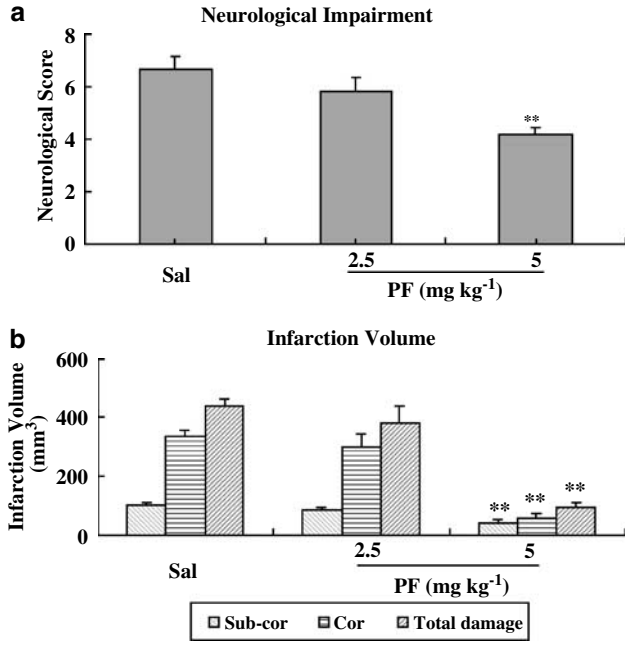

Figure 2 Effect of PF in transient MCA occlusion model. (a) The neurological impairment of ischemic damage. (b) The volume of ischemic damage. PF (2.5 and 5 mg kg^{-1}) was given twice s.c. Each column and vertical bar represents the mean ± s.e.m. of results from eight rats. $^{**}P<0.01$ compared with saline-treated animals (one-way ANOVA followed by Dunnett's *post hoc* comparison).

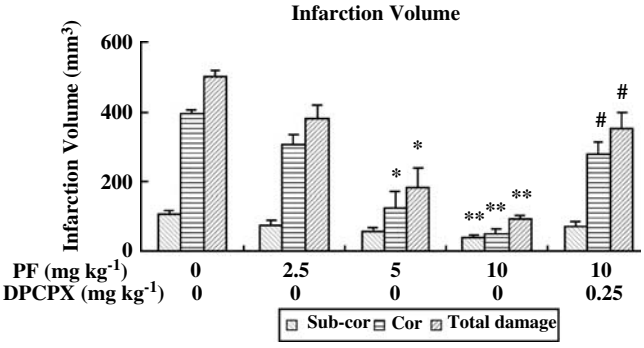

Figure 3 Effect of PF in the permanent MCA occlusion model. PF (2.5, 5 and 10 mg kg^{-1}) was given twice s.c. Each column and vertical bar represents the mean ± s.e.m. of results from 8–10 rats. $^{*}P<0.05$, $^{**}P<0.01$ compared with saline-treated animals (one-way ANOVA followed by Dunnett's *post hoc* comparison). $^{\#}P<0.05$ compared with PF (10 mg kg^{-1})-treated animals (Paired Student's *t*-test).

Effect of PF on the contraction of primary artery in vitro

As shown in Figure 5a and b, either NA or high K^+ concentration could induce contraction of the isolated rabbit primary artery, and the effects could be counteracted by TER treatment or high K^+ concentration washout, respectively. Nevertheless, no influences of PF, even at the dose of 10^{-3} mol l^{-1}, was observed on NA- or high K^+ concentration-induced contraction of isolated rabbit primary artery (Figure 5a and b).

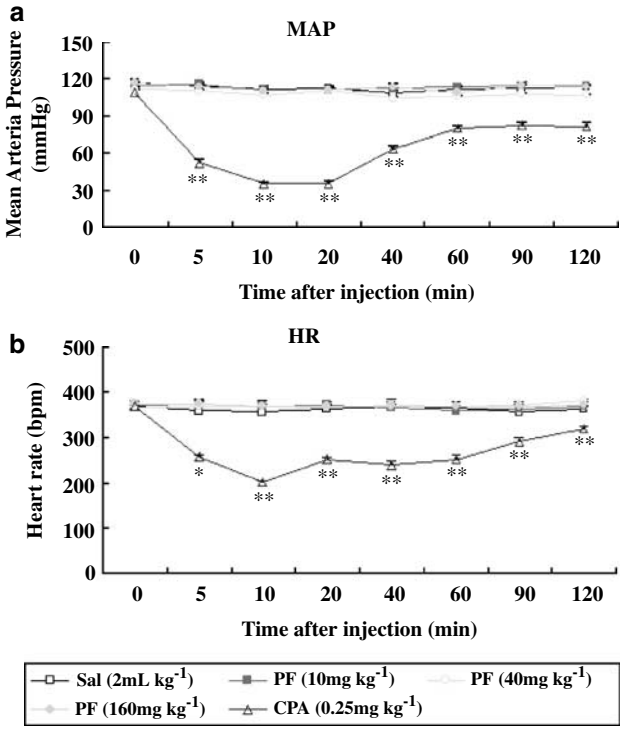

Figure 4 Effect of PF on MAP and HR in conscious rats. (a) MAP, (b) HR. PF (10, 40 and 160 mg kg^{-1}) was injected i.v. Each point and vertical bar represents the mean ± s.e.m. of 8–9 rats. $^{*}P<0.05$, $^{**}P<0.01$ compared with saline-treated animals (one-way ANOVA followed by Dunnett's *post hoc* comparison).

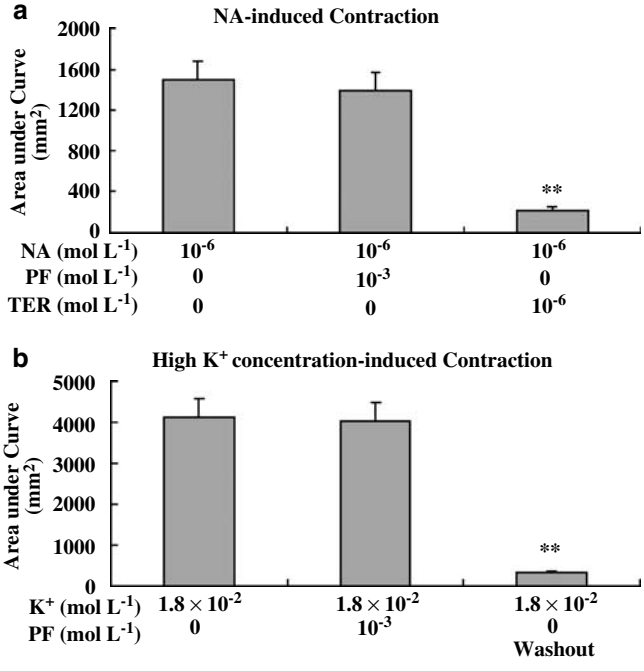

Figure 5 Effect of PF on isolated rabbit primary artery preparation. (a) Effect of PF on NA-induced contraction. (b) Effect of PF on high K^+ concentration-induced contraction. Each column and vertical bar represents the mean ± s.e.m. of results from eight samples. $^{**}P<0.01$ compared with control, respectively (Paired Student's *t*-test).

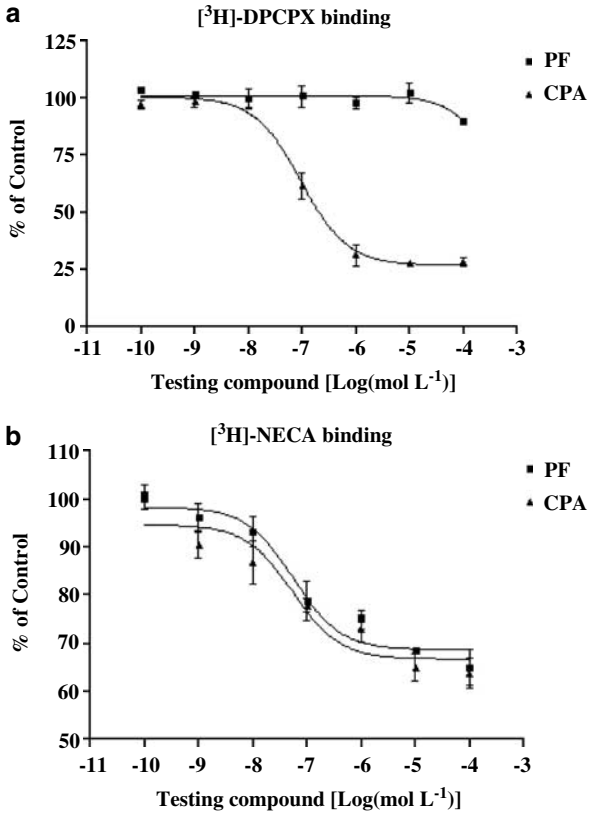

Figure 6 Competitive binding of PF with [^{3}H]DPCPX and [^{3}H]NECA. (a) Competitive binding of PF with [^{3}H]DPCPX to cerebral cortical membrane. (b) Competitive binding of PF with [^{3}H]NECA. Binding was performed as described in Methods. Data shown are triplicate samples with mean ± s.e.m. from two experiments.

Effect of PF on the binding of [^{3}H]DPCPX and [^{3}H]NECA

The data indicated that CPA displaced both the [^{3}H]DPCPX binding with IC_{50} of 58.5 nmol l^{-1} (Figure 6a), and the binding of [^{3}H]NECA with IC_{50} of 13.1 nmol l^{-1} (Figure 6b) to the membrane preparation of the rat cerebrocortex. When compared to that of CPA, PF failed to displace the binding of [^{3}H]DPCPX, even at the highest concentration of 100 μmol l^{-1} (Figure 6a); nevertheless, PF did displace the binding of [^{3}H]NECA to the membrane preparation of the rat cerebrocortex with IC_{50} of 19.5 nmol l^{-1}, which was similar to that of CPA (Figure 6b).

Discussion

Evidence has revealed that activation of A_1R in brain could reduce ischemic injury *in vivo* and hypoxic damage and death *in vitro* (Goldberg *et al*., 1988; Mori *et al*., 1992; Heron *et al*., 1994; Von Lubitz *et al*., 1996). However, the possible therapeutic value of A_1R agonists in treating cerebral ischemia was dubious because of their several severe cardiovascular side effects such as bradycardia and hypotension (Daval *et al*., 1991; Collis & Hourani, 1993). Thus, the preferred strategy for cerebral ischemia might be located in the search of A_1R agonist with less or no cardiovascular side effects (Sweeney, 1997). The key findings of the present studies were that PF was an

Table 2 Physiologic parameters of rats before, during MAC occlusion or 30 min after saline or PF treatment

	n	*pH*	pCO_2 (mmHg)	pO_2 (mmHg)	*Hb* (g/dl)	*Hct* (%)	SO_2%	*Glu* (mmol/l)
Before MCA occlusion	8	7.35±0.01	43.25±2.49	80.45±2.00	13.63±0.63	41.00±1.83	95.53±0.72	10.39±0.84
During MCA occlusion	8	7.36±0.01	41.05±2.67	80.95±2.18	13.83±0.70	41.67±2.03	93.97±0.55	10.76±1.31
30 min after saline injection	8	7.35±0.01	41.77±1.81	83.88±164	14.30±0.80	42.83±2.34	94.42±0.42	11.00±1.04
Before MCA occlusion	8	7.37±0.01	40.65±1.69	83.42±2.22	13.85±0.43	41.50±1.23	94.73±0.63	10.24±0.77
During MCA occlusion	8	7.38±0.02	39.65±3.19	86.95±3.98	13.72±0.55	41.33±1.65	95.05±0.75	11.24±0.64
30 min after PF injection	8	7.34±0.03	40.63±2.74	86.60±3.05	13.85±0.27	41.50±0.76	95.12±0.64	12.15±1.19
Before MCA occlusion	8	7.34±0.02	42.48±1.96	80.02±2.15	13.20±0.56	39.50±1.77	93.63±0.51	11.63±0.97
During MCA occlusion	8	7.34±0.01	41.78±2.25	80.03±1.91	14.47±0.72	43.33±2.17	93.43±0.52	12.70±1.18
30 min after DPCPX+PF injection	8	7.34±0.01	42.63±2.48	82.02±3.91	14.63±0.35	43.83±1.08	93.75±0.69	12.11±0.94

Physiologic data obtained from control and drug-treated groups are presented as the mean±s.e.m. PF (10 mg kg^{-1}) or DPCPX (0.25 mg kg^{-1}) was given subcutaneously. Hb, hemoglobin; Hct, hematocrit; SO_2%, oxygen saturation; Gluc, blood glucose; *n*, number of animals. All animals were maintained at 37±1°C. There were no statistically differences within or between the groups at any time point.

effective compound in reducing cerebral infarction and improving neurobehavioral outcome in transient and permanent MCA occlusion models with little effect on MAP or HR, and that the activation of A_1R might be involved in PF-induced neuroprotection in cerebral ischemia and that PF might bind with A_1R in a manner different from the classical A_1R agonists. In addition, the neuroprotective effects of PF observed in the present study were not to be accounted for by the modification of physiological variables, since these parameters (e.g. body temperature, blood pH, pO_2 and pCO_2, Hb, Hct, or SO_2% or Gluc) were kept within normal physiologic limit (Table 2).

Chinese traditional crude drugs, including the *P. radix* extract and compound prescription, are usually given orally. However, pharmacokinetic studies found that PF, the main component of *P. radix* extract, had a very low bioavailability (3–4%) after oral administration (Takeda *et al*., 1995; 1997). Pharmacokinetic studies also demonstrated that PF had a rapid elimination when it was given i.v. (Takeda *et al*., 1995; 1997). In addition, our preliminary result demonstrated that PF (10 mg kg^{-1}) given i.v. only produced a minor protective effect in transient MCA occlusion model (data not shown). Thus, PF was given s.c. in both transient and permanent MCA occlusion models in the present studies. Although it is unknown whether PF can penetrate through the blood–brain barrier (BBB) after it is given s.c., a recent study demonstrated that PF could quickly penetrate through BBB to reach the brain tissues such as hippocampus after i.v. administration of *P. radix* extract (He *et al*., 2004).

MCA occlusion is a model for producing ischemia-induced damage that has relevance to stroke (Richard *et al*., 2003). However, many compounds that were effective in transient MCA occlusion model failed in clinical trials (Richard *et al*., 2003). To maximize the chances of clinical success, in addition to transient MCA occlusion model, a permanent MCA occlusion model was used in the present studies. The results demonstrated that administration of PF (2.5 and 5 mg kg^{-1}, s.c.) twice at 15 min and 6 h, respectively, after ischemia produced a dose-dependent decrease in neurological score and subcortex nucleus, cortex, and total infarction volume in transient MCA occlusion model. Although administration of PF s.c. at a dose of 5 mg kg^{-1} twice at 15 min and 6 h, respectively, after ischemia resulted in a substantial neuroprotection in transient model, the same treatment with PF only produced a modest effect in permanent occlusion model. When PF was given s.c. at a dose of 10 mg kg^{-1} twice at 15 min and 6 h, respectively, after ischemia, an 80% reduction was found in the volume of damage (Figure 3), suggesting that higher exposure with PF is required to provide neuroprotection in models of permanent ischemia.

It has been known that the primary brain insult in acute cerebral ischemia is largely attributed to the interruption of cerebral blood flow (CBF). The reduction of CBF below 15 ml (100 g min^{-1}) causes cerebral infarction as documented in animal models of stroke (Ginsberg, 2003). By increasing CBF immediately after stroke, vulnerable cells that are destined to die in the ischemic penumbra can be rescued (Niessen *et al*., 2002). Thus, the effect of PF on CBF was determined in the present studies. Our results demonstrated that PF at a dose of 10 mg kg^{-1} (s.c.) had no significant effect on CBF when it was measured with laser Doppler flowmetry (data not shown), suggesting that the neuroprotective effect of PF could not be attributed to its effect on CBF.

In the present studies, we demonstrated that the neuroprotective effect of PF could be abolished by the pretreatment with DPCPX (0.25 mg kg^{-1}, s.c.), a selective A_1 antagonist, suggesting that A_1R might be involved in the neuroprotective effect of PF. However, unlike the classical A_1R agonists that usually induce bradycardia and hypotension (Daval *et al*., 1991; Collis & Hourani, 1993), PF had no significant effect on the MAP and HR of conscious rats and NA- or high K^+ concentration-induced contraction of isolated rabbit primary artery, respectively. In addition, our studies demonstrated that PF had no effect on the isolated rabbit primary artery when administered alone, suggesting that it has no direct effect on blood vessel (data not shown). The result was consistent with the previous finding with isolated rat artery (Tsai *et al*., 1999). Therefore, PF is virtually 'silent' on the cardiovascular system, which is different from the classical A_1R agonists.

To further understand the possible mechanism(s) underlying the functional difference between PF and classical A_1R agonists, competitive binding assays were preformed in the present studies. Our results demonstrated that PF failed to displace the binding of [^{3}H]DPCPX, but displaced the binding of [^{3}H]NECA to the membrane preparation of the rat cerebral cortex. It seems that the binding characteristics of PF are different from those of the classical A_1R agonists, since the classical A_1R agonists such as CPA could displace the binding

of both [^{3}H] DPCPX and [^{3}H]NECA. This difference between PF and classical A_1R agonists might be explained by the finding of Townsend-Nicholson & Schofield (1994), who demonstrated that the threonine residue at position 277 in transmembrane domain VII of A_1R was required for NECA, but not DPCPX, binding. This finding suggests that the binding sites of A_1R for NECA and DPCPX were not overlapping in its entirety. We speculate that PF might bind specifically with binding sites of A_1R only for NECA. To prove this hypothesis, further studies are clearly required.

In conclusion, PF is an effective compound in reducing cerebral infarction and improving the neurobehavioral outcome in transient and permanent MCA occlusion models, with little effect on MAP or HR. The activation of A_1R is involved in PF-induced neuroprotection in cerebral ischemia. PF binds with A_1R in a manner different from the classical A_1R agonists. PF might have potential therapeutic value as an anti-stroke drug.

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