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Analgesic effect of paeoniflorin in rats with neonatal maternal separation-induced visceral hyperalgesia is mediated through adenosine A_1 receptor by inhibiting the extracellular signal-regulated protein kinase (ERK) pathway

Xiao-Jun Zhang [a], Hong-Li Chen [a], Zhi Li [a], Hong-Qi Zhang [a], Hong-Xi Xu [b], Joseph J.Y. Sung [c], Zhao-Xiang Bian [a,*]

[a] School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China
[b] Hong Kong Jockey Club Institute of Chinese Medicine Limited, Kowloon, Hong Kong, China
[c] Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Shatin, Hong Kong, China

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ABSTRACT

Paeoniflorin (PF), a chief active ingredient in the root of *Paeonia lactiflora* Pall (family Ranunculaceae), is effective in relieving colorectal distention (CRD)-induced visceral pain in rats with visceral hyperalgesia induced by neonatal maternal separation (NMS). This study aimed at exploring the underlying mechanisms of PF's analgesic effect on CRD-evoked nociceptive signaling in the central nervous system (CNS) and investigating whether the adenosine A_1 receptor is involved in PF's anti-nociception. *Results*: CRD-induced visceral pain as well as phosphorylated-extracellular signal-regulated protein kinase (p-ERK) and phospho-cAMP response element-binding protein (p-CREB) expression in the CNS structures of NMS rats were suppressed by NMDA receptor antagonist dizocilpine (MK-801) and ERK phosphorylation inhibitor U0126. PF could similarly inhibit CRD-evoked p-ERK and c-Fos expression in laminae I–II of the lumbosacral dorsal horn and anterior cingulate cortex (ACC). PF could also reverse the CRD-evoked increased glutamate concentration by CRD as shown by dynamic microdialysis monitoring in ACC, whereas, DPCPX, an antagonist of adenosine A_1 receptor, significantly blocked the analgesic effect of PF and PF's inhibition on CRD-induced p-ERK and p-CREB expression. These results suggest that PF's analgesic effect is possibly mediated by adenosine A_1 receptor by inhibiting CRD-evoked glutamate release and the NMDA receptor dependent ERK signaling.

1. Introduction

Paeoniflorin (PF), a monoterpene glucoside, is a main active ingredient of the root of *Paeonia lactiflora* Pall (family Ranunculaceae) that is often used in traditional Chinese medicine to alleviate pain, especially visceral pain (Pharmacopoeia Commission of the Ministry of Public Health of PRC, 2005). Besides the well-established analgesic effect of PF for somatic pain (Sugishita et al., 1984; Tsai et al., 2001), our earlier study showed that PF has an analgesic effect on visceral pain caused by colorectal distension (CRD) in the rats with visceral hyperalgesia induced by neonatal maternal separation (NMS) (Zhang et al., 2008).

Previous studies suggest that adenosine A_1 receptor may play an important role in PF's anti-nociceptive effect, at least for somatic pain, as they showed that PF can bind to adenosine A_1 receptor (Liu et al., 2005), and potentiate the analgesic effects of N-cyclopentyl adenosine (CPA), an adenosine A_1 receptor agonist, in the mouse tail pressure test (Liu et al., 2006). It has been shown that adenosine can presynaptically suppress Ca^{2+} influx and postsynaptically activate K^+ channels by acting on adenosine A_1 receptor, and thus mediate neural inhibition by decreasing the activation of NMDA receptor and the release of glutamate (Obrietan et al., 1995; de Mendonca and Ribeiro, 2000; Wardas, 2002). Such a process is most likely associated with adenosine A_1 receptor-mediated anti-nociception. NMDA receptor is involved in the processing of acute nociceptive inputs from viscera (Olivar and Laird, 1999; Traub et al., 2002), and NMDA receptors in the ACC neurons participate in the process of somatic and visceral hyperalgesia (Calejesan et al., 2000; Cao et al., 2008). NMDA receptor activation can initiate phosphorylation of ERK that translocates into nuclei and triggers a series of intracellular changes by activating transcriptional factors such as cAMP response element-binding protein (CREB) (Ji and Woolf, 2001), hence initiates transcriptional and posttranscriptional modulations of target genes and proteins. NMDA receptor-dependant ERK activation in the spinal cord has been shown to contribute to the establishment and long-term maintenance of hyperalgesia (Ji et al., 1999; Daulhac et al., 2006). Our earlier results demonstrated that CRD activated ERK in the CNS structures of NMS rats (Zhang et al., 2009). However, whether it is NMNA receptor-dependent

* Corresponding author. School of Chinese Medicine, Hong Kong Baptist University, HKSAR. Tel.: +852 34112905; fax: +852 34112929.
E-mail address: bzxiang@hkbu.edu.hk (Z.-X. Bian).

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and whether it can be inhibited by adenosine A_1 receptor-activation is unknown.

Experimental evidence has revealed increases in glutamate, the major excitatory amino acid that activates NMDA receptor, in the spinal cord (Zahn et al., 2002; Wen et al., 2003) and periaqueductal gray (PAG) (Silva et al., 2000) in the rats exposed to somatic pain. Glutamate binding sites were discovered in the ACC of rat brain (McDonald et al., 1990; El-Khodor et al., 2004), implicating their involvement in the cognitive and affective components of pain (Gao et al., 2006; Leone et al., 2006). A clinical study showed that painful stimulation elicited dynamic glutamate increase in the ACC, and the increases in glutamate level correlated strongly with the subjective levels of pain experienced by participants (Mullins et al., 2005), suggesting that changes in the levels of this excitatory neurotransmitter in the ACC may be associated with pain intensity. Determination of glutamate concentrations in microdialysates by capillary electrophoresis (CE) coupled with laser-induced fluorescence detection (LIFD) allows a rapid and accurate measurement of neurotransmitters in awake rats, making it possible to correlate nociceptive behavior and transient changes in the levels of extracellular neurotransmitters in CNS sites related to pain in free-moving animals.

Since PF can bind to adenosine A_1 receptor and hence exert antinociceptive effect in response to somatic pain (Liu et al., 2006), and the adenosine A_1 receptor can reduce glutamate release and inhibit NMDA receptor activation, we hypothesized that NMDA receptor possibly participate in CRD-evoked acute visceral pain in rat with visceral hyperalgesia induced by NMS, and PF produces analgesic effect by acting on adenosine A_1 receptor, inhibiting glutamate release and suppressing CRD-evoked NMDA receptor-dependent ERK signaling. To test the hypotheses, the effect of NMDA receptor antagonist dizocilpine (MK-801) on visceral pain and the effect of adenosine A_1 receptor antagonist DPCPX on the analgesic effect of PF were investigated by abdominal withdraw reflex (AWR) test. The protein expression of p-ERK and p-CREB was studied with western blot analysis, and changes of extracellular glutamate concentrations in ACC microdialysates were determined by CE-LIFD.

2. Materials and methods

2.1. Animals

Primiparous timed-pregnant Sprague–Dawley female rats were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong, on gestational days 13–14. All of the experimental protocols were carried out with the approval of the Committee on Use of Human and Animal Subjects in Teaching and Research of Hong Kong Baptist University and according to the Regulations of the Department of Health, Hong Kong, China. We followed the ethical guidelines for investigating experimental pain in conscious animals, recommended by the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Neonatal maternal separation (NMS)

NMS started from postnatal day (PND) 2 until PND14. Dams and their litters were randomly assigned to the NMS and not handled (NH) control groups. NMS was performed as described previously (Barreau et al., 2004). NH pups remained undisturbed in their home cages with the dam. All pups were weaned on PND 22, and female pups were excluded from further procedures to avoid hormonal cycle-induced variations. Pups were then raised in groups until each weighed 250–400 g.

2.3. Drugs and chemicals

PF (purity>98%) was obtained from Tianjing Jianfeng Nature Product Research Limited Company, P. R. China. Morphine, chloralhydrate, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), U0126 ethanolate (U0126), MK-801, 5-carboxyfluorescein succinimidyl ester (CFSE), glutamate, sodium tetraborate, boric acid and N,N-dimethylformamide (DMF) were obtained from Sigma. Isoflurane was obtained from Halocarbon (USA).

2.4. Grouping, tests and drug administration

First, to test whether ERK signaling was involved in CRD-evoked visceral pain of NMS rats and whether it is NMDA receptor dependent, we investigated the analgesic effect of MK-801 and U0126 by AWR test, and studied the influences of MK-801 and U0126 on CRD-evoked p-ERK and p-CREB protein expression by western blot analysis. The NMS litters were randomly divided into 4 groups ($n=4$–6 in each group) to receive intracerebroventricular (i.c.v.) injection of vehicle, U0126 or MK-801. On the day of AWR testing, rats were injected with drugs (U0126: 15 μg 5 μl^{-1} rat^{-1}; and MK801: 20 μg 5 μl^{-1} rat^{-1} i.c.v.) (Song et al., 2003; Zhang et al., 2004) or vehicle (aCSF containing 70% dimethyl sulfoxide) under isoflurane gaseous anesthesia (4% induction, 2% maintenance, Halocarbon, USA). Twenty five minutes after i.c.v. injection, when the rats were fully awake and had adapted to the transparent testing box, the pain threshold pressure was detected as previously described (Zhang et al., 2008). Immediately after AWR test, rats in vehicle+, U0126+ and MK-801+ groups were assigned to receive 10 times of 20-second CRD at 70 mm Hg with 2-minute intervals between stimuli. Rats in vehicle− group did not receive any CRD.

Second, the effects of PF on CRD-evoked p-ERK and c-Fos expression were investigated by IHC staining. Morphine was chosen as the positive control. Rats were administered by i.p. injection of PF (180 mg/kg, labeled as $NMS^+ + PF$), morphine (5 mg/kg, labeled as $NMS^+ + MP$) or saline (labeled as $NMS^+ +$ saline and $NMS^- +$ saline) and allowed to adapt in the test box alone for 25 min. Subsequently, rats in $NMS^+ +$ saline, $NMS^+ + PF$, $NMS^+ + MP$ groups received ten times of 20-second CRD at 70 mm Hg with 2-minute intervals between stimuli. Rats in $NMS^- +$ saline group did not experience any CRD. In order to have a clear comparison, we referred to the IHC data of $NMS^- +$ saline and $NMS^+ +$ saline groups that have labeled as NMS− and NMS+ groups in our earlier publication (Zhang et al., 2009).

Third, to test whether the adenosine A_1 receptor was involved in the analgesic effect of PF, we investigated the influences of DPCPX, a specific adenosine A_1 receptor antagonist, on PF's analgesic effect by AWR test. The effects of DPCPX on PF's inhibition of CRD-evoked p-ERK and p-CREB were investigated by western blot analysis. On the day of AWR test, rats were given i.p. injection of DPCPX (3 mg/kg) (Maione et al., 2007) or vehicle (saline containing 20% dimethyl sulfoxide). PF (180 mg/kg) or saline was administered 10 min later by i.p. 25 min prior to AWR test. Immediately after AWR test, rats in vehicle+ (NMS rats injected with vehicle and saline and subjected to CRD), vehicle + PF (NMS rats injected with vehicle and PF and subjected to CRD), DPCPX+ (NMS rats injected with DPCPX and saline and subjected to CRD) and DPCPX + PF groups (NMS rats injected with DPCPX and PF and subjected to CRD) were given ten times of 20-second CRD at 70 mm Hg with 2-minute intervals between stimuli for collecting western blot analysis samples. Rats in vehicle− group (NMS rats injected with vehicle and saline and did not subject to CRD) did not experience any CRD.

Fourth, changes in the levels of glutamate in the ACC microdialysate of NMS rats were kinetically monitored by microdialysis coupled with CE-LIFD to investigate whether CRD evoked glutamate release, and whether PF suppressed it. After the baseline test, rats in $NMS^+ + PF$ and $NMS^+ +$ saline groups were administered with PF (180 mg/kg, i.p.) or saline (i.p.) respectively 25 min prior to noxious CRD. The control saline group ($NMS^- +$ saline) did not receive any CRD.

2.5. Immunohistochemical (IHC) analysis

Rats were anesthetized and perfused transcardially, and the tissue was prepared as previously described (Zhang et al., 2009). The IHC analysis of p-ERK and c-Fos was investigated in the same batch of animals, and the slides were stained and analyzed at the same time with the NH^-/NH^+ and NMS^-/NMS^+ groups in our previous publication (Zhang et al., 2009) to minimize the animal usage according to the ethical guidelines for investigating experimental pain in conscious animals recommended by the International Association for the Study of Pain (Zimmermann, 1983). Slide images were captured and then analyzed by using the Image J NIH software. Threshold of each image was fixed, and then the numbers of positive nuclei of c-Fos-IR located in the spinal cord and cerebral nuclei were determined bilaterally. The total number of c-Fos-IR nuclei of each area obtained from 4–6 animals in each group was averaged and compared by one-way ANOVA.

2.6. CRD and AWR tests

NH and NMS rats were randomly assigned to receive or not to receive noxious CRD. For the CRD group, a 20-second CRD of 70 mm Hg was delivered ten times, with 2-min intervals between stimuli. The AWR test was performed as previously described (Zhang et al., 2008). Pain threshold pressure was measured by triplicate CRD at 2-min intervals after rats have adapted in a transparent box alone.

2.7. Western blot analysis

Rats were decapitated immediately after noxious CRD. Tissue samples were collected and processed as reported previously (Zhang et al., 2009). Proteins (50 μg/lane) of interest were separated by 12% SDS-PAGE, and transferred onto PVDF membranes (Bio-Rad). Non-specific binding sites were blocked with 5% nonfat milk for 1 h at room temperature, then the blots were incubated at 4 °C overnight with mouse antibody against p-ERK (1:1000 in TBST, Cell Signaling) or beta-actin (1:1000 in TBST, Calbiochem), or rabbit antibody against p-CREB (1:1000 in TBST, Cell Signaling). Membranes were incubated in HRP-conjugated secondary antibodies, and the immunoreaction was detected and analyzed as previous described (Zhang et al., 2009). The results are presented as the ratio of the p-ERK or p-CREB optical density standardized to the optical density of beta-actin.

2.8. Microdialysis and capillary electrophoresis

Microdialysis sample collections were conducted as described in a previous report (Hao et al., 2005). One day prior to the microdialysis experiments, a guide cannula (locking intracerebral guide and stylet, Bioanalytical, USA) was implanted under chloralhydrate (3.5 mg/kg, i.p.) anesthesia at the stereotaxic coordinates: 0.7 mm caudally and 0.7 mm laterally to the bregma (Paxinos and Watson, 2005). The guide was lowered diagonally 1.7 mm from the skull surface at an angle of 60° from the horizontal plane, aiming rostrally. The guide cannula tip was thereby placed just above the left anterior cingulate cortex. The guide was secured with dental acrylic cement (Bioanalytical, USA) and 2 mm screws (Bioanalytical, USA) to the skull. To prevent postoperative inflammation, marbofloxacin (0.2 mg/100 g, i.p.) was used at once after surgery. The microdialysis sampling was conducted after each rat was allowed one day postoperative recovery in individual cages. Rats were given brief ether anesthesia; then a 4-cm-long flexible latex balloon was inserted with the distal end 2 cm from the anus and secured to the base of the tail. A microdialysis probe (Bioanalytical, USA) with a dialysis membrane (2 mm long, 320 μm OD, molecular weight cut-off 7000 Da) was inserted through the guide cannula. Microdialysis was performed by perfusing the dialysis probe with aCSF at a rate of 1 μl/min through a syringe drive (Bioanalytical, USA). Rats were kept in the BAS Raturn system (Bioanalytical, USA) for collection of dialysate samples. Following a wash-out period of 45 min after insertion of the probe, dialysate samples of 5 μl were collected in 5 min-fractions by an autosampler (Bioanalytical, USA) with temperature controlled at 4 °C. Five baseline samples were collected (samples 1–5). Then PF (180 mg/kg, i.p.) or saline was administered. NMS rats were randomly assigned to receive (labeled as NMS^+) or not to receive (labeled as NMS^-) noxious CRD. For the NMS^+ + saline and NMS^+ + PF group, seven 20-second CRD of 70 mm Hg were performed with 2-min intervals between stimuli, and 4 samples were collected during CRD treatment (samples 6–9). Subsequently, eight samples were collected following the noxious CRD (samples 10–17). For the NMS^- + saline group, rats did not experience any CRD, and 17 samples were collected. After collection, the microdialysate samples were stored at −20 °C until quantitative assays were performed.

Glutamate and aspartate were simultaneously determined in rat brain microdialysate by capillary electrophoresis coupled with laser-induced fluorescence detection. Briefly, 5 μl microdialysis sample or different concentrations of standard solution were mixed with 1 μl of 5 mM CFSE solution and 4 μl of 10 mM borate buffer (pH 8.5) for derivatization. Solutions were left to react in the dark at room temperature for 16 h, subsequently diluted 10-fold by adding 90 μl ultrapure water to the derivatized solutions, and stored at −20 °C until injection. A blank control of aCSF was derivatized and diluted in parallel. A Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, CA, USA) equipped with a laser-induced fluorescence detector (Argon Ion 488 nm laser module; Beckman Coulter) was used to perform all capillary electrophoresis separations. Separations were carried out with a 60.2 cm × 50 μm ID fused-silica capillary (effective length: 50 cm, Yongnian Photoconductive Fiber Factory, Hebei, China); the separation buffer was 25 mM borate + 120 mM boric acid.

2.9. Statistical analysis

All data are expressed as mean ± S.E.M. Independent *t*-test and one-way ANOVA followed by post-hoc test with the LSD test were performed to detect the differences among groups. A P value <0.05 was considered statistically significant.

3. Results

3.1. CRD-evoked p-ERK and p-CREB are NMDA receptor-dependent

3.1.1. Analgesic effects produced by MK-801 and U0126

As shown in Fig. 1, the AWR test revealed that U0126 and MK-801 produced significant analgesic effects. The threshold pressures that

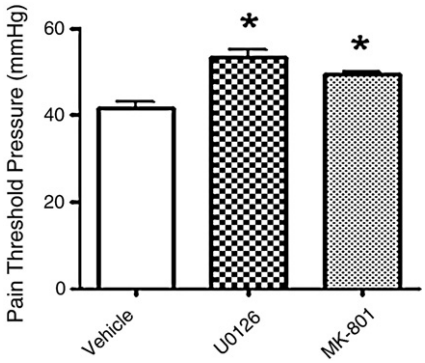

Fig. 1. Statistical comparison of pain threshold pressure of NMS rats subjected to i.c.v. injection of vehicle, U0126 and MK-801. The bars represent mean ± s.e. mean. ($n = 4$–6) *$p<0.01$ vs. vehicle group based on one-way ANOVA.

evoked AWR in response to CRD in U0126 and MK-801 groups were 53.35 ± 1.71 and 49.28 ± 0.70 mm Hg, respectively, significantly elevated in comparison with 41.68 ± 1.36 mm Hg of vehicle group.

3.1.2. MK-801 and U0126 suppressed CRD-evoked p-ERK and p-CREB expression in CNS

Immunoblotting analysis with specific anti-p-ERK antibodies revealed elevated p-ERK expression in the cingulate cortex (Fig. 2-A), thalamus (Fig. 2-B) and lumbosacral dorsal horn (Fig. 2A-C) of NMS rats subjected to CRD (vehicle + group), in comparison with NMS rats without CRD treatment (vehicle− group). U0126 significantly inhibited CRD-elicited p-ERK expression in the thalamus (Fig. 2-B). In comparison with vehicle + group, significant suppression on CRD-evoked p-ERK expression was found in the cingulate cortex (Fig. 2-A), thalamus (Fig. 2-B) and lumbosacral dorsal horn (Fig. 2A–C) of MK-801 + group.

Immunoblotting analysis with specific anti-p-CREB antibodies revealed increased p-CREB expression in the cingulate cortex (Fig. 2-D), thalamus (Fig. 2-E) and lumbosacral dorsal horn (Fig. 2-F) in comparison to the same regions of the vehicle− group. In comparison with vehicle + group, both U0126+ and MK-801+ groups showed significantly suppressed p-CREB expression in the cingulate cortex (Fig. 2-D), thalamus (Fig. 2-E) and lumbosacral dorsal horn (Fig. 2-F).

3.2. PF inhibition of CRD-evoked p-ERK and c-Fos expression

Our previous data showed that CRD increased the protein expression of p-ERK in the laminae I–II of the lumbosacral dorsal horn, central medial thalamic nucleus (CM), paraventricular thalamic nucleus (PV) and ACC (Zhang et al., 2009). In this study, as shown in Table 1, PF significantly attenuated CRD-evoked p-ERK expression in laminae I–II of the lumbosacral dorsal horn and in the ACC. The positive control morphine inhibited CRD-evoked p-ERK expression in laminae I–II and laminae X of the lumbosacral dorsal horn, CeA, CM, PV and ACC. Representative bright field micrographs displaying the p-ERK immunoreactivity in the ACC of the NMS groups with and without CRD, and PF and morphine groups with CRD are shown in Fig. 3.

Our previous study showed that CRD significantly elevated c-Fos expression in laminae I–II of the lumbosacral dorsal horn and in supraspinal structures, including CM, PV, VPL and ACC (Zhang et al., 2009). In this study, as shown in Table 2, PF decreased CRD-induced c-Fos expression in laminae I–II of the lumbosacral dorsal horn, CM, PV and ACC. The effect is similar to morphine in suppressing CRD-evoked c-Fos expression in laminae I–II and X of lumbosacral dorsal horn, CM, PV, VPL and ACC. Typical bright field micrographs revealing c-Fos immunoreactivity in the ACC are shown in Fig. 4.

3.3. PF's analgesic effect and inhibition of ERK cascades are mediated via the adenosine A_1 receptor

3.3.1. Effect of adenosine A_1 receptor antagonist (DPCPX) on PF's analgesia

As shown in Fig. 5, the pain threshold pressure of the vehicle + saline group was 37.44 ± 1.01 mm Hg. In contrast, the pain threshold pressure of the vehicle + PF group was 44.03 ± 0.69 mm Hg, and this represented a significant increase (17.60 ± 1.7%). Pretreatment with DPCPX did not influence the pain threshold of NMS rats. The pain threshold pressure following treatment with DPCPX in the PF group was 39.00 ± 1.14 mm Hg. Under this condition, no significant difference in the pain threshold could be found between the DPCPX + PF group and the DPCPX + saline group. In contrast, when comparing the DPCPX + PF group to the vehicle + PF group, a significant decrease (11.40 ± 1.26%) in pain threshold pressure was found in the DPCPX + PF group.

3.3.2. Effect of DPCPX on PF's inhibition of CRD-evoked p-ERK and p-CREB

Immunoblotting analysis was carried out to determine whether PF's inhibition of CRD-evoked ERK cascades was mediated by the adenosine A_1 receptor. Statistical analysis showed significantly elevated p-ERK expression in the cingulate cortex (Fig. 6-A), thalamus (Fig. 6-B) and lumbosacral dorsal horn (Fig. 6-C) of the vehicle+ group when compared with that of the vehicle− group, indicating that CRD activated ERK by increasing its phosphorylation in these structures. In contrast, significant decreases in p-ERK expression were observed in the cingulate cortex (Fig. 6-A), thalamus (Fig. 6-B) and lumbosacral dorsal horn (Fig. 6-C) of the vehicle + PF group, in comparison with the vehicle+ group, demonstrating that PF pretreatment significantly inhibited CRD-evoked p-ERK activation at the spinal and supraspinal levels.

Significant effects of CRD and PF on p-CREB expression were also observed in the cingulate cortex of NMS rats (Fig. 6-D). The vehicle+ group showed significantly higher p-CREB expression than both the vehicle− group and vehicle + PF+ group, indicating that CRD increased, whereas PF decreased, p-CREB expression in the cingulate cortex. Although CRD tends to elevate p-CREB expression in the thalamus, the change was not statistically significant (Fig. 6-E).

In comparison, the DPCPX + PF group did not differ significantly from the vehicle+ group with regard to p-ERK expression in the cingulate cortex (Fig. 6-A) and thalamus (Fig. 6-B), indicating that DPCPX blocks the inhibitory effect of PF on CRD-evoked p-ERK expression in the cingulate cortex and thalamus. Similarly, we found no significant difference in p-CREB expression between the DPCPX + PF+ and vehicle+ group in the cingulate cortex (Fig. 6-D), suggesting that DPCPX blocked PF's inhibition on CRD-evoked p-CREB expression in this structure.

However, two puzzling phenomena were recorded in the current study. First, decreased p-ERK expression was found in the lumbosacral dorsal horn of both the vehicle + PF+ group and the DPCPX + PF+ group, when compared with the vehicle+ group (Fig. 6-C). Second, decreased p-CREB expression was observed in the thalamus of both the vehicle + PF+ group and the DPCPX + PF+ group when compared with the vehicle+ group (Fig. 6-E). These results indicate that DPCPX may not completely inhibit PF's effect in the lumbosacral dorsal horn and thalamus, but the underlying mechanisms remain to be delineated.

3.4. Effects of CRD and PF on extracellular glutamate in the anterior cingulate cortex (ACC)

As shown in Fig. 7, a significant increase of glutamate was found in the 6th sample of NMS rat subjected to CRD (NMS^+ + saline vs. NMS^- + saline, $p<0.05$) as compared with NMS rats without CRD. This increase occurred during the initial 5 min of noxious visceral stimuli. In contrast, the glutamate concentration of rats that had received PF (180 mg/kg, i.p.) pretreatment showed no significant difference when compared with rats that had not experienced CRD. These results indicate that CRD increased, whereas PF decreased, extracellular glutamate in the ACC.

4. Discussion

4.1. CRD-evoked p-ERK and p-CREB are NMDA receptor-dependent

Recent studies have revealed the involvement of MAPK in nociception and pain hypersensitivity (Obata and Noguchi, 2004; Daulhac et al., 2006). Among the three MAPK family members, ERK is implicated in stress (Trentani et al., 2002) and long-term changes in the CNS (Mcewen, 2000). Evidence highlights the involvement of the ERK MAPK pathway in the NMDA receptor mediated nociceptive signaling. Spinal NMDA receptor-dependent ERK activation contributes to the nociceptive behavior evoked by noxious stimuli (Yamamoto and Yaksh,

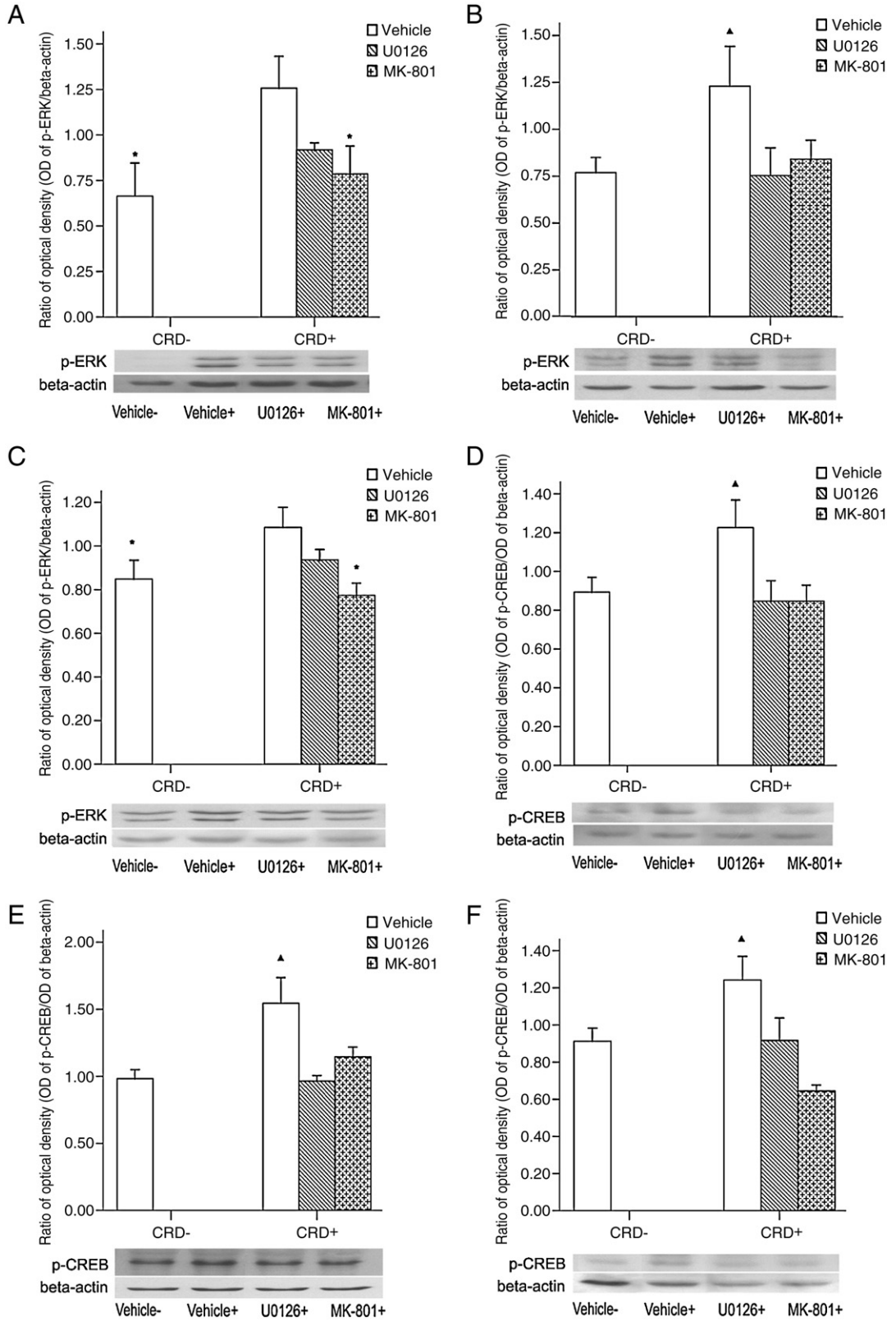

Fig. 2. Western blot analysis of the protein levels of p-ERK in the cingulate cortex (A), thalamus (B) and lumbosacral dorsal horn (C), and p-CREB in the cingulate cortex (D), thalamus (E) and lumbosacral dorsal horn (F) of NMS rats without (CRD−) and with (CRD+) CRD stimulation. Beta-actin proteins were used as the control for protein loading. Data are expressed as mean ± s.e. mean (n = 4–6), indicating the relative ratio of optical density of p-ERK (with typical bands at 42, 44 kDa) or p-CREB (with a typical band at 43 kDa) over optical density of beta-actin (with a typical band at 42 kDa). Vehicle−: vehicle without CRD; vehicle+: vehicle with CRD; U0126+: U0126 with CRD; MK-801+: MK-801 with CRD. Statistical significance of the difference among groups was evaluated by using one-way ANOVA. *p<0.05 vs. vehicle+ group. ▲p<0.05 vs. the other three groups.

Table 1
Immunohistochemical semi-quantification of p-ERK-IR numbers per section per side from the lumbosacral dorsal horn and cerebral nuclei of NH and NMS rats.

	NMS^- + saline*	NMS^+ + saline*	NMS^+ + PF	NMS^+ + MP
Laminae I–II	52.97 ± 8.10▲	78.38 ± 3.99	56.53 ± 3.44▲	34.25 ± 4.57▲
Laminae III–IV	82.70 ± 2.72	84.13 ± 2.64	82.50 ± 5.78	73.00 ± 8.75
Laminae X	29.00 ± 1.47	29.25 ± 2.75	31.13 ± 2.80	18.25 ± 2.39 ▲
CeA	27.08 ± 5.67	38.99 ± 4.69	33.27 ± 4.20	21.97 ± 3.88▲
CM	25.93 ± 4.64▲	47.50 ± 6.06	36.70 ± 3.93	17.13 ± 3.04 ▲
PV	27.67 ± 2.30▲	46.31 ± 4.28	38.80 ± 4.84	28.67 ± 4.48 ▲
ACC	27.25 ± 4.36▲	38.79 ± 6.10	26.50 ± 2.62▲	11.25 ± 2.03 ▲

Statistical analysis of p-ERK expression in lumbosacral spinal cord and cerebral nuclei. Data are expressed as mean ± S.E.M., as determined by the average number of p-ERK-IR nuclei per section per side (n = 4–6). One way ANOVA analysis comparing the p-ERK expression in the NMS rats with (NMS^+ + saline) or without CRD (NMS^- + saline), and NMS rats with CRD but were pretreated with PF (NMS^+ + PF) or morphine (NMS^+ + MP) reveals significantly decreased p-ERK expression in laminae I–II of lumbosacral dorsal horn ($p<0.01$) and ACC ($p<0.05$) of NMS^+ + PF group than the NMS^+ + saline group; as well as markedly decreased p-ERK expression in laminae I–II ($p<0.01$) and laminae X ($p<0.01$) of lumbosacral spinal cord, and in cerebral nuclei including CeA ($p<0.05$), CM ($p<0.01$), PV ($p<0.05$) and ACC ($p<0.01$) of NMS^+ + MP group than the NMS^+ + saline group. ▲$p<0.05$ vs. NMS^+ + saline group, based on one-way ANOVA. *The data of NMS^- + saline and NMS^+ + saline groups have been published earlier, labeled as NMS− and NMS+ in our previous publication, and are referred here to clarify the effects of PF (Zhang et al., 2009).

Table 2
Immunohistochemical semi-quantification of *c-fos*-IR numbers per section per side from the lumbosacral dorsal horn and cerebral nuclei of NH and NMS rats.

	NMS^- + saline*	NMS^+ + saline*	NMS^+ + PF	NMS^+ + MP
Laminae I–II	75.83 ± 6.38▲	102.46 ± 5.31	77.31 ± 3.54▲	61.13 ± 6.95▲
Laminae III–IV	115.67 ± 9.28	114.10 ± 9.29	89.50 ± 5.36	100.33 ± 3.19
Laminae X	51.67 ± 3.55	57.04 ± 7.66	51.13 ± 5.49	32.38 ± 4.32▲
CM	84.80 ± 6.76▲	104.58 ± 4.23	82.40 ± 3.90▲	68.00 ± 2.70▲
PV	95.50 ± 11.14▲	142.80 ± 10.11	97.50 ± 5.95▲	103.00 ± 12.64▲
VPL	207.47 ± 19.45	235.57 ± 28.47	180.80 ± 22.95	128.70 ± 17.56▲
ACC	112.30 ± 19.22▲	203.25 ± 51.47	86.10 ± 13.06▲	86.80 ± 22.18▲

Statistical analysis of *c-fos* expression in lumbosacral spinal cord and cerebral nuclei. Data are expressed as mean ± S.E.M., as determined by the average number of *c-fos*-IR nuclei per section per side (n = 4–6). One way ANOVA analysis comparing the *c-fos* expression in the NMS rats with (NMS^+ + saline) or without CRD (NMS^- + saline), and NMS rats with CRD but were pretreated with PF (NMS^+ + PF) or morphine (NMS^+ + MP) reveals significant PF and MP effect on decreasing CRD-evoked *c-fos* expression in the laminae I–II of lumbosacral dorsal horn, CM, PV and ACC (For all, $p<0.01$). Besides, significant morphine effect on decreasing CRD-evoked *c-fos* expression was also significant in the laminae X of the lumbosacral dorsal horn and VPL (for both, $p<0.01$). ▲$p<0.05$ vs. NMS^+ + saline group, based on one-way ANOVA. *The data of NMS^- + saline and NMS^+ + saline groups have been published earlier, labeled as NMS− and NMS+ in our previous publication, and are referred here to clarify the effects of PF (Zhang et al., 2009).

1992; Coderre and Melzack, 1992) as well as the maintenance of diabetic hyperalgesia (Daulhac et al., 2006). Therefore, we hypothesized that NMDA receptor-dependent ERK cascades may be involved in the functional visceral pain of visceral hyperalgesic NMS rats, and that blockade of NMDA receptor may suppress visceral pain via inhibition of ERK pathways. The results presented here support this hypothesis. Firstly, AWR testing revealed the involvement of NMDA receptor and ERK in the behavioral nociceptive response of NMS rat to CRD by showing that blockade of NMDA receptor and inhibition of ERK phosphorylation produced analgesic effects on CRD-induced visceral pain. Secondly, Western blot analysis showed that CRD upregulated p-ERK and p-CREB expression in the CNS structures involving the lumbosacral dorsal horn, thalamus and cingulate cortex, and confirmed the NMDA receptor-dependent p-ERK and p-CREB activation by revealing that NMDA receptor antagonist MK-801 suppressed CRD-evoked p-ERK and p-CREB expression. Thus, these data demonstrated that the NMDA receptor-dependent ERK pathway is associated with the CRD-evoked behavioral response and neuronal signaling transduction of nociceptive information, and inhibition on the NMDA receptor-dependent ERK signaling is effective to alleviate visceral pain.

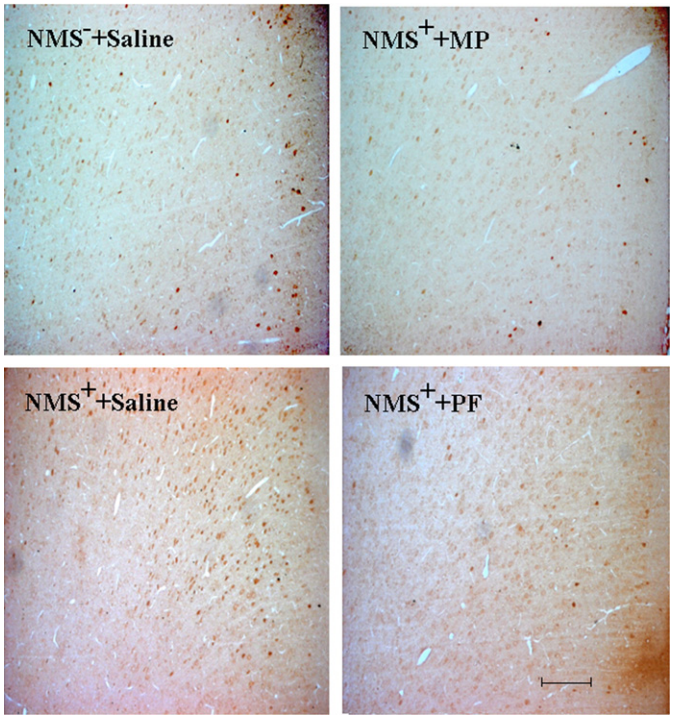

Fig. 3. Representative bright field micrographs of the anterior cingulate cortex (ACC) revealing p-ERK immunoreactivity of NMS rats with (NMS^+ + saline) and without CRD (NMS^- + saline), NMS rats subjected to CRD and treated with morphine (5 mg/kg, i.p.) (NMS^+ + MP), and NMS rats subjected to CRD and treated with PF (180 mg/kg, i.p.) (NMS^+ + PF) (scale bar: 2000 μm).

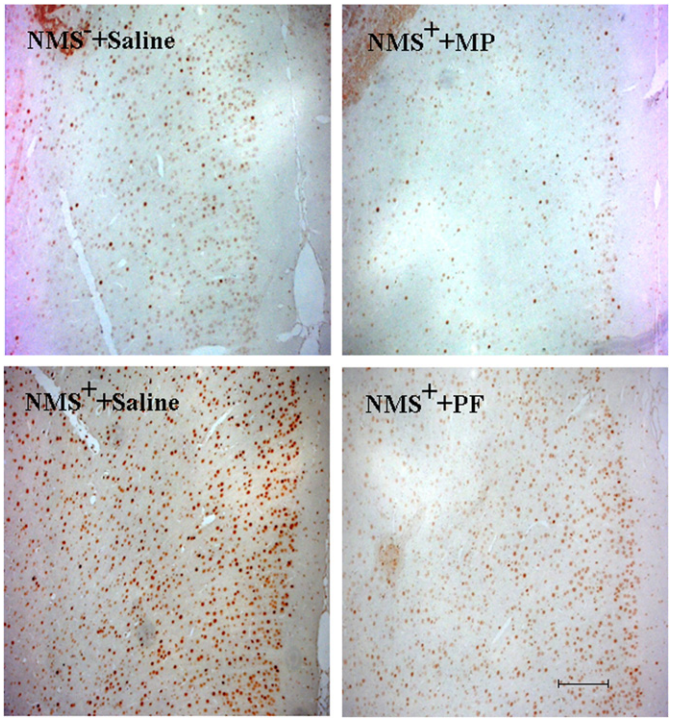

Fig. 4. Representative bright field micrographs of the anterior cingulate cortex (ACC) revealing *c-Fos* immunoreactivity of NMS rats with (NMS^+ + saline) and without CRD (NMS^- + saline), NMS rats subjected to CRD and treated with morphine (5 mg/kg, i.p.) (NMS^+ + MP), and NMS rats subjected to CRD and treated with PF (180 mg/kg, i.p.) (NMS^+ + PF) (scale bar: 2000 μm).

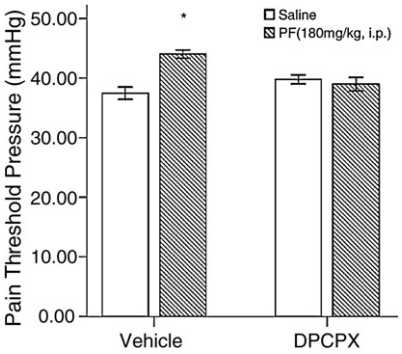

Fig. 5. Statistical comparison of the pain threshold pressure of NMS rats subjected to vehicle or DPCPX as pretreatment and saline or PF as treatment. The bars represent mean ± S.E.M. ($n = 4$–6). *$p < 0.01$ vs. vehicle + saline group, based on one-way ANOVA.

4.2. Blockade of adenosine A_1 receptor inhibited PF's analgesic effect

Our previous results showed nor-BNI (kappa-opioid receptor antagonist), alpha-AMPT (catecholamine synthesis inhibitor), and yohimbine (alpha2-adrenoceptor antagonist) could block the analgesic effect of PF, indicating that PF's analgesic effect may be associated with kappa-opioid receptors and alpha2-adrenoceptors (Zhang et al., 2008). Recent studies revealed that PF could bind to adenosine A_1 receptors (Liu et al., 2005), and hence synergically potentiate antinociceptive effect of adenosine A_1 receptor agonist to somatic pain (Liu et al., 2006). Adenosine A_1 receptor has been suggested to participate in multiple biological activities of PF, such as anti-hypotension (Cheng et al., 1999) and neuronal protection (Liu et al., 2005; Chen et al., 2006). Thus, we postulated that adenosine A_1 receptor may participate in the anti-visceral pain effect of PF. The current results provide experimental support for the hypothesis. In this study, DPCPX, an adenosine A_1 receptor antagonist, blocked PF's inhibition of the CRD-evoked visceromotor response (VMR). This indicates that adenosine A_1 receptor plays a crucial role in PF's analgesic effect. Activation of adenosine A_1 receptor could suppress extracellular levels of glutamate (Quarta et al., 2004) and inhibit NMDA receptor activation (Wardas, 2002). Our earlier study revealed CRD-evoked p-ERK and c-Fos expression in NMS rats (Zhang et al., 2008). The above results showed that CRD-evoked p-ERK expression in NMS rats was NMDA receptor dependent, as both ERK phosphorylation inhibitor U0126 and NMDA receptor antagonist MK-801 could significantly elevate the pain threshold of NMS rats and block CRD-induced ERK phosphorylation in CNS structures. Therefore, it appears that PF can attenuate extracellular glutamate concentration and/or inhibit NMDA receptor by activating adenosine A_1 receptor, which subsequently inhibit CRD-evoked nociceptive signaling and suppress behavioral responses to pain.

4.3. PF inhibited CRD-evoked extracellular glutamate release in the anterior cingulate cortex (ACC)

As a major cortical component of the limbic system, the ACC is functionally in association with affection and pain (Mayer et al., 2006). NMDA receptors are highly expressed in the ACC (McDonald et al., 1990; Wang and Pickel, 2000). As in other regions of the CNS, fast excitatory synaptic transmission within the ACC is mediated by the excitatory amino acids (Wu et al., 2008). The present result revealed a rapid increase of extracellular glutamate in the ACC of NMS rats during the initial 5 min of noxious CRD. The increase of glutamate was transient and highly correlated with the initiation of visceral nociception. It returned to baseline levels swiftly thereafter, although noxious CRD-induced behavioral AWR responses continued. This result is consistent with previous reports of transient elevation of extracellular glutamate in the PAG and VPL, which happened in the initial 2 min and 3.5 min, respectively, of formalin testing (Silva et al., 2000, 2001). It therefore seems probable that glutamate increase is responsible for the acute component of pain, whereas activation of biochemical cascades coupled to metabotropic receptors or the release of other neurotransmitters is responsible for the slow component of pain (Silva et al., 2001). Thus, elevated glutamate is probably a fast response that triggers the subsequent series of cellular and intracellular responses. Since extracellular glutamate is possibly the representation of overflow from the synaptic cleft, the highly efficient reuptake by glutamate transporters on neurons and glia may mask the release of glutamate in the later phase of the pain response (Silva et al., 2001).

The present study showed a strong correlation between PF's inhibition of extracellular glutamate and activation of A_1 receptor. However, extracellular glutamate is regulated not only by neuronal release but also by reuptake in neurons and glial cells, as well as by enzymatic metabolism (Silva et al., 2000). Activation of glutamate transporters might also decrease glutamate in the extracellular space (Shigeri et al., 2004). Whether these possibilities are related to the decreased glutamate in the PF group merits further investigation.

It is suggested that activation of NMDA receptor is associated with visceral hyperalgesia. Microinjection of glutamate in the ACC increased CRD-induced VMR in the rats with visceral hyperalgesia induced by colonic anaphylaxis. In contrast, injection of aminophosphonopentanoic acid, an NMDA receptor antagonist, significantly suppressed the VMR in visceral hypersensitive rats but not in normal rats (Cao et al., 2008). In the present study, the control group of NMS rats that did not experience CRD showed no significant change in extracellular glutamate. Thus, glutamate increase in the ACC is related to nociception caused by CRD. Taken together with previous data, the results clearly demonstrate that glutamate signaling through NMDA receptors in the ACC participated in CRD-evoked visceral nociception in NMS rats.

However, as the cingulate cortex also participates in the affective aspect of visceral pain (Cao et al., 2008), we cannot exclude the possibility that increased glutamate might also be associated with CRD-evoked negative emotion or memory. It is interesting that one previous microdialysis study in freely moving rats demonstrated that increased extracellular glutamate in the PAG during noxious somatic stimulation seemed to be related to persistent pain rather than stress or short-duration noxious stimulation (e.g., pinch), as transient or non-noxious stimuli showed no effect on extracellular glutamate (Silva et al., 2000). Further research is necessary to investigate whether or not the biochemistry is the same in NMS rats.

It has been reported that morphine (5 and 10 mg/kg, i.p.) decreases extracellular levels of glutamate in the ACC of freely moving rats (Hao et al., 2005), suggesting that regulation of glutamate is involved in the neuronal effects of morphine. Our current result showed that PF (180 mg/kg, i.p.) significantly inhibited CRD-evoked glutamate increase, indicating that the analgesic effect of PF may be mediated by down-regulation of CRD-evoked glutamate release. AWR tests confirmed the participation of adenosine A_1 receptor in PF's analgesic effect. Given that activation of adenosine A_1 receptor could result in inhibited glutamate release and NMDA receptor activity (Obrietan et al., 1995; de Mendonca and Ribeiro, 2000), adenosine A_1 receptor probably mediates the process by which PF causes decreased glutamate in the ACC.

The results have established that NMDA receptor activation contributes to noxious CRD-evoked ERK cascades and nociceptive behavior, therefore, the CRD-elevated extracellular glutamate in the ACC of NMS rats possibly triggers intracellular nociceptive signaling through ERK cascades, whereas inhibition of glutamate by PF suppresses nociceptive signaling and alleviates pain.

4.4. PF's inhibition of ERK cascades is mediated via adenosine A_1 receptor

Our previous study showed that noxious CRD could increase p-ERK and c-Fos protein expression in the DRG, in laminae I–II of the

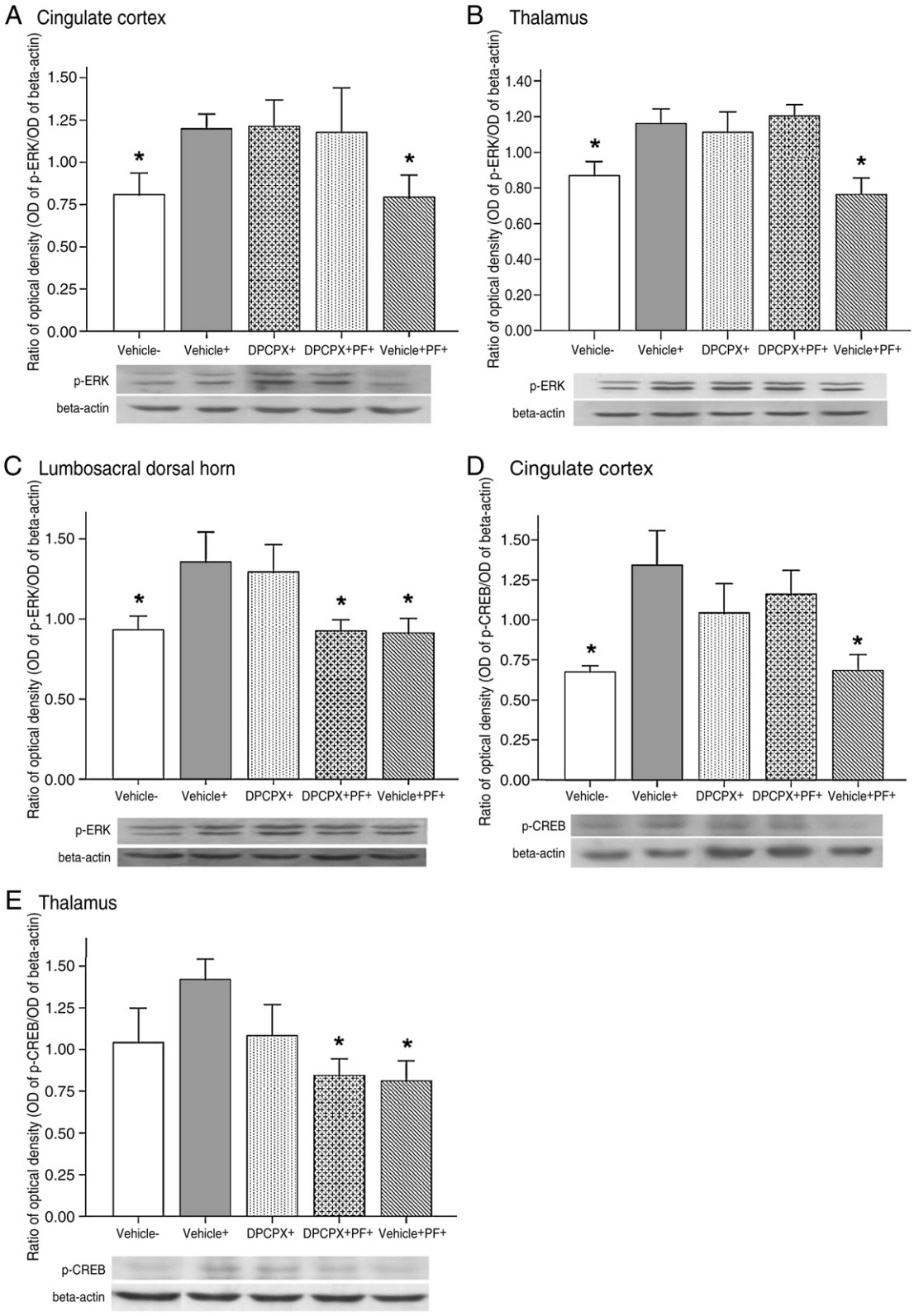

Fig. 6. Western blot analysis of the protein levels of p-ERK in cingulate cortex (A) thalamus (B) and lumbosacral dorsal horn (C), and the protein levels of p-CREB in cingulate cortex (D) and thalamus (E) revealing the influence of DPCPX on PF's inhibition of ERK cascades in NMS rats. Data are expressed in mean ± S.E.M. ($n = 4–5$). Beta-actin proteins are revealed as the control for protein loading. *Y* axis indicates the relative ratio of optical density of p-ERK (with typical bands at 42.44 kDa) or p-CREB (with a typical band at 43 kDa) over optical density of beta-actin (with a typical band at 42 kDa). Vehicle−: NMS rats injected with vehicle and saline but not subjected to CRD; vehicle+, DPCPX+, DPCPX + PF+, vehicle + PF+: NMS rats injected with vehicle/DPCPX and saline/PF and subjected to CRD. Statistical significance of the differences among groups was evaluated by using one-way ANOVA. *$p < 0.05$ vs. vehicle+ group.

lumbosacral dorsal horn and in cerebral parts including CM, PV and ACC (Zhang et al., 2009). The immediate early gene c-Fos has been widely accepted as a marker of neuronal activation following noxious stimuli (Coggeshall, 2005) and represents a useful marker for studying the pharmacology of nociceptive events (Sagar et al., 1988; Bullitt, 1990; Ji and Woolf, 2001; Monnikes et al., 2003). Levels of c-Fos

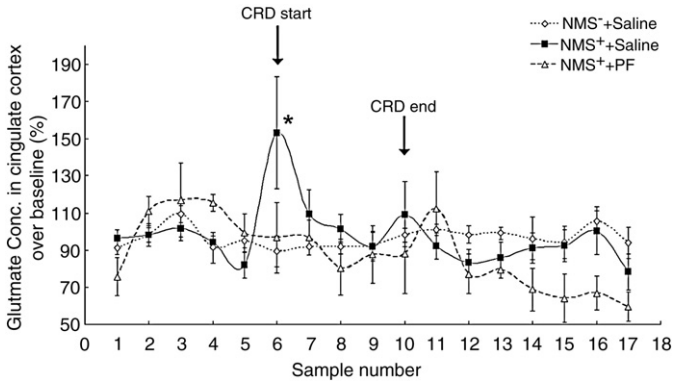

Fig. 7. Percentage of glutamate changes as compared to basal levels in the anterior cingulate cortex of NMS rats without noxious CRD (NMS^- + saline, ◇), with CRD stimuli (NMS^+ + saline, ■) and subjected to PF pretreatment before noxious CRD (NMS^+ + PF, △). Data are expressed as mean ± S.E.M, (n = 3–6). *p<0.05 vs. NMS^- + saline group. The arrow indicates the initiation and end of noxious CRD stimuli. Samples 1–5 were collected during baseline period, samples 6–9 were collected during CRD, and samples 10–17 were collected after CRD.

expression following noxious repetitive CRD could reflect the intensity of the stimulus and the degree of discomfort produced, and could be dose-dependently attenuated by systemic analgesics (Traub et al., 1995).

Our current study showed that PF pretreatment attenuated CRD-induced p-ERK and c-Fos expression in the superficial layer of the lumbosacral dorsal horn and ACC. In the CM and PV, PF significantly reduced CRD-evoked c-Fos expression, but PF did not significantly reduce the CRD-evoked p-ERK expression. A possible explanation is that there may be a magnification of the inhibitory effects of PF, such that even a slight inhibition of ERK, the upstream modulator of c-Fos, may result in a more significant attenuation of downstream c-Fos expression. These findings, then, provide the first evidence supporting the hypothesis that PF exerts its analgesic effect by suppressing CRD-evoked neuronal ERK cascades in the CNS.

In the present study, the inhibitory effect of PF on CRD-induced p-ERK expression in the lumbosacral dorsal horn and thalamus was significantly reversed by adenosine A_1 receptor antagonist DPCPX. Thus, it suggests that PF's inhibition of CRD-evoked ERK cascades at the spinal and supraspinal levels is mediated via adenosine A_1 receptor. DPCPX blocked PF's inhibition of CRD-evoked p-CREB expression in the cingulate cortex, but not in the thalamus. This may be due to activation of CREB that is also regulated by PKA, PKC and CaMK pathways (Johannessen et al., 2004). Thus, DPCPX may not have totally reversed PF's inhibition of CRD-evoked p-CREB.

Inhibition of neuronal ERK is proposed to be a molecular mechanism underlying opioid-mediated neuronal effects (Kawasaki et al., 2006). Morphine could alleviate p-ERK expression elicited by c-fiber stimulation (Kawasaki et al., 2006), and inhibit rat spinal cord c-Fos expression induced by nociceptive somatic (Presley et al., 1990) and visceral stimuli (Traub et al., 1995). Our present study showed that morphine inhibited CRD-induced p-ERK and c-Fos expression in the superficial and deepest layer of the lumbosacral dorsal horn, as well as in cerebral centers involving CM, PV and ACC. These data, consistent with that of previous studies, demonstrate a correlation between the analgesic effect of morphine (on a whole-body level) and inhibition of neuronal p-ERK and c-Fos expression.

5. Limitations

It is known that emotional stress influences the central pain modulation system (Mayer, 2000). Irritable bowel syndrome (IBS) symptoms are exacerbated by stress, and stress reduction is part of the management in IBS therapy (Heitkemper and Jarrett, 2008). One previous study showed that PF has no significant antianxiety and/or anti-depression effects on various behavioral changes induced by stress in experimental animals (Watanabe, 1993). However, deficiency in testifying whether PF has an inhibitory effect on basal neural activity is one of the limitations of the present study. Further investigations will greatly extend our understanding of the central effect of PF.

6. Conclusion

In summary, our results show that (1) adenosine A_1 receptor plays a key role in the analgesic effect of PF on CRD-evoked visceral pain in NMS rats. (2) Noxious CRD evoked a rapid and transient increase of extracellular glutamate in the ACC of NMS rats. (3) PF pretreatment significantly suppressed CRD-induced increase of glutamate and ERK cascades, possibly by activating the adenosine A_1 receptor. (4) The series of neurochemical and intracellular signaling modulations initiated by PF attenuated neuronal and behavioral nociceptive responses. These findings not only shine light on mechanisms of pathogenesis of visceral pain but also help to develop new drugs from herbs for the treatment of visceral pain.

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