Randomized Animal Trial

Antinociceptive Effect of Prostatic Acid Phosphatase in a Rat Model of Cancer-induced Bone Pain

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Background: Cancer-induced bone pain (CIBP) is a severe chronic pain that is less than adequately controlled by conventional analgesics. Prostatic acid phosphatase (PAP) has been considered as a diagnostic marker for prostate cancer and its transmembrane isoform has been reported to play an antinociceptive effect in neuropathic and inflammatory pain. However, it remains unknown whether it has an analgesic effect on CIBP and what are the underlying mechanisms.

Objective: In the present study, we tested whether PAP could alleviate the pain symptoms induced by bone cancer in a rat model.

Study Design: A randomized, double blind, and controlled rat animal trial.

Methods: We first established a rat CIBP model and observed the spinal expression of PAP by immunofluorescence histochemistry and Western blot. Then, PAP (0.1, 0.3, or 1 µg) was intrathecally administered in the CIBP rats in a repeated manner from 15 to 18 days (once per day) after inoculation of tumor cells. On postoperative day (POD) 18, the mechanical paw withdrawal threshold was tested for checking the dose-effect curve and ED50 of the antinociceptive effect of PAP. In an another test, a single dose of ED50 of PAP was intrathecally injected on POD 15 to observe the time course of its effect. Furthermore, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (3 mg/kg), an adenosine A1 receptor antagonist, or dipyridamole (DIP) (10 µg), a nucleoside transporter inhibitor, was administered to the CIBP rats for exploring the analgesic mechanisms of PAP. The concentration of extracellular adenosine was also detected by microdialysis method after intrathecal injection of PAP (0.57 µg) and DIP (10 µg) in the CIBP rats. Finally, an in vivo electrophysiological study of the CIBP rats was performed to observe whether the electrically evoked response of spinal wide-dynamic-range (WDR) neurons could be affected by PAP (0.57 µg), DIP (10 µg), or DPCPX (10 µg).

Results: The expression of PAP in the spinal dorsal horn was significantly reduced in the CIBP rats, and intrathecal injection of PAP dose-dependently attenuated CIBP-induced mechanical allodynia via the adenosine A1 receptor. Simultaneously, intrathecal injection of PAP increased the extracellular concentration of spinal adenosine in the CIBP rats, as well as inhibited the neuronal responses of WDR neurons in deep layers within the spinal dorsal horn through the adenosine A1 receptor. Finally, the analgesic effect of PAP was potentiated by DIP, the nucleoside transporter inhibitor.

Limitations: It's not clear whether PAP's antinociceptive effect is mediated by other signaling molecules besides the adenosine A1 receptor. In addition, the long-term antinociceptive effect of intrathecal PAP is still not clear.

Conclusions: Our study demonstrated that PAP was involved in the maintenance of CIBP and could effectively suppress central sensitization by increasing spinal extracellular adenosine concentrations to exert a significant antinociceptive effect via the adenosine A1 receptor in CIBP rats. Therefore, our experiments suggest that the endogenous enzyme PAP may be a promising candidate for CIBP treatment.

Key words: Cancer-induced bone pain, prostatic acid phosphatase, adenosine, allodynia, spinal dorsal horn, rat

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With the development of cancer treatments, the survival of cancer patients has been significantly improved; however, cancer pain is a challenging medical problem that greatly influences the survivor's quality of life (1-3). Thus, ways to control chronic cancer pain in order to improve quality of life have attracted increasing attention (4). Among all types of cancer pains, cancer-induced bone pain (CIBP) is very common and is mainly due to metastasis (5-8). More than one-third of patients with advanced cancer undergo skeletal metastases and experience severe pain (9). To date, opioid analgesics and other polymodal/multiple modal interventions have been widely used to treat chronic pain. However, the use of opioid analgesics is limited by their tolerability levels and other side effects. Thus, it is an urgent task to find novel and potent drugs for relieving severe cancer pain.

Prostatic acid phosphatase (PAP) was originally thought to only exist in the prostate and has been considered as a diagnostic marker for prostate cancer (10). However, it has recently been reported that a transmembrane isoform of PAP (TM-PAP) is expressed throughout the body, with an especially intensive distribution pattern in the superficial spinal dorsal horn and dorsal root ganglia (11,12). Interestingly, PAP knockout mice show hyperalgesic effects in a neuropathic and inflammatory pain animal model (11). More importantly, intrathecal injection of PAP causes antinociceptive effects and is much more potent than morphine, while side effects and intolerance of PAP are not observed (11). These data suggest that PAP seems to be a potent and effective analgesic toward neuropathic and inflammatory pain. However, until now, our knowledge regarding the mechanism of PAP in pain regulation was very poor (11,12). Whether PAP is appropriate for treating severe and refractory cancer pain remains unknown.

We thus proposed that PAP can alleviate the pain symptoms induced by bone cancer. In order to verify this hypothesis, we detected the expression of PAP in the spinal dorsal horn of rats with cancer pain. The possible analgesic effect of PAP and its antinociceptive mechanism were also explored by combining behavioral, molecular biological, and electrophysiological methods.

OBJECTIVES

In the present study, we tested whether PAP can effectively alleviate the pain symptoms induced by bone cancer and explored the underlying mechanism.

METHODS

Animals

Adult female Sprague Dawley rats, weighing 180 – 220 g, were used in order to successfully establish the rat model of bone cancer pain according to our previous report (13). Rats were housed 3 per cage with food and water freely available and a 12:12 hour light-dark cycle (lights on at 06:00 am). All surgical and testing procedures were approved by the Committee of Animal Care and Use for Research and Education at the Fourth Military Medical University (Xi'an, P.R. China) and followed the ethical guidelines to study experimental pain in conscious animals (14).
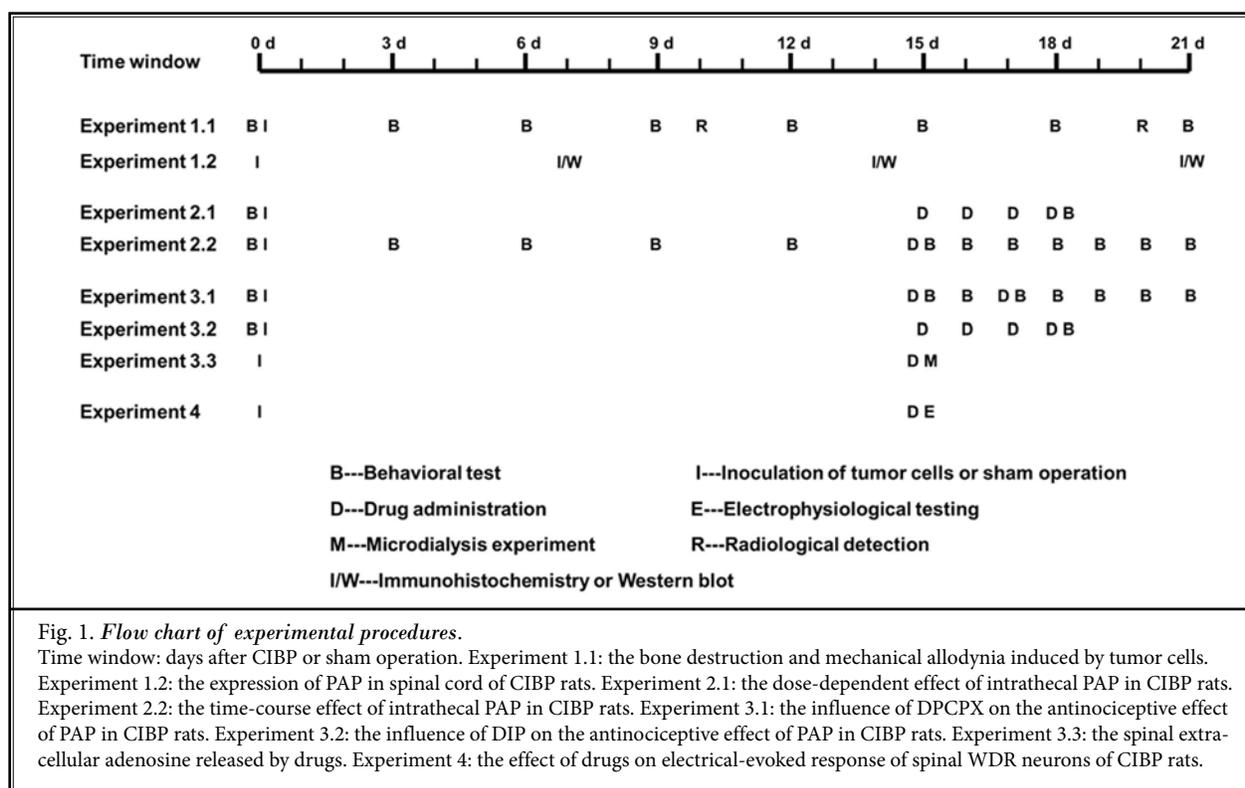
Experiment Procedure

The study comprised 4 experiments (Fig. 1).

Experiment 1: Rat CIBP Model and the Spinal Cord Expression of PAP

1.1 To observe the destruction of tibiae and mechanical allodynia in CIBP rats, 25 rats were divided into a naïve group (n = 8), sham-operated group (n = 8), and CIBP group (n = 9). Radiological imaging was taken on postoperative day (POD) 10 and 20, while paw withdrawal threshold (PWT) was detected on POD 0, 3, 6, 9, 12, 15, 18, and 21.

1.2 To observe the spinal cord expression of PAP, rats were divided into naïve (n = 12), sham-operated (n = 12), and CIBP groups (n = 36). The rats in the CIBP group were humanely killed on POD 7 (n = 12), POD 14 (n = 12), and POD 21 (n = 12). Half of the rats in each group were humanely killed for immunofluorescent staining and the others were used for Western blotting.

Experiment 2: The Antinociceptive Effect of Intrathecal PAP

{2.1 To observe the dose-dependent antinociceptive effect of PAP, 36 CIBP rats were divided into 4 groups, in which different doses of PAP (0.1, 0.3, or 1 µg/rat) or 0.9% of saline was intrathecally injected from POD 15 to 18 (once per day), and the mechanical threshold was tested at one hour after drug injection on POD 18 (n = 9 in each group). Sham-operated rats (injected with heat-treated tumor cells) received intrathecal PAP (1.0 µg/rat) (n = 8) or saline injection (n = 8), respectively.

2.2 In order to observe the temporal effect of PAP, PAP or saline was intrathecally injected on POD 15 when the mechanical allodynia reached its

Time window	0 d		3 d		6 d		9 d		12 d		15 d		18 d		21 d
Experiment 1.1	B I		B		B		B R		B		B		B		R B
Experiment 1.2	I				I/W						I/W				I/W
Experiment 2.1	B I										D D D D B				
Experiment 2.2	B I		B		B		B		B		D B B B B B B B				
Experiment 3.1	B I										D B B D B B B B B				
Experiment 3.2	B I										D D D D B				
Experiment 3.3	I										D M				
Experiment 4	I										D E				

B---Behavioral test   I---Inoculation of tumor cells or sham operation

D---Drug administration   E---Electrophysiological testing

M---Microdialysis experiment   R---Radiological detection

I/W---Immunohistochemistry or Western blot

Fig. 1. *Flow chart of experimental procedures.*
Time window: days after CIBP or sham operation. Experiment 1.1: the bone destruction and mechanical allodynia induced by tumor cells. Experiment 1.2: the expression of PAP in spinal cord of CIBP rats. Experiment 2.1: the dose-dependent effect of intrathecal PAP in CIBP rats. Experiment 2.2: the time-course effect of intrathecal PAP in CIBP rats. Experiment 3.1: the influence of DPCPX on the antinociceptive effect of PAP in CIBP rats. Experiment 3.2: the influence of DIP on the antinociceptive effect of PAP in CIBP rats. Experiment 3.3: the spinal extracellular adenosine released by drugs. Experiment 4: the effect of drugs on electrical-evoked response of spinal WDR neurons of CIBP rats.

peak. Thirty rats were thus divided into 4 groups: sham-operated rats received saline injection (n = 7), sham-operated rats received 0.57 μg (the ED50 for PAP) of PAP injection (n = 7), CIBP rats received saline injection (n = 8), and CIBP rats received 0.57 μg PAP injection (n = 8). To observe the effect of PAP on rats' motor function, 16 naïve rats were divided into 2 groups which received saline or 1.0 μg PAP (n = 8 in each group).

Experiment 3: The Effects of Intrathecal PAP with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or Dipyridamole (DIP) on Pain Threshold and Release of Extracellular Adenosine

3.1 To investigate the influence of DPCPX (antagonist of adenosine A1 receptor) on the antinociceptive effect of PAP, 16 CIBP rats with PAP (0.57 μg, the ED50 for PAP) administration at POD 15 were divided into 2 groups that received intraperitoneal (i.p.) injection of saline or 3 mg/kg DPCPX at POD 17 (n = 8 in each group).

3.2 To assess the effect of DIP (inhibitor of nucleoside transporters) on pain response, 36 CIBP rats were divided into 4 groups: saline group (n = 9), 0.57 μg PAP group (n = 9), 10 μg DIP group (n = 9), and 0.57

μg PAP plus 10 μg DIP combination group (n = 9). Injection of the drugs was carried out from POD 15 to 18 (once per day).

3.3 To detect the spinal extracellular concentration of adenosine, 36 CIBP rats were divided into 4 groups: saline, 0.57 μg PAP, 10 μg DIP, and 0.57 μg PAP plus 10 μg DIP groups (n = 9 in each group). In this microdialysis experiment, drugs were intrathecally injected on POD 15.

Experiment 4: The Effect of PAP on Electrical-evoked Response of Spinal WDR Neurons

4.1 To observe the effect of drugs on central sensitization in cancer pain rats, 32 CIBP rats were divided into 4 groups: saline group (n = 8), 0.57 μg PAP group (n = 8), 10 μg DIP group (n = 8), and 0.57 μg PAP plus 10 μg DIP combination group (n = 8). At POD 15, after getting a stable recording of the wide-dynamic-range (WDR) neuron, drugs in a volume of 50 μl were applied directly onto the exposed surface of the spinal cord.

4.2 To observe the effect of DPCPX, 32 CIBP rats were divided into 4 groups: saline, 0.57 μg PAP, 10 μg DIP, and 0.57 μg PAP plus 10 μg DIP groups (n = 8 in each group). Ten micrograms of DPCPX in

a volume of 50 μl were applied onto the spinal dorsal surface 30 minutes before PAP and/or dipyridamole at POD 15.

Cell Preparation

The Walker 256 rat mammary gland carcinoma cell line was obtained from the Laboratory Animal Center of the Fourth Military Medical University. As described previously (13), 0.5 mL (2 × 107 cells/mL) of cancer cells was injected into the abdominal cavity of the SD rats. After 7 – 10 days, 2 mL of ascitic fluid was extracted from these rats and was centrifuged for 3 minutes at 1500 rpm. The pellet was washed with 10 mL of phosphate-buffered saline (PBS) and centrifuged again for 3 minutes at 1500 rpm. The final pellet was resuspended in one mL of PBS and the cells were counted using a hemocytometer. The cells were diluted to achieve a final concentration of 5 × 105 cells/10 μl of PBS and kept on ice until injection. The sham group consisted of the heat treated (100°C for 20 minutes) cells at the same final concentration.

Establishment of the Rat Model of Bone Cancer Pain

As described previously (13), following complete induction of anesthesia with sodium pentobarbital (50 mg/kg, i.p.), the right hind limb of the rat was shaved and the skin was disinfected with 70% v/v ethanol. A one-cm long rostrocaudal incision was made in the skin over the upper medial half of the tibia. The tibia was carefully exposed with minimal damage to the blood vessels and muscle. A 23-gauge needle was used to pierce from 5 mm below the knee joint medial into the tibial tuberosity. The needle was then replaced with a 50 μl Hamilton syringe containing tumor cells. A 10 μl of Walker 256 carcinoma cells (5 × 105 cells) was slowly injected into the bone cavity. After being kept in situ for 2 minutes, the syringe was then removed and the injection site was closed using bone wax (Ethicon). The muscle and the skin were stitched, with penicillin applied to the wound. The rats were placed on a heated pad, and after they regained consciousness, they were sent to their cages. In the sham-treated group, all the procedures were the same as those for the model group, except for the injection with same volume of heat-treated carcinoma instead.

Bone Radiological Detection

To assess the tibial bone destruction by tumor formation, tibial bone radiographs were obtained. After being anaesthetized with sodium pentobarbital (45 mg/kg, i.p.), the rats were placed in the prone position and exposed to an E-COM Digital Radiographer System (E-COM Technology Co. Ltd., Guangdong, China) on POD 10 and 20.

Immunohistochemistry

After being anesthetized with an overdose of sodium pentobarbital, the rats were perfused through the ascending aorta with l00 mL of 0.9% (w/v) saline, followed by 500 mL of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lumbar segments of the spinal cord of CIBP rats were removed immediately, placed into the same fresh fixative for 2 hours (4°C), and then saturated with 30% (w/v) sucrose in 0.1 M PB (pH 7.4) overnight at 4°C. Transverse frozen spinal sections (30 μm thick) were cut with a cryostat (Leica CM1800; Heidelberg, Germany) and collected serially in 3 dishes. Each dish contained a complete set of serial sections that were processed for immunofluorescence staining. One of the dishes was selected randomly. The sections in the dish were rinsed in 0.01 M PBS (pH 7.3) 3 times (10 minutes each), blocked with 2% normal goat serum in 0.01 M PBS containing 0.3% Triton X-100 for one hour at room temperature (RT, 20 – 25°C), and then used for immunofluorescence staining. The sections were incubated overnight at 4°C with chicken anti-PAP (15) (1:2,000; Aves Labs, Tigard, OR, USA) in 0.0l M PBS containing 5% (v/v) normal donkey serum (NDS), 0.3% (v/v) Triton X-100, 0.05% (w/v) NaN3, and 0.25% (w/v) carrageenan (PBS-NDS, pH 7.4). The sections were washed 3 times in 0.01 M PBS (10 minutes each) and incubated for 4 hours at RT with the biotinylated goat anti-chicken IgG antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA), and then incubated in fluorescein isothiocyanate (FITC)-labeled avidin D (1:200, catalog number: A-2001, Vector Laboratories, Burlingame, CA, USA) in PBS for 2 hours. Images were obtained using a confocal laser microscope (FV1000; Olympus, Tokyo, Japan). The specificity of the staining was tested on the sections in another dish by omission of the specific primary antibodies. No immunoreactive products were found on these sections (data not shown).

Western Blotting

As described previously (13), in order to observe the time course of PAP change, rats were sacrificed in the bone cancer group at POD 7, 14, and 21. All rats were quickly humanely killed and the whole lumbar

spinal cords were removed and the dorsal parts were harvested onto the dry ice. The selected region was homogenized with a hand-held pestle in sodium dodecyl sulfate (SDS) sample buffer (10 mL/mg tissue). The electrophoresis samples were heated at 100°C for 5 minutes and loaded onto 10% of SDS-polyacrylamide gels with standard Laemmli solutions (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were electroblotted onto a polyvinylidenedifluoride membrane (PVDF, Immobilon-P, Millipore, Billerica, MA, USA). The membranes were then placed in a blocking solution containing Tris-buffered saline with 0.02% Tween (TBS-T) and 5% nonfat dry milk for one hour, and incubated overnight under gentle agitation with chicken anti-PAP (1:2,000; Aves Labs, Tigard, OR, USA) and rabbit anti-GAPDH (1:5000; Sigma) antibodies. Bound primary antibodies were detected with the biotinylated goat anti-chicken IgG antibody (1:1,000; Vector Laboratories, Burlingame, CA, USA) for PAP or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) for GAPDH. Then, the membranes were incubated with avidin HRP (1:2000; Aves Labs, Tigard, OR, USA). Between each step, the membranes were washed with TBS-T. All reactions were detected by the enhanced chemiluminescence (ECL) detection method (Amersham). The densities of protein blots were analyzed by using Labworks Software (Ultra-Violet Products, Cambridge, UK). The densities of PAP and GAPDH immunoreactive bands were quantified with background subtraction protocol. The PAP levels were normalized against GAPDH levels and expressed as a fold change compared to the control.

Paw Withdrawal Threshold (PWT) Testing

As we described previously (13,16), animals were habituated to the testing environment for 3 days (one hour per day) before testing. On the testing day, they were placed into the inverted plastic boxes (30 × 30 × 50 cm³) on an elevated mesh floor, and habituated for 30 minutes. The ipsilateral hind paw was pressed with a logarithmic series of 8 calibrated Semmes-Weinstein monofilaments (von-Frey hairs; Stoelting, Kiel, WI, USA). Log stiffness of the hairs was determined by the log10 method (milligrams × 10) (17). The filaments had the following log-stiffness values (value in grams is given in parentheses): 4.17 (1.479 g), 4.31 (2.041 g), 4.56 (3.630 g), 4.74 (5.495 g), 4.93 (8.511 g), 5.07 (11.749 g), 5.18 (15.136 g), and 5.46 (28.840 g). The

range of monofilaments (1.479 – 28.840 g) produced a logarithmically graded slope that was used to interpolate the 50% response threshold of stimulus intensity, which was expressed as log10 (milligrams × 10) (18). Each filament was applied 10 times, and the minimal value that caused more than 5 obvious withdrawals was recorded as the PWT. The behavioral responses were used to calculate the 50% PWT by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method, as previously described in detail (17). The behavioral testing was blind with respect to previous treatment. The percentage of the anti-allodynia was calculated according to the following equation:% Antiallodynia = 100 – 100 × (baseline – post-Drug) / (baseline – post-Saline) (19).

Rotarod Test

The rotarod testing method used was similar to that described previously (19). The animals were placed on an UgoBasile 7650 Rotarod accelerator treadmill (UgoBasile, Varese, Italy), set at the minimum speed, for training sessions of 1 – 2 minutes at intervals of 30 – 60 minutes. Then, the animals were placed onto the rotarod at a constant speed of 25 RPM. As the animal took a grip of the drum, the accelerator mode was selected on the treadmill. Thereafter, the time was measured from the start of the acceleration period until the rat fell off the drum. The cut-off time was 30 seconds. Each rat was tested 30 minutes before drug administration as the control performance and then was tested once a day for 4 days during the drug administration. The time that the animal remained on the rotarod was recorded and expressed as a percentage of that animal's own mean control performance.

Intrathecal Catheter Insertion and Drug Administration

The intrathecal implantation was performed as previously reported (20). Briefly, a guide cannula (20 gauge, 0.9 mm × 38 mm) was inserted into the intrathecal space between the L5 and L6 vertebrae through a back skin incision. PE-10 tubing was inserted through the guide cannula. The catheter was tied in a loose knot and sutured on the back under the skin. The external end of the tube was passed subcutaneously and secured to the back of the neck. Rats were allowed to recover for 3 – 5 days before behavioral testing. Only the animals that were neurologically normal were used in the following behavioral observation. In the following experiments, because secretory PAP

protein is commercially available and has the same N-terminal catalytic region to TM-PAP (11), pure human (h) PAP protein (the secretory isoform) (Chemicon) was intrathecally (i.t.) administered as previously reported (21,22). In addition, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Sigma) and dipyridamole (DIP; Sigma) were used for further investigation on the mechanism of the analgesic effect of PAP. DPCPX was dissolved in 5% DMSO and diluted with 0.9% saline for i.p. injection, one hour before behavioral measurements. DIP was dissolved in 0.9% saline slightly acidified with HCl. All the drugs were injected in a volume of 10 µl, followed by a 10 µl saline flush.

Electrophysiological Study

Electrophysiological studies on CIBP rats were carried out at POD 15. At this stage, the mechanical allodynia was stable and reached the highest level. The rats were anaesthetized with sodium pentobarbital (45 mg/kg, i.p.) and a laminectomy was performed to expose the spinal cord at the L1–3 vertebral level. Single unit extracellular recordings were made at the L4–5 spinal cord segments with glass micropipettes filled with 0.5 M sodium acetate containing 2% Pontamine Sky Blue (impedance = 10 – 15 MΩ at 1000 Hz). The microelectrode was inserted perpendicularly into the dorsal horn from a point about midway between the midline and the medial edge of the dorsal root entry zone. The electrode was controlled by a microdrive to measure the neuron depth relative to the spinal cord surface. An electrical search stimulation (15 mA, one Hz, one ms pulse) was applied via 2 stainless steel needles inserted into the skin of the ipsilateral hind paw in order to find neurons that responded to the stimulation during microelectrode progression. When a single unit was discovered, only WDR neurons that responded to both gentle brushing and a noxious pinch applied on the hind paw were used, as previously described (23). In CIBP rats, only WDR neurons showed the significant increased responses to stimuli, so we focused on the WDR cells (24). After one WDR neuron was identified, the threshold stimuli intensity for C-fiber response was first detected. To do this, 3 consecutive single pulses separated by at least 20 seconds were applied and the lowest stimuli intensity that could evoke stable C-fiber responses was used. Then, 3 trains of stimuli (16 pulses with a 2 ms width, 0.5 Hz) were given at 10 minute intervals, with the intensity 3 times more potent than the C-fiber threshold intensity. According to the response time, the evoked responses were identified as $A\beta$- (0 – 20 ms), $A\delta$- (20 – 90 ms), C-fiber response (90 – 300 ms), and post-discharge activity (300 – 800 ms). As in previous research (23), since the C-response and post-discharge activities were considered to be nociception-related, the C-response and post-discharge activities of WDR neurons were used in the present study. Deep neurons were identified according to depth (500 – 1200 µm) (24). Drugs in a volume of 50 µl were applied directly onto the exposed surface of the spinal cord. Prior to administration of drugs, the neuronal responses of 3 stable control responses (< 10% variation for all parameters) were averaged to pre-drug baseline values. One neuron per animal was characterized.

Microdialysis and High Performance Liquid Chromatography (HPLC)

CIBP rats at POD 15 were used for this experiment. A microdialysis probe (MAB 7.80.10, MAB, Stockholm, Sweden) and PE-10 tubing were inserted into the lumbar intrathecal space from the L5 and L6 vertebrae. All rats were allowed to recover from implantation for one day. Only the animals that were neurologically normal after implantation surgery were used for the following experiments. The microdialysis probe was connected to a syringe pump (CMA 402, Stockholm, Sweden) and the outlet cannula was connected to the microfraction collector (CMA 142, Stockholm, Sweden). The probe was perfused with artificial cerebrospinal fluid (ACSF) at a flow rate of 2 µl/minute. After a 60-minute washout period and two 10-minute baseline samples, 10 µl of saline or drugs was injected through the intrathecal catheter, followed by a 10 µl saline injection. Dialysis samples were then collected every 10 minutes for 30 minutes and frozen at -80°C. Sample analysis was measured by HPLC. A Rainin A1 autosampler onto a Luna C18 column (250 × 4.6 mm) (Phenomenex, Torrance, CA) was used at a flow rate of one mL/minute with a mobile phase consisting of 10 mM ammonium phosphate, pH 6.0, with 15% methanol. Adenosine was determined using a Rainin Dynamax Model UV-D II absorbance detector at 254 nm.

Statistical Analysis

All data were collected by researchers blinded to the surgery and reagents used. Data were presented as mean ± standard error of the mean. The Student's T-test and one-way ANOVA followed by the Bonferroni post hoc tests were used for analysis. In all cases, $P < 0.05$ was considered as significantly different.

RESULTS

Tumor Cells Induced Bone Destruction and Mechanical Allodynia

Ten days after inoculation with cancer cells, small radiolucent lesions in the proximal epiphysis of the tibia ipsilateral to the injection site could be detected. Twenty days after injection, major loss of medullary bone and some destruction of cortical bone of the proximal epiphysis were observed. No radiological change was found in sham-operated animals treated with heat-killed cells or in the contralateral tibiae of rats injected with Walker 256 cells (Fig. 2A–D).

Cancer-induced pain is characterized as mechanical allodynia (25). In this study, rats injected with cancer cells displayed a slight but not significant decrease in PWT within 6 days following intratibial injections (PWT at POD 6: naive group: 5.36 ± 0.06, sham group: 5.29 ± 0.06, CIBP group: 5.22 ± 0.09; one-way ANOVA, $F_{(2, 22)} = 0.97$; $P > 0.05$). However, from POD 9, rats injected with cancer cells began to display a significant, progressive reduction in PWT compared to those in the naive
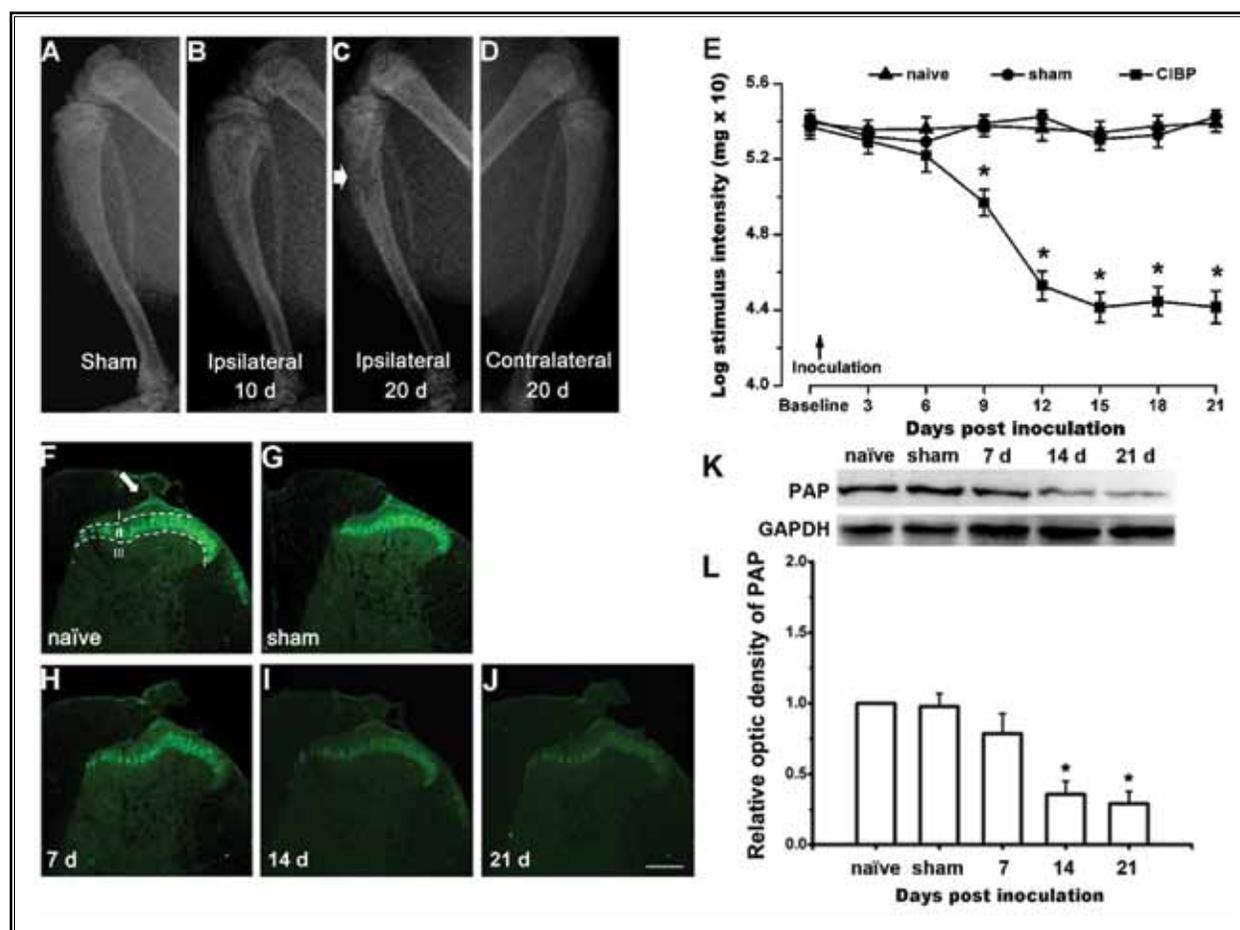

Fig. 2. *Rats with CIBP showed allodynia and reduced expression of PAP in the spinal dorsal horn.*
A-D, Radiographs showing the tibia inoculated with heat-killed Walker 256 cells (A) or 10 d (B) and 20 d (C) after live Walker 256 cell inoculation. The arrow shows structural destruction of the proximal bone. The contralateral tibia after Walker 256 cell inoculation is shown in (D). (E) The paw withdrawal thresholds (PWTs) of naive rats and rats that received intratibial inoculations of Walker 256 tumor cells (CIBP) or heat-killed cells (sham). Log stiffness of the hairs was determined by log10 (milligrams × 10). The PWT was decreased from 9 days to 21 days after cancer cell inoculation in rats. *, $P < 0.05$ vs. the sham rats. PAP-IP structures were densely located in the spinal cord dorsal horn of the naive rats (F) and sham operated rats (G). Dotted lines present the layers of spinal cord dorsal horn and arrow points to the dorsal root entry zone (DREZ) (F). Compared with the sham rats, the intensity of the PAP-IP fibers and terminals of CIBP rats was not reduced in the ipsilateral spinal dorsal horn until 14 days after cancer cell inoculation (H–J). In accordance with the immunohistochemical results, Western blot analysis showed the downregulation of PAP in the spinal dorsal part of rats with CIBP (K–L). *, $P < 0.05$ compared to the sham group. Scale bars = 200 µm.

or sham-operated groups (PWT at POD 9: naive group: 5.38 ± 0.06, sham group: 5.39 ± 0.05, CIBP group: 4.97 ± 0.07; one-way ANOVA, $F_{(2, 22)}$ = 17.06; $P < 0.05$) (Fig. 2E).

PAP Expression in the Spinal Dorsal Horn of CIBP Animals

PAP immunopositive (PAP-IP) structures were densely located in the lamina II of the spinal dorsal horn (15). There was no significant change of PAP-IP terminals between naive (Fig. 2F) and sham rats (Fig. 2G). Compared with sham rats, the intensity of PAP-IP fibers and terminals of CIBP rats was apparently reduced in the ipsilateral spinal dorsal horn 14 days and 21 days after cancer cell inoculation (Fig. 2I-J). In accordance with the immunohistochemical results, Western blot analysis showed that PAP in the spinal dorsal part was also downregulated in CIBP rats from 14 days after cancer cell inoculation (naive group: 1.00 ± 0.00, sham group: 0.97 ± 0.08, 7 day group: 0.77 ± 0.15, 14 day group: 0.36 ± 0.10, 21 day group: 0.29 ± 0.09; one-way ANOVA, $F_{(4, 25)}$ = 11.79; $P < 0.05$) (Fig. 2K-L).

The Antinociceptive Effect of Intrathecal Injection of PAP

In order to detect the antinociceptive effects of PAP on CIBP rats, 3 different concentrations of PAP were intrathecally injected from 15 days to 18 days (once per day) after cancer cell inoculation, and the mechanical threshold was tested one hour after PAP injection on the eighteenth day. The results showed that 0.1 µg of PAP had no obvious effect on CIBP-induced mechanical allodynia ($P > 0.05$). However, higher doses of PAP (0.3 and 1 µg) significantly increased the pain threshold, compared with that of the CIBP-saline group (sham-saline group: 5.38 ± 0.06, sham-PAP (1 µg) group: 5.29 ± 0.06, CIBP-saline group: 4.42 ± 0.07, CIBP-PAP (0.1 µg) group: 4.52 ± 0.09, CIBP-PAP (0.3 µg) group: 4.73 ± 0.08, CIBP-PAP (1 µg) group: 4.98 ± 0.07; one-way ANOVA, $F_{(5, 46)}$ = 28.74; $P < 0.05$) (Fig. 3A). The data points were fit with a sigmoid: $f = 1/(1+exp((ED50-E)/K))$, in which the ED50 (the dose of PAP that caused a 50% antinociceptive effect) equals to 0.57 µg (Fig. 3B). Furthermore, the pain threshold of sham rats was not affected by intrathecal PAP injection even with the administration of 1 µg of PAP ($P > 0.05$) (Fig. 3A). All these results suggested that PAP plays a dose-dependent analgesic effect on CIBP rats.

Then the time course of PAP's effect was observed. At 15 days after cancer cells inoculation, when the me-

chanical allodynia reached the highest level, a single dose of 0.57 µg (ED50) PAP was intrathecally injected, which caused an obvious inhibition of the cancer-induced mechanical allodynia at 16 days (sham-saline group: 5.31 ± 0.06, sham-PAP group: 5.29 ± 0.06, CIBP-saline group: 4.42 ± 0.10, CIBP-PAP group: 4.77 ± 0.10; one-way ANOVA, $F_{(3, 26)}$ = 29.72; $P < 0.05$) and the analgesic effect lasted to 17 days, compared to that of CIBP-saline rats (sham-saline group: 5.32 ± 0.05, sham-PAP group: 5.35 ± 0.06, CIBP-saline group: 4.43 ± 0.08, CIBP-PAP group: 4.86 ± 0.06; one-way ANOVA, $F_{(3, 26)}$ = 40.26; $P < 0.05$) (Fig. 3C).

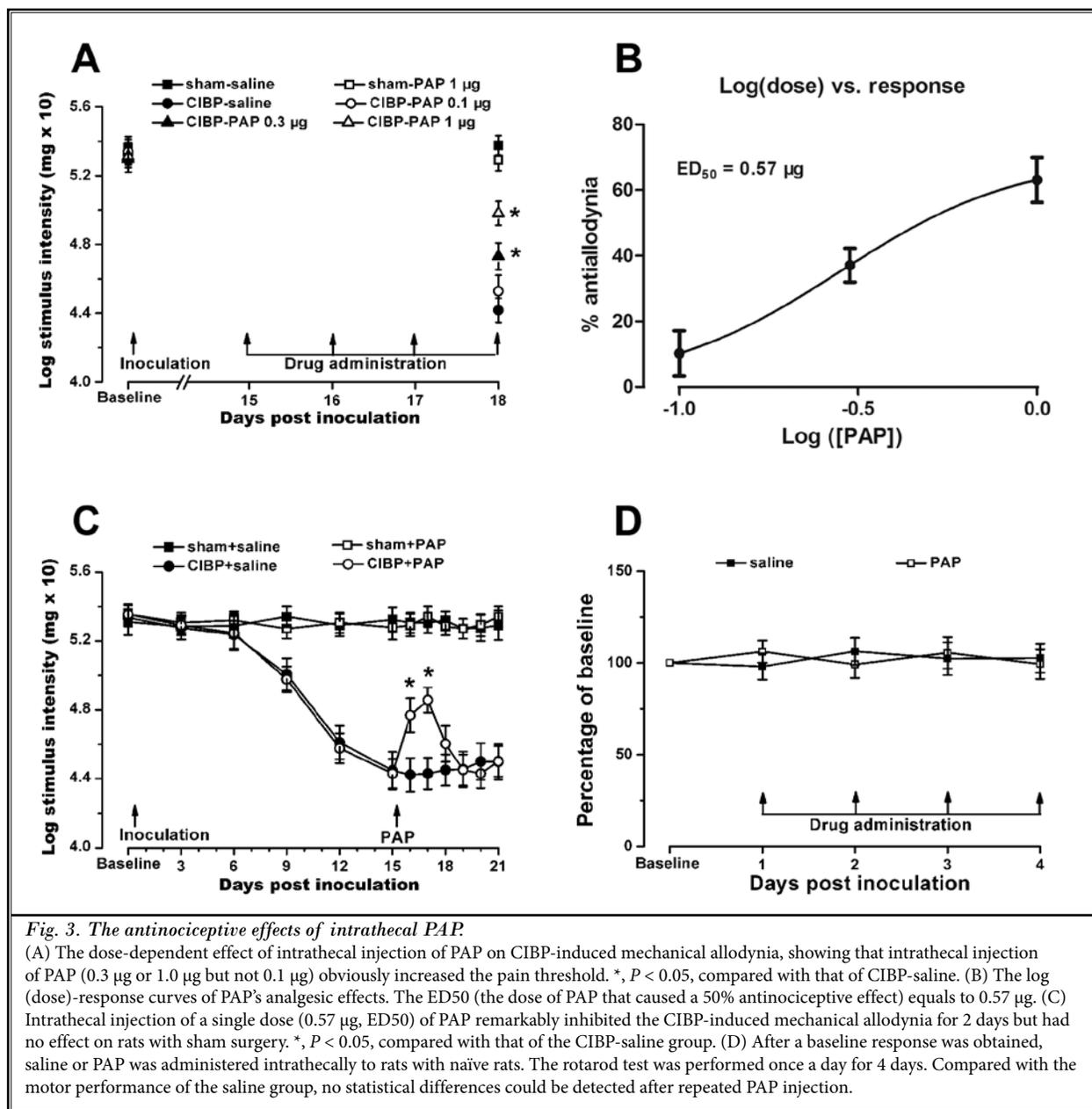## Effects of Drugs on Motor Functions as Indicated by the Rotarod Test

Nociceptive behavioral results can be influenced by motor dysfunctions. In order to exclude the possibility that motor function could be influenced by PAP intrathecal injection, a rotarod performance test was applied on naïve rats. The results showed that repeated intrathecal injection (totally 4 times, once per day) of PAP (1.0 µg) did not affect the motor behavior, compared with saline injection ($P > 0.05$) (Fig. 3D).

The Analgesic Effects of PAP Were Mediated by the Release of Extracellular Adenosine

The mechanism of the analgesic effect of PAP at the spinal level is unknown. One possible mechanism is that PAP can induce the release of adenosine in the spinal cord (26). We thus tested whether PAP's analgesic effect is related to the activity of adenosine.

DPCPX, a selective adenosine-1 receptor (A1R) antagonist, was applied to check whether PAP's analgesic effect can be regulated. After intraperitoneal injection of DPCPX, the antinociceptive effect of PAP was totally blocked, suggesting that A1R is important for PAP's effect (PWT at POD 17: PAP-saline group: 4.86 ± 0.09, PAP-DPCPX group: 4.46 ± 0.09; n = 8 for each group; t-test, $P < 0.05$) (Fig. 4A).

We then intrathecally injected dipyridamole, which is an inhibitor of nucleoside transporters and thus increases the concentration of extracellular adenosine by preventing adenosine from flowing into cells. In rats with CIBP, a combination application of dipyridamole (10 µg) and PAP (0.57 µg) remarkably enhanced PAP's analgesic effect, compared with that in the PAP injection group (saline group: 4.43 ± 0.08, dipyridamole group: 4.50 ± 0.09, PAP group: 4.79 ± 0.09, PAP-dipyridamole group: 5.09 ± 0.06; n = 9 for each group; one-way ANOVA, $F_{(3, 32)}$ = 16.66, $P < 0.05$) . Meanwhile,

Fig. 3. The antinociceptive effects of intrathecal PAP.
(A) The dose-dependent effect of intrathecal injection of PAP on CIBP-induced mechanical allodynia, showing that intrathecal injection of PAP (0.3 μg or 1.0 μg but not 0.1 μg) obviously increased the pain threshold. *, P < 0.05, compared with that of CIBP-saline. (B) The log (dose)-response curves of PAP's analgesic effects. The ED50 (the dose of PAP that caused a 50% antinociceptive effect) equals to 0.57 μg. (C) Intrathecal injection of a single dose (0.57 μg, ED50) of PAP remarkably inhibited the CIBP-induced mechanical allodynia for 2 days but had no effect on rats with sham surgery. *, P < 0.05, compared with that of the CIBP-saline group. (D) After a baseline response was obtained, saline or PAP was administered intrathecally to rats with naïve rats. The rotarod test was performed once a day for 4 days. Compared with the motor performance of the saline group, no statistical differences could be detected after repeated PAP injection.

single dipyridamole (10 μg) application did not show any apparent analgesic effect (*P* > 0.05) (Fig. 4B).

The behavioral results strongly suggested that PAP increases the extracellular adenosine concentration and thus causes the antinociceptive effect. To further confirm this hypothesis, we then directly detected the concentration of spinal extracellular adenosine by using the microdialysis method in conscious animals. Dipyridamole (10 μg), PAP (0.57 μg), or saline was intrathecally injected. It was found that PAP and dipyridamole could

apparently increase the concentration of adenosine within 10 minutes after injection, compared with that of the saline group (saline group: 122.11 ± 26.45%, PAP group: 304.20 ± 34.20%, dipyridamole group: 238.44 ± 34.94%, PAP-dipyridamole group: 527.55 ± 65.50%; n = 9 for each group; one-way ANOVA, $F_{(3, 32)} = 25.83$, *P* < 0.05) (Fig. 4C). Furthermore, intrathecal injection of dipyridamole immediately following PAP injection caused a more powerful effect on the release of adenosine, compared with that of the single PAP injection
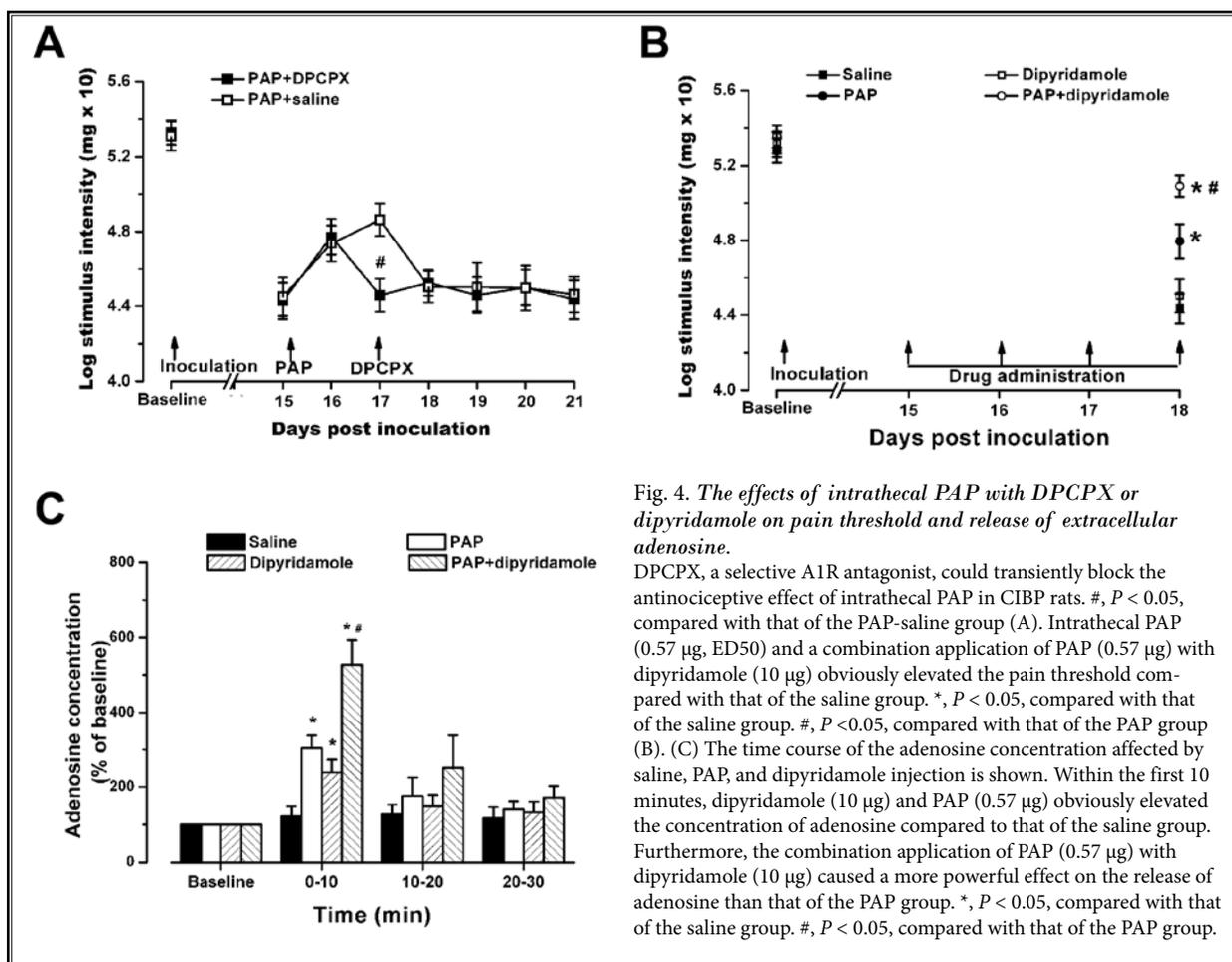
Fig. 4. *The effects of intrathecal PAP with DPCPX or dipyridamole on pain threshold and release of extracellular adenosine.*
DPCPX, a selective A1R antagonist, could transiently block the antinociceptive effect of intrathecal PAP in CIBP rats. #, $P < 0.05$, compared with that of the PAP-saline group (A). Intrathecal PAP (0.57 µg, ED50) and a combination application of PAP (0.57 µg) with dipyridamole (10 µg) obviously elevated the pain threshold compared with that of the saline group. *, $P < 0.05$, compared with that of the saline group. #, $P <0.05$, compared with that of the PAP group (B). (C) The time course of the adenosine concentration affected by saline, PAP, and dipyridamole injection is shown. Within the first 10 minutes, dipyridamole (10 µg) and PAP (0.57 µg) obviously elevated the concentration of adenosine compared to that of the saline group. Furthermore, the combination application of PAP (0.57 µg) with dipyridamole (10 µg) caused a more powerful effect on the release of adenosine than that of the PAP group. *, $P < 0.05$, compared with that of the saline group. #, $P < 0.05$, compared with that of the PAP group.

group (PAP-dipyridamole group vs PAP group, $P < 0.05$) (Fig. 4C). No further change of adenosine release was observed from 10 to 30 minutes after even a single or combined drug administration (Fig. 4C, $P > 0.05$).

The Effect of PAP on an Electrically Evoked Response of Spinal WDR Neurons

We then applied in vivo extracellular recordings to check the direct effect of PAP on the electrically evoked responses (C-fiber and postdischarge) of WDR neurons in the spinal dorsal horn. It has been found that the frequency of the C-fiber and postdischarge responses of WDR neurons are all greatly enhanced in rats with CIBP compared with rats with sham surgery, in accordance with previous reports (data not shown) (24,27,28). Bath application of PAP (0.57 µg) or dipyridamole (10 µg) on the spinal cord significantly inhibited the electrically evoked C-fiber responses (saline group: 108.38 ± 8.51%, PAP group: 69.75 ± 7.28%,

DIP group: 73.13 ± 8.95%, combination group: 40.76 ± 9.67%; n = 8 for each group; one-way ANOVA, F (3, 28) = 10.26; $P < 0.05$) and the postdischarge responses (saline group: 105.16 ± 8.99%, PAP group: 61.47 ± 8.02%, DIP group: 74.09 ± 7.16%, combination group: 28.49 ± 7.57%; n = 8 for each group; one-way ANOVA, F (3, 28) = 16.00; $P < 0.05$) (Fig. 5A and 5C). Furthermore, the inhibitory effect of a combination application of PAP with dipyridamole on the postdischarge was stronger than that of the PAP application ($P < 0.05$) (Fig. 5A and 5C). Finally, pretreatment with DPCPX (10 µg) reversed the attenuation of the evoked neuronal responses induced by PAP and/or dipyridamole application on C-fiber responses (saline group: 108.12 ± 9.01%, PAP group: 82.88 ± 12.52%, DIP group: 92.08 ± 9.04%, combination group: 75.10 ± 11.30%; n = 8 for each group; one-way ANOVA, F (3, 28) = 1.80; $P > 0.05$) and postdischarge responses (saline group: 106.16 ± 9.24%, PAP group: 77.97 ± 11.22%, DIP group: 89.53
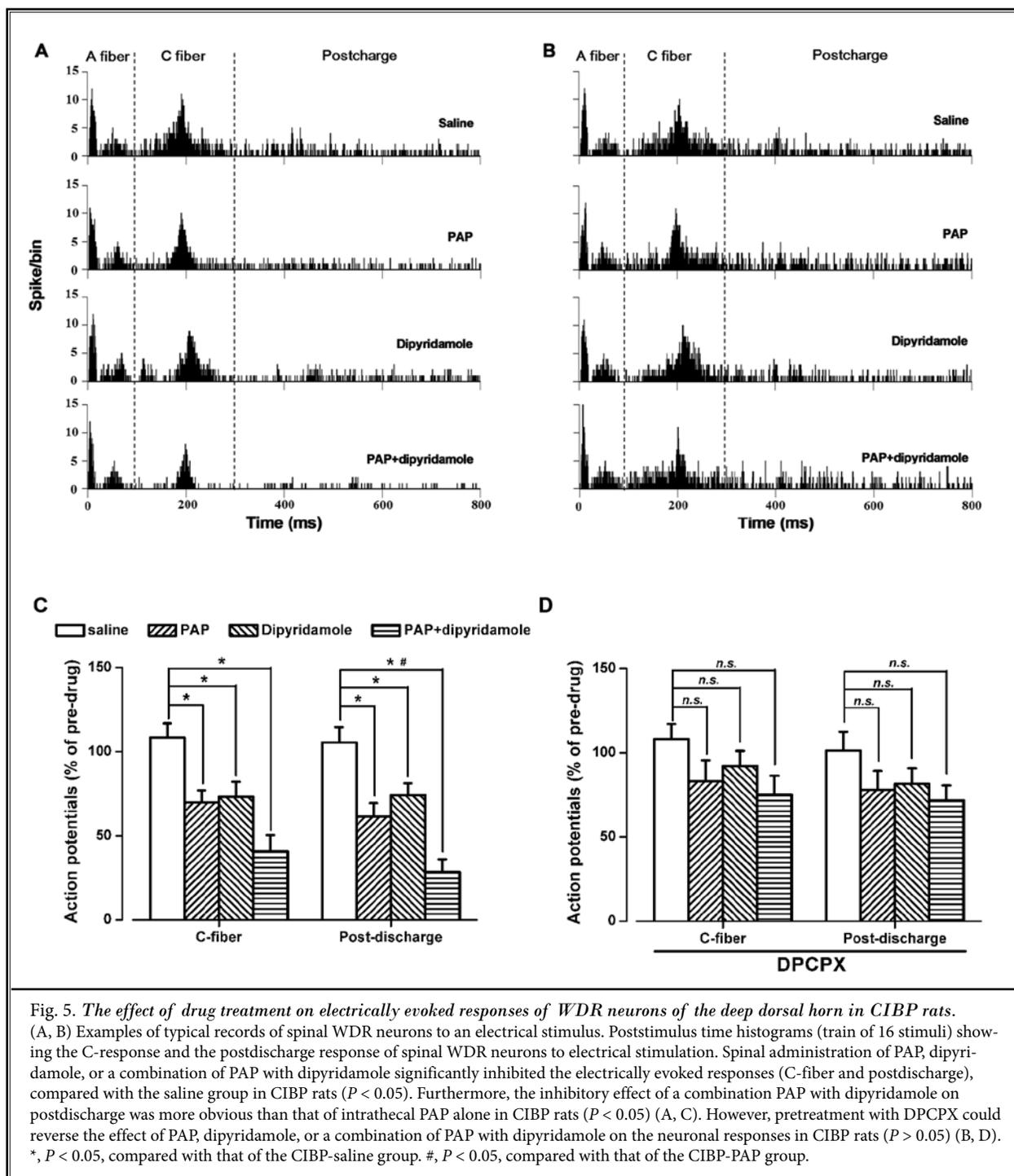
Fig. 5. *The effect of drug treatment on electrically evoked responses of WDR neurons of the deep dorsal horn in CIBP rats.*
(A, B) Examples of typical records of spinal WDR neurons to an electrical stimulus. Poststimulus time histograms (train of 16 stimuli) showing the C-response and the postdischarge response of spinal WDR neurons to electrical stimulation. Spinal administration of PAP, dipyridamole, or a combination of PAP with dipyridamole significantly inhibited the electrically evoked responses (C-fiber and postdischarge), compared with the saline group in CIBP rats ($P < 0.05$). Furthermore, the inhibitory effect of a combination PAP with dipyridamole on postdischarge was more obvious than that of intrathecal PAP alone in CIBP rats ($P < 0.05$) (A, C). However, pretreatment with DPCPX could reverse the effect of PAP, dipyridamole, or a combination of PAP with dipyridamole on the neuronal responses in CIBP rats ($P > 0.05$) (B, D). *, $P < 0.05$, compared with that of the CIBP-saline group. #, $P < 0.05$, compared with that of the CIBP-PAP group.

± 9.16%, combination group: 71.69 ± 8.92%; n = 8 for each group; one-way ANOVA, $F_{(3, 28)} = 2.45$; $P > 0.05$) (Fig. 5B and 5D).

Discussion

The purpose of our study was to observe the roles of PAP in a rat model of CIBP. The present study indicated

the following: (1) PAP was intensively distributed in the spinal dorsal horn and was significantly reduced in the spinal dorsal horn after CIBP. (2) Intrathecal injection of PAP significantly attenuated mechanical allodynia as well as inhibited the neuronal responses of spinal WDR neurons in rats with CIBP, which was mediated through adenosine A1 receptor by increasing the concentration of extracellular adenosine.

TM-PAP is a transmembrane form of PAP; however, its function is not clear (12). In previous works, it has been found that TM-PAP-IP fibers extensively exist in the lamina II of the spinal cord, while TM-PAP-IP neuronal cell bodies are restricted to the small-diameter dorsal root ganglion neurons. All these morphological data suggest that TM-PAP should be involved in pain regulation. In the present study, we confirmed the distribution pattern of TM-PAP in the spinal dorsal horn and found that the expression of TM-PAP in the spinal dorsal horn was greatly decreased in CIBP rats. Meanwhile, the decrease of spinal PAP paralleled the development of the CIBP induced mechanical allodynia, suggesting that PAP expression in the spinal cord is important for maintaining the normal mechanical pain threshold. The downregulation of PAP might cause the disinhibitory influence on pain transmission in CIBP rats. This hypothesis was further confirmed by the finding that intrathecal injection of PAP could obviously reverse the cancer pain-induced allodynia.

The analgesic effect of PAP in CIBP rats could be blocked by the A1 receptor inhibitor DPCPX, which strongly suggests that the antinociceptive effect of PAP depends on A1 receptor. These results are consistent with previous data carried out on neuropathic pain models (11,21,29). Moreover, our study observed that in conscious rats, the release of extracellular adenosine induced by intrathecally administered PAP could be improved by the extracellular adenosine reuptake inhibitor dipyridamole. Interestingly, although dipyridamole could not exert an analgesic effect by itself, it could enhance the analgesic effect of PAP. These results strongly suggested that extracellular adenosine is closely related to the antinociceptive effect of PAP.

Adenosine A1 receptor is a Gi/o-coupled receptor, which is widely distributed throughout the body and extensively expressed on neurons and astrocytes (26). In chronic pain, astrocytes can be activated and release a large number of cytokines, which in turn act on neurons to form the cross-talk between neurons and astrocytes, and then further contribute to pain transmission (30). Therefore, after intrathecal PAP administration, it

is possible that the increased extracellular adenosine (confirmed in the present study by the microdialysis results) could act on the A1 receptor on both nociceptive neurons and astrocytes and block the transmission of pain signals.

In the spinal dorsal horn, there are 2 types of neurons related to noxious stimuli. Most of the neurons in the superficial layer are nociceptive-specific (NS) neurons that respond only to strong noxious stimulation (31), whereas the vast majority of neurons in deep layers are WDR neurons that respond to both non-noxious and noxious temperature and mechanical stimuli (32). Compared to NS neurons, WDR neurons were considered to be the major interconnecting neurons related to the nociceptive transmission (33) and the hyperexcitability of WDR neurons is considered to be associated with central sensitization in the process of chronic pain (24,33,34). Thus, we only observed the neuronal response of WDR neurons of deep layers in the cancer pain state. Meanwhile, the C-response and postdischarge activities of WDR neurons were observed because they are closely related with the generation and maintenance of chronic pain (23). In our study, we found that PAP could effectively suppress the C-response and postdischarge of WDR neurons, which is increased in the cancer pain state (24,27,28). These results suggested that the intrathecal PAP injection-induced analgesic effect is due to the suppression of the ongoing central sensitization mediated by spinal cord WDR neurons. Simultaneously, the effect of PAP on WDR neuronal responses could be blocked by the A1 receptor antagonist DPCPX and was potentiated by dipyridamole. The electrophysiological results are in accordance with the behavioral and microdialysis results, further suggesting that the inhibition effect of PAP on WDR neurons might be mediated by adenosine. Interestingly, although most of the PAP and adenosine A1 receptors were located in spinal superficial layer considered to be closely related to nociceptive transmissions (35), in our study, PAP could inhibit the hyperexcitable neuronal responses of spinal deep layer by enhancing the release of spinal adenosine. Thus, this may indicate that spinal superficial dorsal horn is the origin of a spinal-supraspinal loop which transmitted nociceptive information to brain and also is the origin of spinal circuits which directly alters the excitability of deep dorsal horn neurons (36,37).

Actually, based on the current experiment data, we couldn't exclude the possibility that other components, except for adenosine A1 receptor, could be involved in

the analgesic effect of PAP. Furthermore, besides the central analgesic effect, PAP could exert an antinociceptive effect in periphery. Hurt et al (38) found that injection of PAP into the popliteal fossa has dose-dependent antinociceptive effects in mouse models of acute and chronic pain. The results from present and previous works indicate that PAP could exert antinociceptive effect by both central and peripheral mechanisms.

Limitations

Besides adenosine A1 receptor, the other components about the analgesic mechanism of PAP could not be excluded. In addition, the long-term antinociceptive effect of intrathecal PAP is not clear yet.

Conclusions

Our studies demonstrated that PAP is involved in maintaining CIBP and effectively alleviates pain symptoms. The mechanism could be that PAP participates in promoting the release of extracellular adenosine. Thus, clinical application of PAP may be promising for the treatment of serious bone cancer pain.

Acknowledgement

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