Introduction

Chronic pain is conceptualized as a disease process itself. It affects people worldwide and severely affects the life quality of patients, becoming an escalating health problem (1). Chronic pain can be classified into inflammatory pain and neuropathic pain by the underlying mechanism (2). In clinical practice, the treatment of inflammatory pain generally responds to opioid medications, nonsteroidal anti-inflammatory drugs (NSAIDs), and acetaminophen (3). Neuropathic pain from injury or disease of the nervous system, including the peripheral nerve, dorsal root ganglion (DRG), spinal cord or brain, is usually more difficult to treat than inflammatory pain (4, 5) and its control is often refractory to opioids and requires larger doses or more than one drug, which may otherwise induce hazardous side effects (1). Therefore, the clinical treatment of chronic pain by NSAIDs, steroids, antidepressants, antiepileptics, or/and opioid medications are still unsatisfactory in most cases and remains a challenge for doctors and researchers who have committed themselves to search for novel analgesics with potent analgesic potency and no or relatively minor side effects.

Chronic pain may involve a mix of both inflammatory and neuropathic components. It was reported that both inflammatory and neuropathic pain processes involve numerous neurotransmitters, neuromodulators, and receptors of primary afferent neurons relay pain signals from the periphery to the central nervous system (2, 3). Substance P (a well-known tachykinin peptide) encoded by the preprotachykinin-A (PPT-A) gene, as an important neurotransmitter and/or a neuromodulator, is synthesized in the DRG and released from primary afferent neurons for conveying nociceptive information and transmitting pain (6 – 8). Interestingly, Guan et al. (9) indicated that

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Dragon’s Blood Inhibits Chronic Inflammatory and Neuropathic Pain Responses by Blocking the Synthesis and Release of Substance P in Rats

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Abstract. As a traditional Chinese medicine, dragon’s blood (DB) is widely used in treating various pains for thousands of years due to its potent anti-inflammatory and analgesic effects. In the present study, we observed that intragastric administration of DB at dosages of 0.14, 0.56, and 1.12 g/kg potently inhibited paw edema, hyperalgesia, cyclooxygenase-2 (COX-2) protein expression, or preprotachykinin-A mRNA expression in carrageenan-inflamed or sciatic nerve–injured (chronic constriction injury) rats, respectively. A short-term (15 s or 10 min) pre-exposure of cultured rat dorsal root ganglion (DRG) neurons to DB (0.3, 3, and 30 μg/ml) or its component cochinchinenin B (CB; 0.1, 1, and 10 μM) blocked capsaicin-evoked increases in both the intracellular calcium ion concentration and the substance P release. Moreover, a long-term (180 min) exposure of cultured rat DRG neurons to DB or CB significantly attenuated bradykinin-induced substance P release. These findings indicate that DB exerts anti-inflammatory and analgesic effects by blocking the synthesis and release of substance P through inhibition of COX-2 protein induction and intracellular calcium ion concentration. Therefore, DB may serve as a promising potent therapeutic agent for treatment of chronic pain, and its effective component CB might partly contribute to anti-inflammatory and analgesic effects.

Keywords: analgesic, anti-inflammation, dragon’s blood, cyclooxygenase-2, synthesis and release of substance P
substance P could evoke delta-opioid receptor membrane trafficking in cultured DRG neurons and demonstrated that deletion of the PPT-A gene reduced stimulus-induced surface insertion of delta-opioid receptors and abolished delta-opioid receptor–mediated spinal analgesia and morphine tolerance. Moreover, increases in either intracellular calcium ion concentration or cyclooxygenase-2 (COX-2) expression were demonstrated to be involved in the rapid or slow release of substance P evoked by noxious stimulus from cultured DRG neurons (8, 10, 11). The approaches described above would be suitable for elucidating the molecular mechanism of chronic pain induced by noxious stimuli which are important for understanding the role of substance P in mature somato sensory systems and for developing new therapeutic strategies to treat chronic pain.

Chinese dragon’s blood (DB), a dark red resin, is a traditional Chinese medicine obtained from Dracaena cochinchinensis (Lour.) S.C. Chen (12, 13). DB is widely used as an external wash to promote healing of wounds and to stop bleeding, and also internally as the “blood-activating drug” to treat various pains, post partum bleeding, internal traumas, and menstrual irregularities in China (14, 15). Furthermore, DB has been documented to reduce the body-torsion reaction of mice induced by acetic acid (14). Whole cell electrophysiological experiments indicated that DB and its components could suppress tetrodotoxin-sensitive voltage-gated sodium currents in rat DRG neurons (16, 17).

However, there is still no direct evidence for anti-inflammatory and analgesic effects of DB on chronic pain. One of the purposes of the present investigation was therefore to characterize the antinociceptive and analgesic effects of DB on carrageenan- and chronic constriction injury (CCI)-induced pain responses in vivo. Furthermore, pharmacological effects of DB and its available component cochinchininen B (CB) on bradykinin- and capsaicin-induced substance P release were separately evaluated in vitro.

Materials and Methods

Materials

The following drugs were used: bradykinin (Peptide Institute, Inc., Osaka); collagenase, phosphoramidon, bacitracin, captopril, diclofenac, capsaicin, capsazepine, HOE140, and carrageenan (Sigma Chemical, St. Louis, MO, USA); [125I]Tyr8-substance P (81.4 TBq/mmol; New England Nuclear, Boston, MA, USA); Dulbecco’s modified Eagle’s medium and trypsin (Invitrogen, Burlington, ON, Canada); DB (Xishuanbanna Yulin Pharmaceutical Co., Kunming, Guangxi, China); CB (Guangxi Institute of Chinese Medicine and Pharmaceutical Co., Kunming, Guangxi, China); DB (Xishuanbanna Yulin Pharmaceutical Co., Kunming, Guangxi, China). The following drugs were used: bradykinin (Peptide Institute, Inc., Osaka); collagenase, phosphoramidon, bacitracin, captopril, diclofenac, capsaicin, capsazepine, HOE140, and carrageenan (Sigma Chemical, St. Louis, MO, USA); [125I]Tyr8-substance P (81.4 TBq/mmol; New England Nuclear, Boston, MA, USA); Dulbecco’s modified Eagle’s medium and trypsin (Invitrogen, Burlington, ON, Canada); DB (Xishuanbanna Yulin Pharmaceutical Co., Kunming, Guangxi, China); CB (Guangxi Institute of Chinese Medicine and Pharmaceutical Co., Kunming, Guangxi, China).

Carrageenan-induced inflammatory pain

The carrageenan test was used for determination of anti-inflammatory activity (18). Young adult male Wistar rats were divided into six groups: control (distilled water-treated) group, carrageenan group, positive group (diclofenac-treated), and DB-treated groups (low-, middle-, and high-dose groups). The rats in the DB-treated groups were given one intragastric administration of DB (0.14, 0.56, and 1.12 g/kg; suspended in distilled water), and the rats in the positive group were intraperitoneally injected with an equal volume of diclofenac solution (10 mg/kg, stock solution: 1 mg/ml in distilled water). The rats in the control and carrageenan groups were intraperitoneally injected with an equal volume (10 ml/kg) of distilled water. All groups received intragastric administration of the test agents for 5 consecutive days. Two days after the DB treatment, paw edema was induced in the rats by a 100-μl intradermal injection of 2-carrageenan (1%) into the subcutaneous tissue of the right hind paw. Hyperalgesia to thermal stimulation and paw edema were separately measured using a Plantar test (Model 37370; Ugo Basile, Varese, Italy) and a plethysmometer (Model 7159, Ugo Basile). Subsequent readings of the same paw were carried out at 0, 1, 2, 3, 4, 12, 24, 48, and 72 h after the carrageenan injection. After the corresponding point measurement, the rats were sacrificed via rapid decapitation, and their hind paw skins were dissected and rapidly fixed for 24 h at 22°C with 4% paraformaldehyde (Sigma) for immunohistochemical staining. All animal procedures were performed in accordance with the Guide for Animal Experimentation, South-Central University for Nationalities, and the Committee of Research Facilities for Laboratory Animal Sciences, South-Central University for Nationalities, China.

Peripheral nerve injury

A CCI of the sciatic nerve was performed in adult male Wistar rats anesthetized with trichloroacetaldehyde monohydrate (450 mg/kg, i.p.; Wako, Osaka) according to previously described methods (19). Young adult male Wistar rats (6–9 weeks of age) were divided into groups in the same way as in the carrageenan test and received intragastric administration of the test agents for 30 consecutive days. Two days after the DB treatment, the left sciatic nerves were exposed at the mid-thigh portion, where three ligatures of 4–0 chromic gut (Ethicon, Brussels, Belgium) were tied loosely. As the sham control, rats were subjected to a surgical procedure to expose the sciatic nerve without any ligation. The wound was immediately closed with silk sutures and topical antibiotic was applied at once. The CCI-induced pain threshold
was assessed every week for five weeks after the operation by a series of 11 von Frey filaments (ranging from 0.16 to 15 g force). To isolate the lumbar DRGs, some rats in the corresponding groups were sacrificed via rapid decapitation four weeks after the operation.

**Real-time PCR for determining PPT-A mRNA expression**

Total RNA harvested from the L5 DRGs of rats by the acid guanidinium thiocyanate – phenol – chloroform extraction method was separately subjected to reverse transcription into cDNA by using a Superscript kit (TaKaRa Bio, Dalian, China) according to the manufacturer’s protocol. Quantitative real-time PCR was performed on a Thermal Cycler Dice TP800 system (TaKaRa Bio, Otsu) by using SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer’s protocol. Quantitative real-time PCR was performed according to the manufacturer’s instructions (21). Next, 4-μm-thick sections were cut and mounted on glass microslides (Matsunami, Osaka). Briefly, the paraffin-embedded sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to water. The sections were then washed twice in 10 mM phosphate-buffered saline (PBS) and incubated overnight at 4°C with rabbit anti COX-2 antibody (1:1,000 dilution, Sigma), followed by washing twice with PBS and incubation for 60 min at room temperature with Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes, Eugene, OR, USA). Finally, the sections were washed three times in PBS and visualized by an inverted fluorescence microscope (Eclipse Ti, Nikon). Quantification of COX-2 immunofluorescence was performed with the use of image analysis software (NIS-Elements AR 3.0, Nikon) from 7 separate experiments. For the quantification in each experiment, three equal-sized fields of each photograph per group were randomly chosen.

**Immunohistochemical staining of COX-2**

The hind paw skins of rats fixed at each time point after carrageenan injection were embedded in paraffin blocks. Next, 4-μm-thick sections were performed with a standard immunoperoxidase technique [Histofine Simple Stain Rat MAX-PO (MULTI) kit; Nichirei, Tokyo] according to the manufacturer’s instructions (20). Briefly, the sections were incubated overnight at 4°C with rabbit anti-COX-2 antibody (1:1,000 dilution, Sigma). After the treatment with Histofine simple stain rat MAX-PO (MULTI), color development (brown) was performed using a DAB substrate kit (Nichirei), and the sections were counterstained with hematoxylin (blue). Then, multispectral imaging analyses of slides were performed by using an Eclipse Ti microscope (Nikon, Tokyo) with a Nuance Multispectral Imaging System (Cambridge Research and Instrumentation Inc., Woburn, MA, USA) according to the method instructions (20). Spectral optical density data were automatically acquired from 420 – 720 nm in 10-nm increments. Spectral unmixing was accomplished by Nuance software v1.42 and pure spectral libraries of individual chromogens. Nonspecific background staining was subtracted from each image individually. For the quantification in each experiment, three equal-sized fields of each photograph per group were randomly chosen.

**Immunofluorescence staining of COX-2**

The isolated lumbar DRGs from these sham-operated and CCI model mice were embedded in paraffin blocks according to the method instructions (21). Next, 4-μm-thick sections were cut and mounted on glass microslides (Matsunami, Osaka). Briefly, the paraffin-embedded sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to water. The sections were then washed twice in 10 mM phosphate-buffered saline (PBS) and incubated overnight at 4°C with rabbit anti COX-2 antibody (1:1,000 dilution, Sigma), followed by washing twice with PBS and incubation for 60 min at room temperature with Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes, Eugene, OR, USA). Finally, the sections were washed three times in PBS and visualized by an inverted fluorescence microscope (Eclipse Ti, Nikon). Quantification of COX-2 immunofluorescence was performed with the use of image analysis software (NIS-Elements AR 3.0, Nikon) from 7 separate experiments. For the quantification in each experiment, three equal-sized fields of each photograph per group were randomly chosen.

**Isolation and culture of rat DRG cells**

DRGs of young adult Wistar rats (6 – 9 weeks of age) were dissociated into single cells by the treatment of enzymes (collagenase and trypsin) according to a previously described method (22). Then, the cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum, 4 mM glutamine, 1% penicillin/streptomycin, and 30 ng/ml nerve growth factor, and plated on polyethyleneimine and laminin-coated dishes (5 – 6 DRGs/35 mm dish for measurement of substance P content) and on glass coverslips (1 DRG/well for measurement of intracellular calcium ion concentration) with a silicon rubber wall (FlexiPERM; Heraeus Biotechnology, Hanau, Germany). The cultures were maintained at 37°C in a water-saturated atmosphere with 5% CO2 for 5 days before the initiation of experiments.

**Determination of effective component CB in DB**

DB obtained from Dracaena cochinchinensis (Lour.) S.C.CHEN was completely soluble in 100% ethanol. A solution of DB in ethanol (30 mg/ml) was prepared prior to experiments. The content of CB (possible effective component, ref. 17) in DB were determined by HPLC (Agilent 1200; Agilent Technologies, Santa Clara, CA, USA) under the following conditions: areserved phase column (Ultimate®XB-C18, 4.6 mm x 250 mm, 5 μm; Welch Materials, Ellicott, MD, USA), a mobile phase composed of acetonitrile and 1% acetic acid (37/63), an ultraviolet detector at 275 nm, and at a flow rate of 1.0...
ml/min. Under the above conditions, the chromatograms of CB and DB are shown in Fig. 3, and the retention time of CB was near 15.02 min. To determine the content of CB, 30 mg/ml of DB (1 ml) was diluted with 49 ml of the mobile phase. The resultant solution was filtrated with a filter membrane (0.45 μm) to remove all suspended particulates (solids) before injecting into HPLC. The linearity of the response was verified over the range of 4.0 – 64.0 μg/ml, and the correlation equation (y = 270.44x – 19.792) was the following with a correlation coefficient of 0.9998 (Fig. 3C).

Measurement of substance P content

Except for some DRG cells treated by peptidase inhibitors alone (as a control), other DRG cells were separately exposed to various drugs (DB and CB) in DMEM (serum-free) containing peptidase inhibitors (1 μM phosphoramidon, 4 μg/ml bacitracin, and 1 μM captopril) for 30 min at 37°C in a water-saturated atmosphere with 5% CO₂. After being washed two times with 1 ml of Krebs-HEPES buffer (110 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.7 mM D-glucose, 5 mM HEPES at pH 7.4), the cells pretreated with various drugs were continuously stimulated by capsaicin (for 10 min in Krebs-HEPES buffer) or bradykinin (for 180 min in serum-free DMEM) at 37°C. Thereafter, the substance P content collected from Krebs-HEPES buffer or DMEM was measured by using a highly sensitive radioimmunoassay (23).

Measurement of intracellular calcium ion concentration

The cultured DRG cells plated on glass coverslips were incubated in 5 μM of fura-2 acetoxymethyl ester (Biotium, Hayward, CA, USA) dissolved in a modified Mg²⁺-free Hanks balanced salt solution (HBSS; 2.6 mM CaCl₂, 15 mM HEPES, pH 7.4) containing 0.05% pluronic-F127 (Sigma) for 40 min in a dark room at 37°C (22) and then rinsed (3 times) with extracellular solution and allowed to de-esterify for more than 30 min before the initiation of experiments. Neurons were continuously perfused with various experimental solutions (150 μl/well) through polyethylene tubes connected to a HPLC pump (HPG-3400SD; Dionex, Noida, India); complete changes of bath solution occurred in 3 s. Every 4 s, the fura-2 signal fluorescence intensity changes in calcium ion concentration were monitored for 12 min with the excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm by using an inverted fluorescence microscope (Eclipse Ti, Nikon) equipped with a variable filter wheel (Sutter Instrument Company, Novato, CA, USA) and digital CCD camera (Digital Sight DS-QiMC, Nikon). The fluorescence output was digitized and analyzed with the Nikon image analysis system (NIS-Elements AR 3.0). The 340/380 emission ratio was used to determine the intracellular calcium concentration. Cell diameter was determined by calibrated video image.

Statistics

The data are presented as the mean ± S.E.M. Statistical analyses were performed by using a one-way analysis of variance (ANOVA) with Dunnett multiple comparisons. Significance was set at a value of P < 0.05.

Results

Effect of DB treatment on the carrageenan-induced inflammatory pain conditions

To investigate whether a treatment with DB produces inhibitory effects against inflammatory pain conditions, the levels of carrageenan-evoked paw edema and hyperalgesia in rats treated with or without DB were examined (Fig. 1). It was observed that λ-carrageenan (1%) evoked a significant increase in the paw edema of rats along a bell-shaped, time-dependent curve during the period from 1 to 72 h after the carrageenan injection (Fig. 1A). The maximal response of carrageenan-injected rat paw edema (230.1% ± 25.6% in comparison to 100% ± 16% for control at 0 h, n = 7, P < 0.001) occurred at 2 h after the injection. Conversely, λ-carrageenan (1%) decreased pain threshold of the same paw to thermal stimulation along a time-dependent curve during the same period (Fig. 1B). The maximal response of pain threshold decrease evoked by carrageenan (7.8 ± 0.8 s for carrageenan group in comparison to 17.4 ± 2.9 s for the control group, n = 7, P < 0.01) also occurred at 2 h after the injection. In comparison to carrageenan alone, the intragastric administration of DB attenuated levels of paw edema (159.6% ± 2.8% for the DB-L group, 125% ± 5.1% for the DB-M group, and 123.4% ± 4.8% for the DB-H group, n = 7) and pain threshold (10.6 ± 0.8 s for the DB-L group, 10.9 ± 1.0 s for the DB-M group, and 15.0 ± 2.1 s for the DB-H group, n = 7) induced by carrageenan in a dose-dependent manner (0.14, 0.56, and 1.12 g/kg). In comparison to the control, the use of DB at the three different doses exhibited a tendency to increase pain threshold (17.9 ± 1.1, 18.5 ± 1.1, and 21.7 ± 2.5 s, respectively) of rats (carrageenan-uninjected, 0 h, 17.2 ± 0.8 s) as shown in Fig. 1B. After the corresponding point measurement, hind paw skins of the rats were dissected and rapidly fixed for immunohistochemical staining of COX-2 (Fig. 1C). We observed that carrageenan exhibited a bell-shaped, time-dependent curve of COX-2 protein expression during the period from 1 to 72 h after the injection (data not shown). The maximal response of
carrageenan-induced COX-2 expression occurred at 2 h after the injection (Fig. 1: C and D) by a multispectral imaging analysis. In this experiment, we therefore examined the possible inhibitory effect of DB on carrageenan-induced inflammatory pain conditions in rats. A) Inhibition of carrageenan-induced paw edema by DB. Changes of edema volume in the ipsilateral hindpaw of rats (n = 7 per group) were measured at 0, 1, 2, 3, 4, 12, 24, 36, 48, and 72 h after the carrageenan injection. B) The mean thermal withdrawal latency (in seconds) measured in the ipsilateral hindpaw of rats. Responses were measured at 0, 1, 2, 3, 4, 12, 24, 48, and 72 h after the carrageenan injection. C) Representative multispectral images for immunohistochemical staining of COX-2 in the ipsilateral hind paw skins at 2 h after carrageenan injection. Brown is positive COX-2 staining and blue is hematoxylin counterstain. Left panel: RGB images of six groups, middle panel: unmixed DAB images of left panel, right panel: unmixed hematoxylin images of left panel. D) Determination of spectral optical density of COX-2 expression in the the ipsilateral hind paw skins of panel C. The data are obtained from 7 (A, B or C, D) separate experiments. DB-L, dragon’s blood at low dose; DB-M, dragon’s blood at middle dose; DB-H, dragon’s blood at high dose. **P < 0.01 and ***P < 0.001. Bar: 50 μm.
induced COX-2 expression at 2 h after the injection and observed that the levels of carrageenan-induced COX-2 expression were dose-dependently inhibited by DB treatment. As observed in Fig. 1, a comparison of inhibitory effects on those responses indicated that DB (middle- and high-dose) possessed a significantly greater potency than (127.7% ± 10.4% for paw edema and 12.1 ± 1.4 s for pain threshold) diclofenac (10 mg/kg, ref. 24). Together with the results shown in Fig. 1, these findings indicate that DB might have practical utility for preventing the carrageenan-evoked inflammatory pain conditions through the inhibition of COX-2 expression.

Effect of DB treatment on the CCI-induced neuropathic pain conditions

To evaluate effects of DB upon nociceptive responses associated with neuropathic pain behaviors, anti-allodynic effects were assessed in the CCI model of peripheral nerve injury. As shown in Fig. 2A, CCI surgery produced a decrease in the mechanical pain threshold during five weeks after loose ligation of the sciatic nerve, compared to pre-surgery levels of the rats. In CCI rats (69.3% ± 4.2% for paw edema and 12.1 ± 1.4 s for pain threshold) diclofenac (10 mg/kg, ref. 24). Together with the results shown in Fig. 2, these findings indicate that DB might have practical utility for preventing the carrageenan-evoked inflammatory pain conditions through the inhibition of COX-2 expression.

The immunofluorescent staining data (Fig. 2: C and D) showed that the CCI-induced up-regulation of COX-2 expression (312% ± 26% for the CCI group in comparison to 100% ± 20% for the sham group, n = 7) was significantly inhibited by DB in a dose-dependent manner at four weeks after operation. Compared with the diclofenac group (183% ± 14% of sham, n = 7), DB at high dose (166% ± 29% of the sham group, n = 7) exhibited a stronger inhibitory tendency on the up-regulation of COX-2 expression induced by CCI. Similarly, CCI-induced up-regulation of PPT-A mRNA expression (269% ± 28% for the CCI group in comparison to 100% ± 16% for the sham group, n = 7) in the ipsilateral L5 DRG from the same specimen described above was attenuated by DB in a dose-dependent manner. Compared with the diclofenac group (138% ± 6% of the sham group, n = 7), DB at high dose (132% ± 13% of the sham group, n = 7) exhibited a more potent inhibitory tendency on the up-regulation of PPT-A mRNA induced by CCI. Together with the results shown in Fig. 2, these findings indicate that DB induces an inhibitory effect against CCI-induced neuropathic pain behaviors accompanying strong inhibition of COX-2 protein expression and PPT-A mRNA expression.

Effects of DB and its components on the capsaicin- and bradykinin-induced substance P release

To clarify the possible pharmacological mechanism(s) of DB on the behavior responses of inflammatory pain and neuropathic pain, we further examined effects of DB and its possible effective component CB on the rapid or slow release of substance P induced by capsaicin or bradykinin from cultured DRG neurons. According to the determination, results of the effective component CB in DB shown in Fig. 3, the concentration of CB was calculated as 2.8 μM in 30 mg/ml of DB solution. Therefore, we selected 0.1 to 10 μM as an appropriate concentration range for the effective component CB in the present study. As shown in Fig. 4A, a short-term (10 min) exposure of DRG neurons to capsaicin (100 nM; 113 ± 6 pg/dish for the capsaicin group in comparison to 44 ± 9 pg/dish for the control, n = 10 or 11, respectively) increased substance P release in a TRPV1 antagonist (50 ± 10 pg/dish, n = 9), capsazepine-sensitive manner. The rapid release of substance P evoked by capsaicin was dose-dependently attenuated by a 10-min pretreatment with DB (0.3, 3, and 30 μg/ml; 128 ± 20, 73 ± 20, and 55 ± 10 pg/dish, n = 6, respectively). As expected, CB (0.1, 1, and 10 μM) exhibited a more potent inhibitory effect (66 ± 9, 46 ± 7, and 85 ± 12 pg/dish, n = 5, respectively) on the capsaicin-evoked rapid release of substance P. On the other hand, a long-term exposure of DRG neurons to bradykinin (1 μM; 251 ± 20 pg/dish, n = 4)
Inhibition of SP Synthesis and Release by DB

Fig. 2. Inhibitory effects of DB on the CCI-induced neuropathic pain conditions in rats. A) Return of CCI-induced pain threshold by DB in the ipsilateral hind paw of rats. B) Representative immunofluorescence photomicrographs for COX-2 (green) in the ipsilateral L4 – 6 DRGs of CCI rats at day 28 after the operation. C) Representative immunofluorescence photomicrographs for COX-2 (green) in the ipsilateral L5 DRGs of CCI rats treated with or without DB or diclofenac at four weeks after the operation. D) Fluorescence determination of COX-2 expression in the ipsilateral L5 DRGs of panel C. E) PPT-A mRNA expression in the ipsilateral L5 DRGs of panel C. The data are obtained from 7 separate experiments. *p < 0.05, **p < 0.01, and ***p < 0.001. Bars: 25 μm.
increased substance P release in a bradykinin B₂-receptor antagonist (59 ± 8 pg/dish, n = 4), HOE140-sensitive manner (Fig. 4B). The slow release of substance P evoked by bradykinin was dose-dependently attenuated by DB (0.3, 3, and 30 μg/ml; 189 ± 25, 165 ± 17, and 141 ± 23 pg/dish, n = 4, respectively). Similarly, CB (0.1, 1, and 10 μM) also exhibited certain inhibitory effects (143 ± 17, 182 ± 5, and 226 ± 22 pg/dish, n = 4, respectively) on the
bradykinin-evoked release of substance P. However, DB and CB used alone did not have any effect on the amount of spontaneous release of substance P from cultured DRG neurons (data not shown).

**Inhibition of capsaicin-induced increase in intracellular calcium concentration by DB or CB**

Previous observations indicated that intracellular calcium ions are involved in capsaicin-evoked substance P release from cultured DRG neurons (11). To further examine the possible beneficial effect of DB on capsaicin-induced increase in intracellular calcium ion concentration loaded with fluorescent indicator (fura-2), we therefore selected DB (3 μg/ml) and CB (0.1, 1, and 10 μM) as appropriate experimental conditions according to the inhibition of capsaicin-induced substance P release by DB shown in Fig. 4. Acute exposure to capsaicin (100 nM, 15 s) evoked a transient increase in intracellular calcium ion concentrations in cultured small-sized DRG neurons (Fig. 5A). After the pretreatment with capsaizpine (1 μM, 5 min), a selective antagonist of the TRPV1, the capsaicin-evoked intracellular calcium ion transient was completely blocked, indicating that it was probably mediated through the TRPV1 (Fig. 5B). As expected, a 5-min pretreatment with DB (3 μg/ml) attenuated the capsaicin-evoked intracellular calcium ion transient increase (Fig. 5C). Similarly, the three concentrations of effective component CB exhibited certain inhibition on the capsaicin-evoked transient increases in intracellular calcium ion concentration. However, both DB and CB used alone caused a weak tendency to increase intracellular calcium ion concentration in cultured small-sized DRG neurons (Fig. 5: C, D, E, and F).

**Discussion**

The current approach was intended to determine possible pharmacological mechanisms underlying the action of DB on various noxious stimuli–induced chronic pain responses by using both molecular and pharmacological tools. Firstly, we have focused our attention on the pharmacological effects of DB on paw edema, thermal hyperalgesia, COX-2 protein expression, and PPT-A mRNA expression in carrageenan-inflamed or sciatic nerve-

![Fig. 5](image-url)
injured (CCI) rats. Secondly, we turned our attention to examine possible inhibitory effects of DB and its effective components on intracellular calcium ion level and substance P release induced by capsaicin or bradykinin in cultured rat DRG neurons.

Carrageenan-induced paw edema is a useful inflammatory pain model characterized as a biphasic event with involvement of various mediators to produce some inflammatory responses (25). The first phase of edema (0 – 1 h) is not inhibited by non-steroidal anti-inflammatory drugs, while the second phase (1 – 72 h for the present study) is correlated with the elevated production of kinins and prostaglandins (26, 27). In the present study, we demonstrated that being similar to diclofenac, the intragastric administration of DB not only elevated nociceptive threshold of normal rats, but also dose-dependently attenuated carrageenan-evoked paw edema and thermal hyperalgesia. Especially, DB exhibited a more potent inhibitory effect on carrageenan-induced COX-2 expression than diclofenac. Indeed, diclofenac was considered as one of the few NSAIDs of first choice in the treatment of acute and chronic painful and inflammatory conditions (28). In addition, COX-2 is an inducible enzyme that increases in the peripheral and central nervous systems following tissue damage and the resulting inflammatory process. Recent phytochemical studies on DB revealed the presence of flavonoids (including CB) and steroids (29, 30). Therefore, the inhibition of COX-2 expression by DB seems to be evoked by CB, one of the low molecular weight compounds among the flavonoids from DB. However, the exact target molecule(s) of DB that evoked the inhibition of carrageenan-induced COX-2 expression in hindpaw skins of rats should be examined in future experiments. Therefore, these findings suggest that DB may have both anti-nociceptive and antihyperalgesic effects through preventing the induction of COX-2 expression.

In order to estimate the possible pharmacological effect of DB on neuropathic pain conditions, the CCI model of the rat sciatic nerve was chosen in this study. The CCI model was the most common chronic pain model that could finely mimic many features of clinical neuropathic pain, such as lumbar disk herniation (31). In the present study, the observations from the mechanical pain threshold test, immunofluorescence staining, and qRT-PCR determination showed significant increases in mechanical allodynia, COX-2 induction, and PPT-A mRNA expression in the L5 DRG of CCI neuropathic rats (Fig. 2). We selected DRG as an appropriate specimen because DRGs plays an important role in initiation, transmission, and maintenance of the neuropathic pain (19). Here we further found that DB, as well as diclofenac, exhibited strong inhibitory effects on the CCI-induced increases in COX-2 induction, PPT-A mRNA expression, and nociceptive sensitization. Our data are in agreement in part with the previous reports of Matsunaga et al. (32) who showed that the inhibition of COX-2, but not COX-1, by selective inhibitors attenuates hyperalgesia in neuropathic rats. Therefore, these results suggest that DB may also have preventive and therapeutic activity on neuropathic pain conditions through inhibition of nociceptive states arising from nerve injury. Based on the behavior results (Figs. 1 and 2) of two pain models in the present study and the observations described above, we propose a hypothesis that DB might directly exert its analgesic effect to deter the nociceptive reception and transmission accompanying its anti-inflammation effect through its possible effective components. However, the potential mechanism through which DB exerts suppressive effects on hyperalgesia induced by CCI remains unclear.

DB plays an important role in initiation, transmission, and maintenance of the neuropathic pain, as it involves neuropeptides, ion channels, and Gq-protein-coupled receptors (8, 33, 34), and therefore a cell culture model using DRG neurons was chosen in this study. Our previous observations indicated that both extracellular and intracellular calcium ions are required for the capsaicin- or bradykinin-evoked substance P release through the activation of TRPV1 or bradykinin B2 receptor from cultured DRG neurons (8, 23). Moreover, various observations indicated that spinal prostaglandin E2 release and thermal hyperalgesia evoked by intrathecal injection of substance P can be reversed by spinal COX-2 inhibition (35 – 37). Furthermore, increases in either intracellular calcium ion concentration or COX-2 expression were demonstrated to be involved in the capsaicin-evoked rapid or bradykinin-evoked slow release of substance P from cultured DRG neurons (8, 10, 11). In addition, it was demonstrated that CB (one small-molecular compound of flavonoids from DB; see Fig. 3) could inhibit the whole-cell current induced by capsaicin (15). Thus, we assume that the suppression of the synthesis and release of substance P by DB is probably due to the inhibition of intracellular calcium ion concentration or the release of substance P in cultured DRG neurons (Figs. 4 and 5). However, it is yet unknown what other effective components are contained in DB in addition to CB. Of course, the identification of new effective components in DB for reducing chronic inflammatory and neuropathic pain will be confirmed in future experiments.

In summary, this study first demonstrates that the tra-
ditional Chinese medicine DB can attenuate local inflammatory edema and hyperalgesia induced by hindpaw injection of carrageenan in rats through the inhibition of COX-2 expression and \( \text{PPT-A} \) mRNA expression. Both DB and its effective component CB exhibit remarkable inhibitory effects on the intracellular calcium ion levels and substance P release induced by capsaicin or bradykinin. These observations provide novel evidence that DB, by blocking the synthesis and release of substance P through a peripheral mechanism by inhibition of COX-2 induction and intracellular calcium ion concentrations, may exert its anti-inflammatory and analgesic effects. Therefore, DB may be a promising potential therapeutic drug for reducing chronic inflammatory and neuropathic pain, and its effective component CB might partly contribute to the anti-inflammatory and analgesic effects.

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