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Original Article

Anti-nociceptive Effect of Patchouli Alcohol: Involving Attenuation of Cyclooxygenase 2 and Modulation of Mu-Opioid Receptor*

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ABSTRACT **Objective:** To explore the anti-nociceptive effect of patchouli alcohol (PA), the essential oil isolated from *Pogostemon cablin* (Blanco) Bent, and determine the mechanism in molecular levels. **Methods:** The acetic acid-induced writhing test and formalin-induced plantar injection test in mice were employed to confirm the effect *in vivo*. Intracellular calcium ion was imaged to verify PA on mu-opioid receptor (MOR). Cyclooxygenase 2 (COX2) and MOR of mouse brain were expressed for determination of PA's target. Cellular experiments were carried out to find out COX2 and MOR expression induced by PA. **Results:** PA significantly reduced latency period of visceral pain and writhing induced by acetic acid saline solution (P<0.01) and allodynia after intra-plantar formalin (P<0.01) in mice. PA also up-regulated COX2 mRNA and protein (P<0.05) with a down-regulation of MOR (P<0.05) both in *in vivo* and *in vitro* experiments, which devote to the analgesic effect of PA. A decrease in the intracellular calcium level (P<0.05) induced by PA may play an important role in its anti-nociceptive effect. PA showed the characters of enhancing the MOR expression and reducing the intracellular calcium ion similar to opioid effect. **Conclusions:** Both COX2 and MOR are involved in the mechanism of PA's anti-nociceptive effect, and the up-regulation of the receptor expression and the inhibition of intracellular calcium are a new perspective to PA's effect on MOR.

KEYWORDS Chinese medicine, patchouli alcohol, analgesic effect, mu-opioid receptor, cyclooxygenase 2

Pachouli alcohol (PA) is a natural compound isolated from *Pogostemon cablin* (Blanco) Bent (Patchouli). Patchouli is a common Chinese medicine mainly used for the treatment of gastrointestinal disorders and diarrhea induced by infections.[1,2] The chemical structure of PA is shown in Figure 1. Pharmacological studies have shown that PA can regulate gastrointestinal functions[3] and protect the gastric mucosa from alcohol injury.[4] In addition, PA was reported to have an anti-inflammatory effect[5] that suppressed the lipopolysaccharide-induced increase in cyclooxygenase 2 (COX2).[6-8] PA has antibacterial and antiviral activities,[9,10] protects skin from ultraviolet damage,[11] and has anti-tumor activity.[12] Patchouli oil showed an anti-depressive effect with the excitation of the central nervous system.[13] In our preliminary study, we found a significant anti-nociceptive effect of PA in the acetic acid-induced writhing test in mice with decreased expression of COX2 in the mouse brain, which implies that PA is also an analgesic agent.

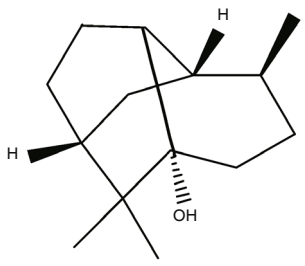

Figure 1. Chemical Structure of PA

Although it is known that PA inhibits the expression of COX2, it is unknown whether it

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has other targets to achieve this analgesic effect. Therefore, we performed experiments *in vivo* and *in vitro* to determine the mechanism of its analgesic action with the goal of providing experimental evidence for a new class of analgesics.

METHODS

Animals

Institute of Cancer Research (ICR) mice, male, specific pathogen free, weighing 25±2 g were purchased from Vital River Laboratories (Beijing, China). The animals were housed in temperature- and humidity-controlled rooms, maintained on a 12-h light/dark cycle and provided with unlimited amounts of rodent chow and drinkable water. The laboratory animal facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University approved all animal protocols used in this study (No. 16-DLJ-Pach).

Drugs and Chemicals

PA (PubChem CID 10955174 in NCBI: http://pubchem.ncbi.nlm.nih.gov/compound/10955174), purity of 98% according to high performance liquid chromatography (HPLC), was gifted by Prof. XU Li-zhen, Institute of Medicinal Plants, Chinese Academy of Medical Sciences. PA was dissolved in dimethyl sulphoxide (DMSO) and diluted in normal saline before administration (pH 7.2). Acetic acid was purchased from Beijing Chemical Plant (Beijing, China). Aspirin was purchased from Xiehe Pharmaceutical Company (Shijiazhuang, China). [D-Ala2,N-MePhe4,Gly-ol]-enkephalin (DAMGO, No. ab120674), a mu-opioid receptor (MOR) selective agonist,[14] and naloxone, an opioid receptor antagonist, were purchased from Abcam Trading (Shanghai) Company Ltd. (China).

Experimental Procedures *In Vivo*

Acetic Acid-Induced Writhing Test in Mice

Sixty mice were divided into 5 groups randomly (n=12 in each group): normal, PA, model, PA plus model and aspirin plus model groups. The model groups received a celiac injection of acetic acid saline solution (0.6% acetic acid in normal saline) 30 min after the subcutaneous injection of drugs.[15,16] The control and PA groups were injected with normal saline. The incubation time for body twisting and the duration of the writhing reaction of each mouse for the next 15 min were observed immediately after the injection of acetic acid saline solution. For mice that did not exhibit a writhing reaction, 15 min was recorded as the incubation time. The mouse brain was isolated and stored at –80 ℃ for mRNA and protein determination. The inhibition rate = (the model group–medicine group)/model group × 100%. Based on our preliminary experiments, the dosages of PA and aspirin were set at 200 and 100 mg/kg, respectively.

Intraplantar Formalin-Induced Allodynia Test in Mice

Another 65 mice were divided into 5 groups randomly using randomized block design (SPSS12.0 software, USA): normal (n=12), PA (n=12), model (n=16), PA plus model (n=16) and aspirin plus model (n=13) groups. The model groups of mice were injected with formalin solution (3.8% formalin in normal saline) for intraplantar administration (20 μL per mouse) 30 min after the subcutaneous injection of drugs.[17-19] The mouse plantar licking time was recorded. The control and PA groups were injected with normal saline. The mouse brain was isolated and stored at –80 ℃ for mRNA and protein determination. The inhibition rate = (the model group – medicine group)/model group × 100%. PA and aspirin were used at the dosages of 200 and 100 mg/kg, respectively.

Cell Culture *In Vitro*

The C17.2 cell line (a stem cell of the mouse cerebellum)[20,21] was presented by Prof. XIE Wei-dong, Life Sciences Division, Graduate School at Shenzhen, Tsinghua University and cultured in Dulbecco's modified Eagle medium containing 15% bovine serum albumin (BSA) and maintained at 37 ℃ in a humidified incubator containing 5% CO_2. The PC12 cell line was purchased from the Cell Center, Chinese Academy of Medical Sciences (Beijing, China), and cultured in RPMI1640 containing 10% BSA and 5% horse serum and maintained at the same humidified incubator. The cytotoxicity of PA was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay performed according to the methods described previously.[22] In *in vitro* experiments, PA was used at a concentration of 1–25 μg/mL, a safe range according to the MTT assay. DMSO was used as the vehicle control.

Calcium Imaging

A confocal assay for calcium was employed

in the C17.2 cells and the PC12 cells. Cells were cultured for 2 days, incubated with the fluorescent probe fluo-3AM (S1056; Beyotime, Haimen, China) for 1 h at 37 ℃, and then incubated for 30 min at 37 ℃ in Hank's buffer.[23] Images were taken with a Zeiss LSM 710 META confocal microscope (Carl Zeiss, Germany) and analyzed by Zen Light Edition Software. The cultured cells were illuminated at 488 nm, and an emission at 525–530 nm was detected.

Quantitative Analysis of Intracellular Calcium Concentration

Cells were cultured for 2 days and incubated with PA and other agents for 3 h. Then, cells were ultrasonicated to collect cytoplasm and total protein for calcium determination. Calcium was detected according to the instructions of the kit (Zhongsheng Beikong BioTech Inc., Beijing, China) and determined using BIO-RAD680 enzyme-linked immunosorbent assay (ELISA, Bio-Rad Laboratories, Inc., Hercules, California, USA) at 570 nm. Total protein was determined according to the manufacturer's instructions (Zhongsheng Beikong BioTech Inc., Beijing, China). The calcium content was described as μg/mg protein. According to the preliminary experiments, the concentration of PA was 5 μg/mL, naloxone was 1 μg/mL, and DAMGO was 2 μg/mL.

Real-Time PCR

The mRNA and protein determination was performed using q-PCR and Western blot assay as described in detail previously.[24,25] In q-PCR analysis, all primer sequences were designed by NCBI GenBank and produced by Sangon Biotechnology Ltd. (Shanghai, China). The primers are listed in Table 1. β-actin served as an internal control. A light-cycler 480II was employed for real-time PCR analysis (Roche, Shanghai, China).

Western Blot

Primary antibodies against COX2 (P35354, rabbit monoclonal antibody, 74 kDa) were purchased from Bioworld Technology (Nanjing), Co. Ltd. (China). MOR (BS-3623R, rabbit polyclonal antibody, 50 kDa) was purchased from BIOSS (Beijing, China), and TNF-α (ab199013, mouse monoclonal antibody, 17 kDa) was purchased from Abcam Trading (Shanghai) Co. Ltd. (China). Primary antibodies against IL-1β (PO1584, mouse monoclonal antibody, 19 kDa) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primary antibody of β-actin (SC-47778, mouse monoclonal antibody, 43 kDa) and NF-κB (SC8008, mouse monoclonal antibody, 65 kDa) were purchased from Santa Cruz Biotechnology Inc. (Texas, USA). Secondary antibodies of goat anti-mouse IgG-HRP (BE0102) and goat anti-rabbit (BE0101) IgG-HRP were purchased from Bioeasy (Beijing) Technology Co. Ltd. (China). The targeted proteins were visualized with the super signal west femto luminol/enhancer sSolution (No. 1856189, Thermo-Scientific, Rockford, Illinois, USA), and the intensity of visualized bands was analyzed using Quantity One software (Bio-rad). Data were expressed by the ratio to β-actin.

Table 1. Primers Sequences Used for Real-Time PCR

Gene	Forward	Reverse
COX2	5'-TTCAACACACTCT ATCACTGGC-3'	5'-TGTTGACAGTGGTATT TCTGGTG-3'
MOR	5'-CCAGGGAACATC AGCGACTG-3'	5'-GTTGCCATCAACGTG GGAC-3'
NF-κB	5'-ATGGCAGACGAT GATCCCTAC-3'	5'-TGTTGACAGTGGTATT TCTGGTG-3'
IL-1β	5'-GAAATGCCACCT TTTGACAGTG-3'	5'-TGGATGCTCTCATCAG GACAG-3'
TNF-α	5'- GGGATCAAATC CAAGCCTGC-3'	5'-GTGAGGGTGGATGCT CAGTG-3'
β-actin	5'-GTGACGTTGAC ATCCGTAAAGA-3'	5'-GCCGGACTCATCGTA CTCC-3'

Notes: NF-κB: nuclear factor κB; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor α

Statistical Analysis

All values were expressed as mean ± standard deviation ($\bar{x} \pm s$). Data were statistically analyzed using one-way analysis of variance (ANOVA) with *F* value determination. The *F* test was performed using Excel 2013 (Microsoft, Way Redmond, WA, USA). Student's *t*-test between two groups was performed after the *F* test. *P* values below 0.05 were considered statistically significant.

RESULTS

Acetic Acid-Induced Writhing Test in Mice

In the body writhing test, the incubator time and writhing action times indicated a relatively strong anti-nociceptive effect of PA. Model mice exhibited significant body writhing in a short time period after acetic acid saline injection, while PA extended the incubation period of body writhing and reduced the frequency of writhing action. Aspirin demonstrated a stronger inhibitory effect (Figure 2).

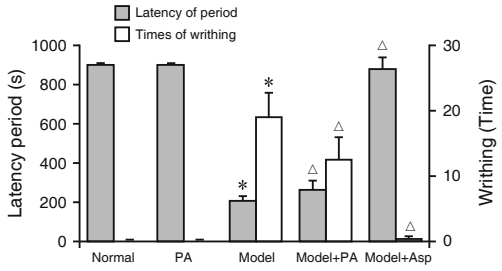

Figure 2. Latency Period of Visceral Pain and Writhings in Mice (n=12, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. normal group; △P<0.01 *vs*. model group

Effects of PA on Protein Expressions of COX2, MOR and Inflanmatory Factors in Mice Brain

COX2 protein levels in the mouse brain were detected and discovered that PA inhibited COX2 in the same way as aspirin. In addition, PA up-regulated the transcription and expression of MOR, while aspirin did not have the same effect. Further examination of the expression of related inflammatory factors reported the down-regulation of IL-1β and TNF-α by PA, with a decrease of NF-κB. Additionally, aspirin showed the down-regulated effect of IL-1β and TNF-α without the inhibition of NF-κB expression (Figure 3).

Effect of PA on Plantar Formalin-Induced Allodynia in Mice

Model mice exhibited long durations of foot licking. PA significantly reduced the plantar licking time, which indicated that pain was reduced. Aspirin also inhibited pain (Figure 4A). Protein expression in the brains of mice showed that the levels of MOR and COX2 were significantly increased in the model mice and that PA and aspirin could inhibit COX2 expression. In addition, PA did not affect the increase of the MOR expression, but aspirin down-regulated MOR. PA also facilitated the expression of MOR in normal mouse brains. The expression of both mRNA and protein expressions of MOR in the PA+model group increased compared with control groups. And they showed no significant difference compared with model groups. In MOR protein expression of the PA+model group looks like lower than that of the model group but they showed no statistical significance due to its standard deviation (Figure 4B, 4C).

Effect of PA on Calcium Ion Concentration in Cytoplasm

PA can significantly reduce intracellular calcium ion concentration in both cell lines, and naloxone, an MOR antagonist, also decreased the intracellular calcium level, while the MOR agonist DAMGO increased calcium (Figures 5A and 5B). To further evaluate PA's effect on intracellular calcium, the calcium ion concentration in cells was measured. The equation of the calcium standard curve was determined as $Y=1.4509X-0.00183$ ($R^2=0.9968$, Figure 5C). Naloxone impeded the decrease of the intracellular calcium level induced by PA, while DAMGO also changed the effect by which PA reduced the intracellular calcium ion concentration (Figure 5D).

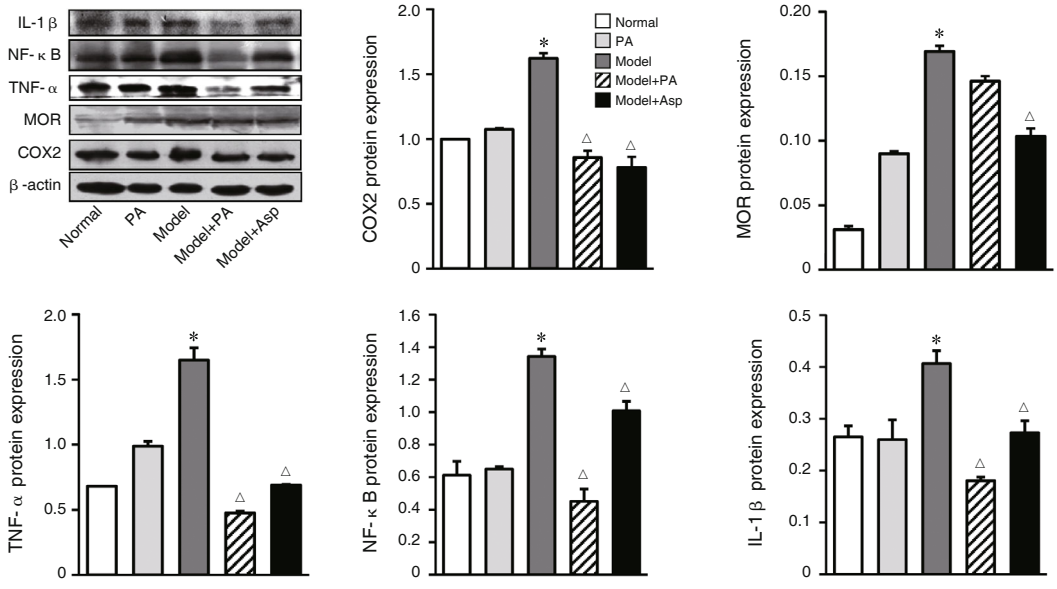

Figure 3. Protein Expressions of COX2, MOR and Pro-inflammatory Cytokines after PA Treatment on Acetic Acid-Induced Visceral Pain in Mice Brain (n=12, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. normal group; △P<0.01 *vs*. model group

Chin J Integr Med 2019 Jun;25(6):454-461

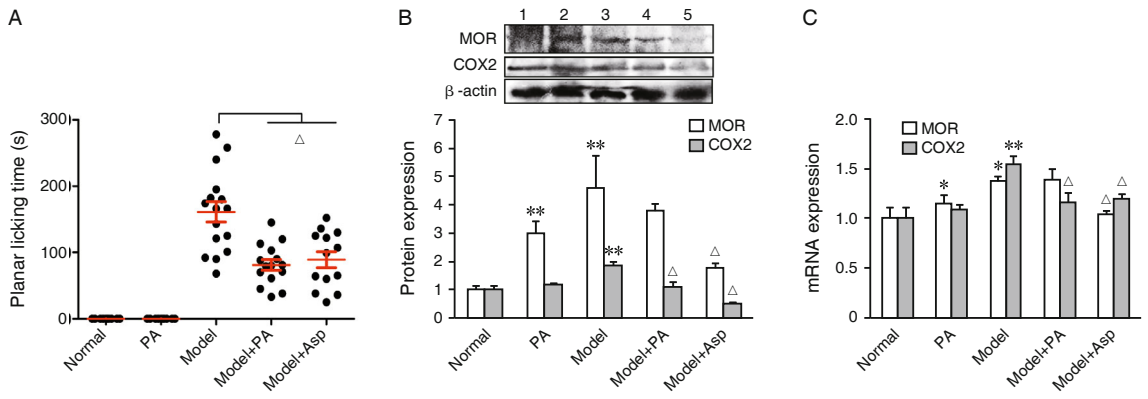

Figure 4. Effect of PA on Plantar Formalin-Induced Allodynia in Mice ($\bar{x} \pm s$)

Notes: A: Allodynia of mice after intra-plantar formalin. B-C: Protein expressions of COX2 and MOR. D: mRNA expressions of COX2 and MOR. 1: normal, 2: PA, 3: model, 4: model+PA, 5: model+aspirin. *n*=10 in normal control and PA groups; *n*=16 in model and model+PA groups; *n*=13 in model+aspirin group. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. normal group; $^{\triangle}P<0.01$ *vs*. model group

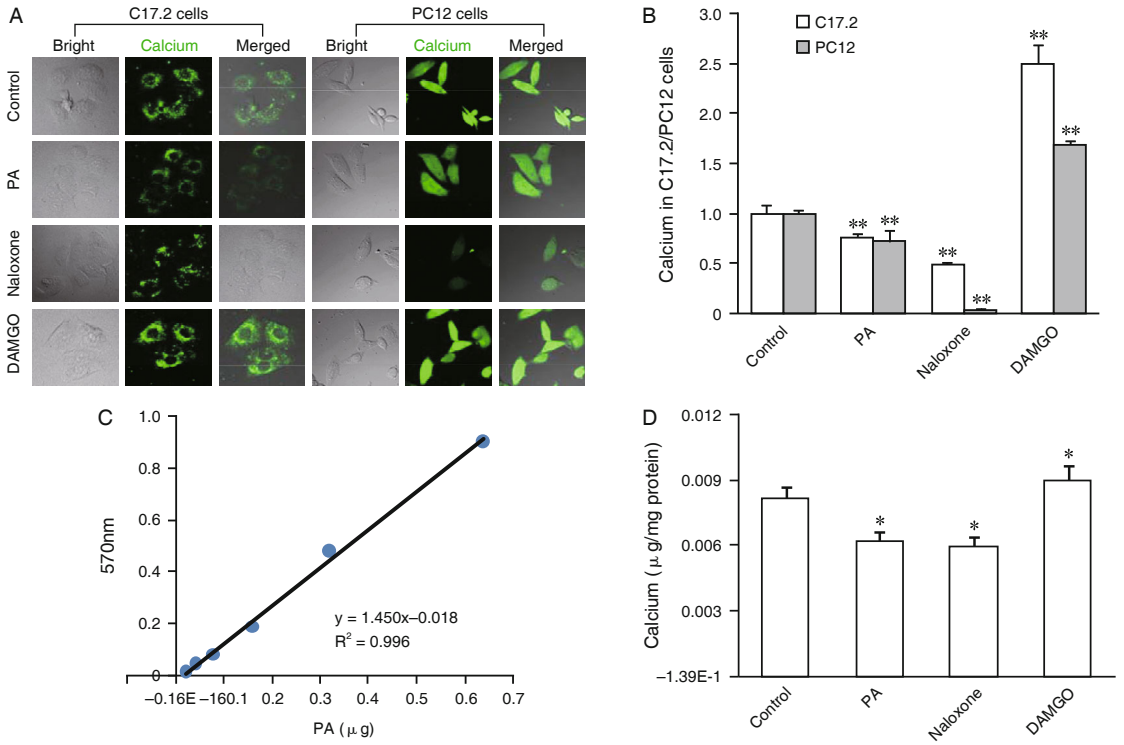

Figure 5. Intracellular Calcium after PA Administration *In Vitro*

Notes: A, B: Effect of PA on Fura3 image indicating calcium in C17.2 cells. Calcium in the cytoplasm using confocal assay. C, D: Quantitative analysis of intracellular calcium after administration of PA and other agents in PC12 cells. C: Standard curve of calcium. D: Concentration of calcium induced by PA, naloxone and DAMGO. Data are shown as $\bar{x} \pm s$ from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.05$ *vs*. normal group

Effect of PA on COX2 and MOR Expression *In Vitro*

PA enhanced the expression of MOR protein and suppressed the expression of COX2 consisting of the results in the mouse experiments (Figure 6). However, naloxone reduced MOR expression, which showed the antagonist naloxone could inhibit the expression of MOR. Notably, the agonist DAMGO attenuated COX2 expression and celecoxib suppressed MOR expression.

DISCUSSION

In this study, we showed a new mechanism by which PA displayed an anti-nociceptive effect by both reducing COX2 expression and modulating MOR. In the *in vivo* experiments, we observed that PA was able to reduce the painful reaction in the acetic acid-induced writhing test in mice. PA prolonged the potential duration of writhing and decreased the writhing attack, which demonstrated its anti-nociceptive

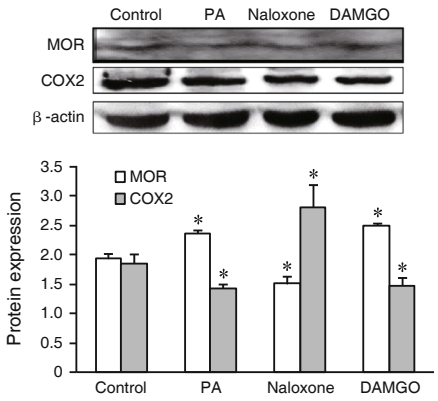

Figure 6. Protein Expression of MOR and COX2 after Administration of Different Agents in PC12 Cells (n=3, $\bar{x} \pm s$)

Note: $^{*}P$<0.05 *vs*. control group

effect. Meanwhile, we found that PA could significantly decrease COX2 transcription and expression in the mouse brain and simultaneously up-regulated the expression of MOR in the brain, which suggested that MOR is involved in the mechanism of PA anti-nociception in mice. However, aspirin had no effect on MOR expression, although it showed a stronger anti-nociceptive effect in the acetic acid-induced writhing test in mice, which suggested different mechanisms of anti-nociceptive action between PA and aspirin. Furthermore, we compared the differences between PA and aspirin by examining the expression of related inflammatory factors in the brain. In the mouse brain, PA and aspirin could down-regulate the expression of IL-1β, TNF-α and NF-κB compared to the model control; however, PA showed a stronger inhibitory effect on IL-1β, TNF-α and NFκB than that of aspirin, which was consistent with the literature.[26] Meanwhile, we found that PA promoted the expression of MOR, thereby revealing a new mechanism underlying PA analgesia. The intraplantar formalin-induced allodynia test showed that the positive effect of PA was consistent with previous results, which confirmed that the anti-nociceptive effect of PA was correlated to both COX2 and MOR.

Previous literature has not explored opioid receptors in this context.[27] The present study has shown that MOR expression was distinctly upregulated in the mouse brain after chemical stress. Our findings suggest that the expression of opioid receptors could be upregulated in response to stimulation by chemical agents, which implied that the brain initiated an analgesic mechanism upon recognizing the inflammatory stimulus. In addition, the relationship was mediated by signaling between inflammatory factors and analgesic receptors; further study is required.

COX2, an inflammatory cyclooxygenase, promotes the inflammatory response and induces pain by mediating IgE.[28] As a non-steroidal anti-inflammatory drug, aspirin inhibits COX2 and relieves pain.[29] It is classified as an anti-inflammatory analgesic. Our present results suggest that PA is also an anti-inflammatory molecule, which is consistent with previous findings.[3,6,26]

Opioids are regarded as some of the most effective analgesics, but they present some unwanted side effects, such as addiction. Opioid systems are critical in the modulation of pain behavior and anti-nociception.[30] Opioids inhibit neurotransmitter release by suppressing calcium currents and activating opioid receptors.[31,32] A reduction in calcium currents sensitive to P/Q-type, N-type, and L-type channel blockers was induced by active opioid receptors.[33] This opioid receptor-induced inhibition was mediated by binding of the dissociated Gβγ subunit directly to the channel.[34,35] Typically, the up-regulation of MOR affects intracellular calcium concentration through calcium channels, thereby regulating its excitability.[36,37] We utilize the calcium ion concentration as a phenotype symbol to study PA's role in the function of MOR. The results of calcium imaging and intracellular calcium concentration analysis demonstrated that the reduction of intracellular calcium ion concentration by PA was closely related to the anti-nociceptive effect. There was a relationship between MOR regulation and the reduction of intracellular calcium ion concentration. Further experiments using the MOR agonist and inhibitor suggest that PA prefers the MOR modulator like opioids. Because the excitation of MOR and changes in the concentration of intracellular calcium involve a complex regulatory process,[38] PA's effect on intracellular calcium still requires further study.

In the present experiment, we noted that PA treatment alone could increase MOR protein expression of normal mice, consisting with that MOR protein expression of PC12 cells *in vitro*. In the groups of PA treatment on normal mice, the protein expression of COX2 showed no decrease compared with control groups. However, PA could suppress the protein expression of COX2 in PC12 cells *in vitro*, showing the

complex differences *in vivo* and *in vitro* that need to study further.

Together, these results provide insight into COX2 and MOR expression during analgesia induced by acetic acid and formalin and its association with PA's anti-nociceptive effects. These findings demonstrate that PA can decrease COX2 and other inflammatory factors and regulate the activation of MOR in the mouse brain. Simultaneously, PA reduces the intracellular calcium ion concentration, which produces an anti-nociceptive effect. This new information on the role of the downregulation of COX2 in the anti-inflammatory effect and the modulation of MOR in the anti-nociception effect of PA may promote the development of a new candidate analgesic drug.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

Yu X and Du LJ conceived and designed the experiments. Yu X, Wang XP, Yan XJ, Jiang JF, Xing DM and Guo YY performed the experiments. Yu X, Wang XP and Lei F analyzed the data. Jiang JF and Xing DM contributed to reagents/materials/analysis tools. Yu X, Wang XP and Du LJ wrote the manuscript. All authors provided edits to the manuscript.

Acknowledgments

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