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Role of peripheral polyamines in the development of inflammatory pain

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ABSTRACT

Polyamines (putrescine, spermidine and spermine) are aliphatic amines that are produced by the action of ornithine decarboxylase (ODC) in a rate-limiting and protein kinase C (PKC)-regulated step. Because high levels of polyamines are found in the synovial fluid of arthritic patients, the aim of the present study was to identify the role of peripherally produced polyamines in a model of inflammatory pain induced by adjuvant. The subcutaneous injection of Complete Freund's adjuvant (CFA, 50 μL/paw) caused the development of mechanical allodynia and edema. Moreover, it increased ODC expression and activity and PKC activation. Administration of the selective ODC inhibitor DFMO (10 μmol/paw) attenuated the development of allodynia and edema and decreased ODC activity in both control and CFA-treated animals. Furthermore, administration of the PKC inhibitor GF109203X (1 nmol/paw) reduced allodynia and ODC activity in animals injected with CFA. A subcutaneous injection of putrescine (10 μmol/paw), spermidine (3–10 μmol/paw) or spermine (0.3–3 μmol/paw) into the rat paw also caused mechanical allodynia and edema. The present results suggest that endogenously synthesized polyamines are involved in the development of nociception and edema caused by an adjuvant. Moreover, polyamine production in inflammatory sites seems to be related to an increase in ODC activity stimulated by PKC activation. Thus, controlling polyamine synthesis and action could be a method of controlling inflammatory pain.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous and small aliphatic amines present in almost all cells [1]. Putrescine is formed from the decarboxylation of ornithine by ornithine decarboxylase (ODC; E.C 4.1.1.17) in a rate-limiting step in polyamine synthesis [2,3]. Putrescine is converted into spermidine and spermine by spermidine synthase and spermine synthase, respectively, which are two aminopropyl transferases [1,2]. ODC is a protein with a short half-life (10 min-1 h in mammals), and its activity and expression are highly regulated by the availability of polyamines and protein kinase C (PKC) [4–6]. Besides being endogenously synthesized, an exogenous supply of polyamines is provided through dietary intake and intestinal absorption from bacterial metabolism [7].

Polyamines are involved in several biological processes, including the control of neuronal excitability and memory improvement and learning in the central nervous system [1,8]. Previous studies have shown that a diet deficient in polyamines and supplemented with an antibiotic (to reduce the level of microflora-derived polyamines) relieves the hyperalgesia induced by incisions, inflammation or neuropathy in rats. This effect seems to be mediated by N-methyl-D-aspartate receptor (NMDA) in the spinal cord [9,10]. Accordingly, some studies have shown that spermine administered in the spine (by the intrathecal route) produces nociceptive behavior in rodents [11,12]. Apart from their pro-nociceptive action at the spinal cord, the role of peripheral polyamines in pain is unknown.

Interestingly, increased levels of polyamines are found in tissues and synovial fluid from patients with osteo-, rheumatoid, posttraumatic and infectious arthritides [13,14]. Moreover, it has been demonstrated that the activity and expression of ODC are increased in colonic tissue from Inflammatory Bowel Disease (IBD) patients [15]. These findings indicate that endogenously produced polyamines could play a role in inflammatory pain. Therefore, the present study investigated the role of peripheral, endogenous polyamines in nociception and edema in the early phase of CFA-induced inflammation.

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2. Material and methods

2.1. Animals

Experiments were performed on adult male Wistar rats (weight 250–300 g) bred in our animal house. The animals were housed in a controlled temperature (22 ± 2 °C) with a 12 h light/dark cycle. They were given standard lab food and water *ad libitum*. The animals were habituated to the experimental room for at least 30 min before the experiments. The experiments were performed in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [16]. The number of animals and the intensities of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. The Committee on the Use and Care of Laboratory Animals at our university approved this study (no. 23081.012331/2009-81).

2.2. Drugs and treatments

The following drugs and chemicals were used in this study: putrescine, spermidine, spermine, DL-α-difluoromethylornithine-hydrochloride hydrate (DFMO, 50 μl/paw, an ODC inhibitor), a PKC inhibitor (GF109203X, 50 μl/paw), phosphate-buffered saline (PBS), and Complete Freund's Adjuvant (CFA, 1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate). All drugs and chemicals were obtained from Sigma (St. Louis, USA). The drug solutions were prepared in PBS except for GF109203X, which was prepared in 0.1% of dimethyl sulphoxide (DMSO).

To assess the role of peripheral, endogenous polyamines or PKC activation in inflammatory pain, CFA (50 μl) was administered subcutaneously (s.c.) under the plantar surface of the right hind paw, and a separate group of animals received an s.c. injection of vehicle (PBS). Different groups were also pre-injected s.c. with the selective ODC inhibitor DFMO (1–10 μmol/paw) or the PKC inhibitor GF109203X (1 nmol/paw) 1 h before CFA administration. Higher doses of these drugs were not used because of their solubility limits.

To determine whether exogenously administered polyamines were capable of producing nociception or edema, putrescine, spermine or spermidine (50 μl, 0.003–10 μmol/paw) was also administered under the plantar surface of the right hind paw s.c., and a separate group received an s.c injection of vehicle (PBS). Higher doses of polyamines were not used because they elicited spontaneous nociception (observed as hind paw licking, lifting and shaking/flinching), which made nociception measurements difficult.

The effects of the drugs on nociception and edema were assessed from 0.5 to 24 h after drug administration. The drug doses and times of administration were based on pilot studies and literature [17].

2.3. Nociception assessment

Mechanical allodynia was measured as described previously by Chaplan et al. [18] and was considered an indicator of nociception. Rats were placed individually in clear Plexiglas boxes (9 cm × 7 cm × 11 cm) on elevated, wire-mesh platforms to access the ventral surface of the hind paws. The paws were touched with one in a series of seven von Frey hairs (6–100 g). The von Frey hairs were applied perpendicular to the plantar surface of the paws with sufficient force to cause a slight buckling against the paws and were held for approximately 2 s. The 50% withdrawal threshold was determined using the up-and-down method of Dixon [19]. In this paradigm, testing was initiated with the 15-g hair. Stimuli were always presented consecutively, whether ascending or descending. Withdrawal thresholds were verified at several time points after polyamine or CFA injection (from 0.5 to 24 h) and were compared with baseline values (before drug administration).

2.4. Edema formation assessment

The edema induced by different agents was considered as the increase in paw thickness, measured by a digital caliper (Mytutoio, Japan), as described previously by Milano et al. [20]. Paw thicknesses were verified at several time points after polyamine or CFA injection (0.5–24 h) and compared to baseline values (before drug administration).

2.5. ODC activity

After behavioral observation, paw-skin samples proximal to the point of injection were collected to perform ODC activity analysis according to Tabib [21] with minor modifications. The samples were homogenized in buffer (10 mM Tris–HCl, pH 7.5) containing 2.5 mM of DL-dithiothreitol (DTT) and 0.1 mM of ethylenediaminetetraacetic acid (EDTA). Following a 10-min incubation at 4 °C, the homogenates were centrifuged at 35,000 × *g* for 45 min at 4 °C, and the supernatants were collected. The protein in the supernatants was measured using the Bradford [22] method. To perform these reactions, 200 μl (0.5 mg/ml of protein) of the supernatants was added to assay buffer (1 M Tris–HCl (pH 7.5), 250 mM of DTT, 2 mM of pyridoxal phosphate, 20 mM of L-ornithine, and 0.1 m of Ci/ml L-[1-^{14}C]-ornithine), and the mixtures were incubated at 37 °C for 30 min in capped-glass tubes. A filter-paper humidifier was attached to the top of each tube in 0.25 ml of 1 M hyamine hydroxide. Sulphuric acid (250 μL, 5 M) was added to the reactions, and the mixtures were incubated for 30 min at 37 °C. The filter papers were then collected to measure the $^{14}CO_2$ released from [^{14}C]-ornithine. ODC activity was expressed as $^{14}CO_2$%, compared with the control group (PBS-treated paws). The mean activity of ODC in the control group was 7.1 ± 0.7 pmol/min/mg protein.

2.6. ODC expression

We also performed Western blot analysis to verify the expression of ODC protein in the paw-skin samples. The assay was performed as described previously [17] with minor modifications. The paw tissues were homogenized in 300 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM DTT and 2 mM of sodium orthovanadate). After centrifugation (13,000 × *g* for 30 min at 4 °C), the supernatants containing the cytosolic fraction were collected. The protein contents were determined by the method of Bradford [22] using bovine serum albumin (BSA) as the standard. Protein (70 μg) as mixed in loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol and 0.04% bromophenol blue) and boiled for 5 min. Proteins were separated in 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes according to the manufacturer's instructions (PerkinElmer, USA). Proteins were stained on the PVDF membrane with a solution of 0.5% actin and 1% glacial acetic acid in water, and this served as the loading control [23]. The membranes were then dried, scanned and quantified with the PC version of Scion Image. The membranes were washed, blocked with 1% BSA in TBS-T (0.05% Tween 20 in Tris-borate saline) and incubated for 10 min with diluted (1:150), primary antibodies against ODC (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were processed using a SNAP i.d. system (Millipore, USA). Blots

were washed three times with TBS-T and incubated with an alkaline phosphatase-coupled secondary antibody (1:3000) for 10 min. The protein bands were visualized with the 5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium system (BCIP/NBT). The membranes were dried, scanned and quantified again, and the ODC Western blot showed a faint background that was corrected in the image analysis.

2.7. PKC activation

Phospho-PKC is the catalytically active form of PKC [24]. We performed Western blot analysis on the paw-skin samples to observe possible changes in PKC activation. This was verified as an increase in the detection of the phosphorylated form of PKC and the ratio between the phosphorylated and total forms of PKC after drug administrations. The method was similar to that described previously in Kassuya et al. [25] with some modifications. The paw tissues were homogenized in a lysis buffer containing 50 mM HEPES (pH 7.9), 1 M KCL, 1 M $MgCl_2$, 0.1 M EDTA, 0.1 M NaF, 10 mg/ml aprotinin, 1 M β-glycerolphosphate, 200 mM phenylmethanesulphonyl fluoride, 1 M DTT and 0.1 M of sodium orthovanadate. After centrifugation (13,000 × *g* for 30 min at 4 °C), the supernatants containing the cytosolic fraction were collected. For PKC activation, the samples (70 μg protein) were incubated for 10 min with 1:150 dilutions of primary antibodies to PKC and phospho-PKC (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were visualized with a BCIP/NBT system. The membranes were dried, scanned and quantified with the PC version of Scion Image. The PKC Western blot showed a faint background that was corrected in the image analysis.

2.8. Statistical analyses

The results are expressed as the mean ± SEM. A Student's *t*-test and one-way or two-way analysis of variance (ANOVA) were used to analyze the statistical significance between groups followed by Bonferroni's or Student–Newman–Keuls' (SNK) post hoc tests. The level of significance was set at $P < 0.05$, and F values demonstrated treatment versus time interactions. The values of ED_{50} (the dose of drug necessary to produce 50% of the nociceptive response relative to the maximum effect) are reported as the geometric means and their respective 95% confidence limits (GraphPad Software 5.0, Graph Pad, USA). The ED_{50}, E_{max} (maximal effect) and I_{max} (maximal inhibition) were calculated based on the responses of the control group. To meet ANOVA assumptions, mechanical allodynia data were subjected to Log transformation before statistical analysis.

3. Results

3.1. Effects of endogenous polyamine production on mechanical allodynia and paw edema in rats with adjuvant inflammation

To investigate the role of endogenous polyamines in mechanical allodynia and edema, we tested the effect of the selective ODC inhibitor DFMO in CFA-induced inflammation. We observed a decreased mechanical threshold (mechanical allodynia) and increased paw thickness (paw edema) from 0.5 to 24 h after s.c. administration of CFA (50 μl/paw), compared with baseline values (Fig. 1A and C). Pre-treatment with s.c. DFMO (10 μmol/paw) reduced the mechanical allodynia induced by CFA significantly

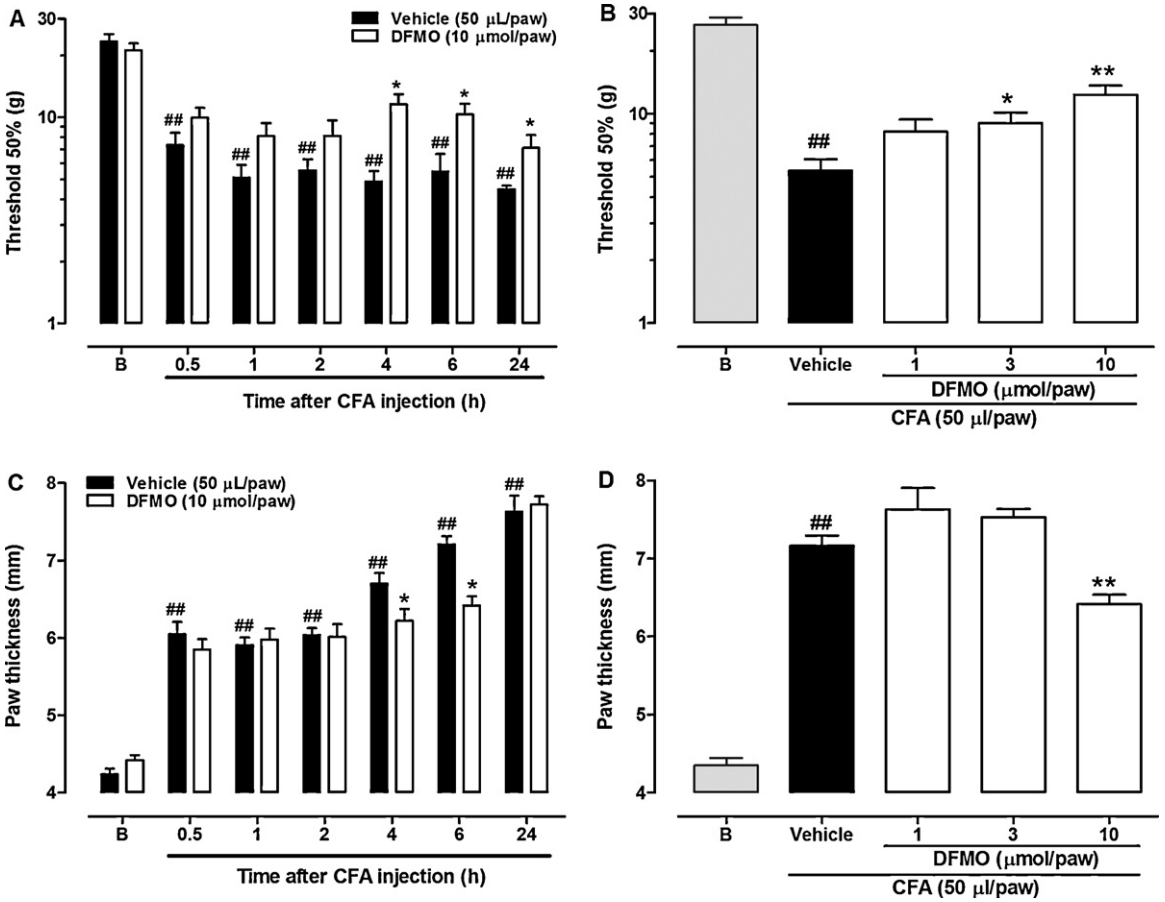

Fig. 1. Time-courses (A and C) and dose–response curves (B and D) for the ODC inhibitor DFMO's effects on mechanical allodynia (A and B) and paw edema (C and D) induced by CFA. The dose–response curves for allodynia or edema were assessed at 4 or 6 h, respectively, after CFA injection. Data represented as mean ± SEM from 9 to 12 rats. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to baseline (B) values. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle group (one-way ANOVA followed by SNK test).

from 4 h to 24 h, compared with the vehicle group [$F(1,23) = 4.136$, $P < 0.0008$]. The maximal inhibition (I_{max}) observed 4 h after administration was 29 ± 5% with a dose of 10 μmol/paw (Fig. 1A and B). Moreover, we also observed that DFMO attenuated the edema formation induced by CFA injection after 4 h [$F(1,23) = 214.9$, $P < 0.0001$] and 6 h [$F(1,23) = 6.440$, $P < 0.0001$]. The most pronounced effect was observed 6 h after CFA treatment when the I_{max} was 28 ± 4%, which was obtained with a dose of 10 μmol/paw (Fig. 1C and D). We also observed that CFA-induced thermal hyperalgesia was not altered by DFMO treatment (results not shown).

3.2. Effects of exogenously administered polyamines on mechanical allodynia and edema in non-arthritic rats

After inhibiting endogenous polyamine synthesis and reducing inflammatory pain in arthritic rats, we tested the ability of polyamines to induce nociception and edema in non-arthritic rats. Peripheral s.c. administration of putrescine (10 μmol/paw), spermidine (10 μmol/paw) or spermine (1 μmol/paw) caused mechanical allodynia starting at 0.5, 1 and 0.5 h, respectively, after injection and persisted for 6, 4 and 4 h, respectively (Fig. 2A–C). The peak effects for mechanical allodynia were observed at 2, 1 and 2 h

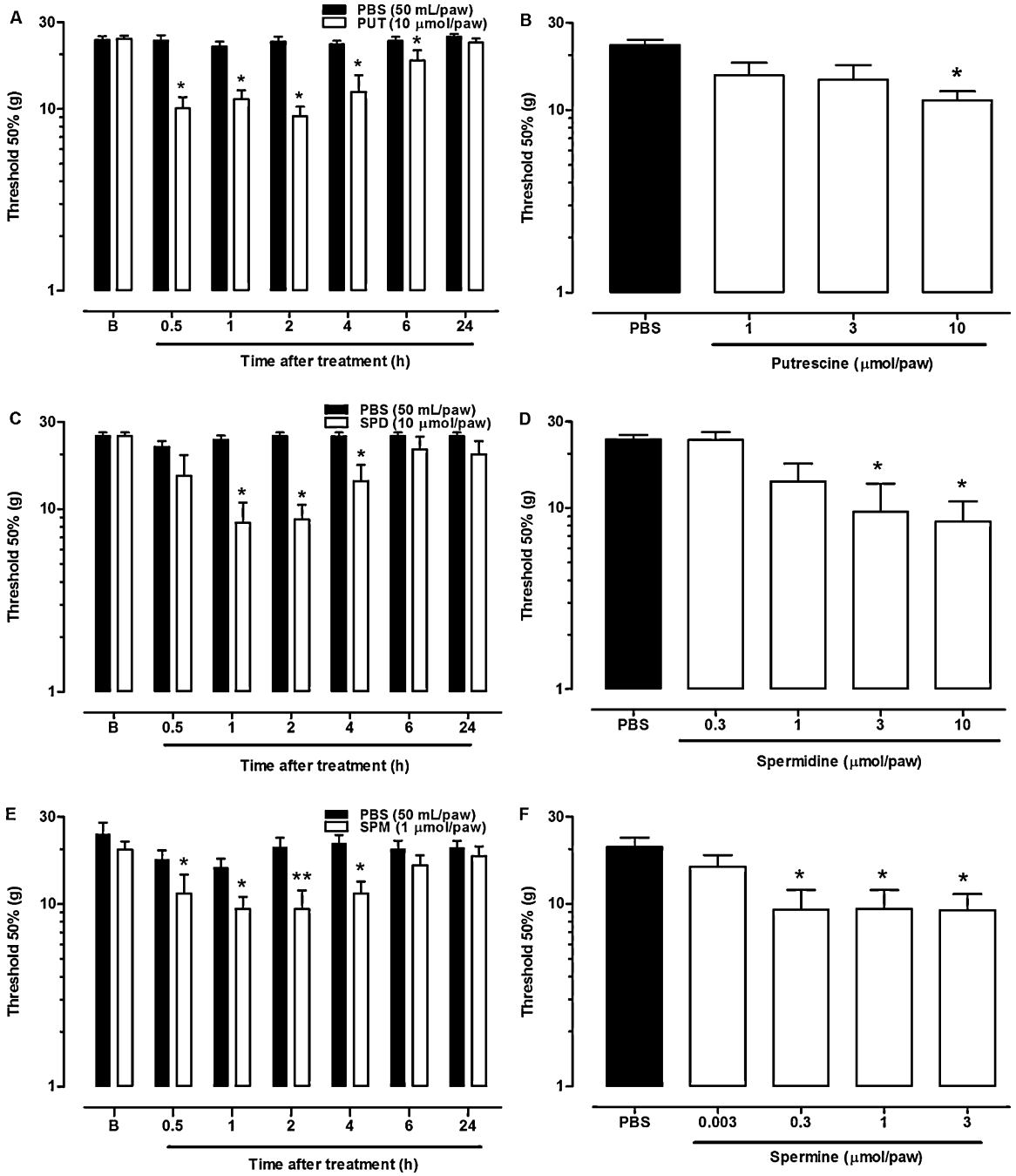

Fig. 2. Time-courses (A, C and E) and dose–response curves (B, D and F) for the induction of mechanical allodynia in rats by peripheral injection of putrescine (PUT), spermidine (SPD) or spermine (SPM). Each data point represents the mean ± SEM from 7 rats for A and B, 5 rats for C and D and 9 rats for E and F. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle (PBS) group (one-way ANOVA followed by SNK test).

for putrescine, spermidine or spermine injections, respectively. Respective maximal effects (E_{maxs}) were 39 ± 5%, 65 ± 10% and 43 ± 9% of the mechanical threshold, compared with the control group (Fig. 2). Because the allodynias induced by putrescine, spermidine and spermine were not dose-dependent (Fig. 2B, D and F), the ED_{50} values were not calculated for these polyamines.

Similar to nociception, s.c. administration of putrescine (10 μmol/paw), spermidine (10 μmol/paw) or spermine (1 μmol/paw) also induced paw edema starting 0.5 h after injection and persisted for 6 h for all polyamines (Fig. 3A–C). The peak effect for edema was also observed 30 min after injection for all polyamines, and E_{maxs} of 31 ± 3%, 49 ± 3% and 29 ± 3% of the paw thickness increase were observed, compared with the control group, for putrescine, spermidine or spermine, respectively (Fig. 3). Moreover, similar to nociception, edema induced by these compounds (Fig. 3D and F) was not dose-dependent, and therefore, ED_{50} values were not calculated.

3.3. ODC expression and activity in inflamed tissue from rats with adjuvant inflammation

Because we observed that inhibiting polyamine synthesis reduced inflammatory pain, we directly evaluated ODC activity after CFA injection. When compared with vehicle-treated rats, CFA significantly increased the ODC activity at 2 h (24 ± 2%), 4 h (38 ± 5%) and 6 h (50 ± 15%) after injection (Fig. 4A). Moreover, we verified that the dose of DFMO (10 μmol/paw) that reduced CFA-induced nociception and edema was also effective in fully preventing the increase in ODC activity observed 4 h after CFA administration (Fig. 4B). DFMO (10 μmol/paw) pre-treatment also reduced the

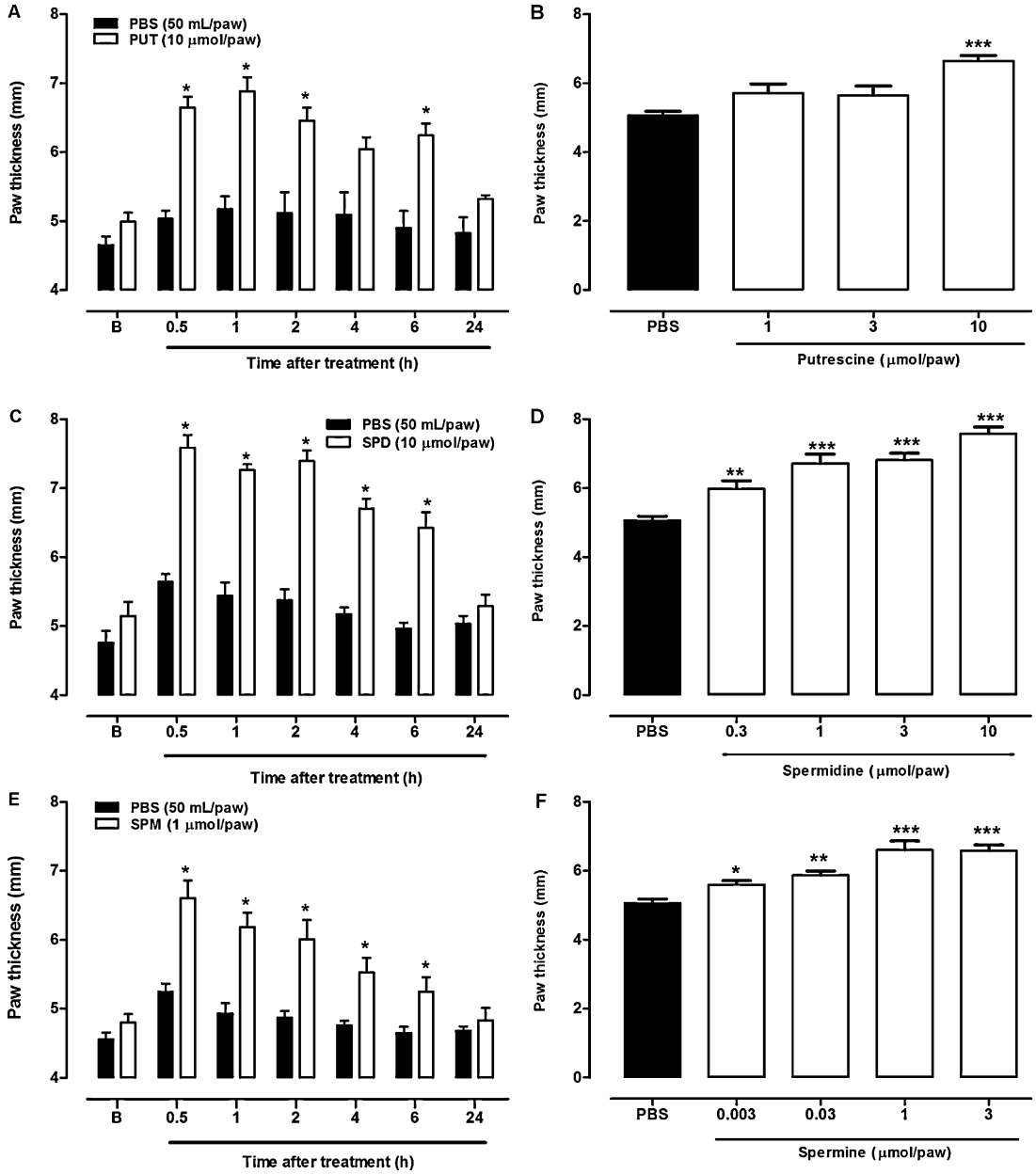

Fig. 3. Time-courses (A, C and E) and dose–response curves (B, D and F) for the induction of paw edema in rats by the peripheral injection of putrescine (PUT), spermidine (SPD) or spermine (SPM). Each data point represents the mean ± SEM from 7 rats for A and B, 5 rats for C and D and 9 rats for E and F. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to vehicle (PBS) group (one-way ANOVA followed by SNK test).

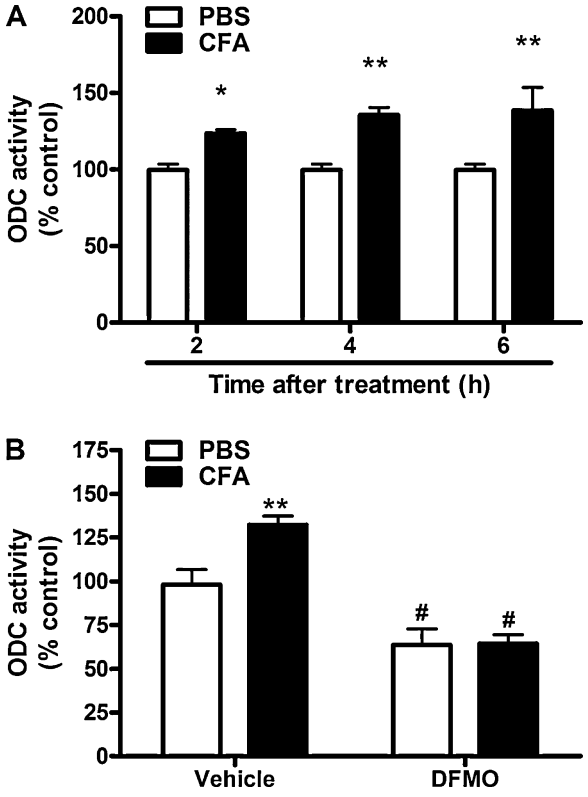

Fig. 4. (A) Effect of CFA on ODC activity in injected paw tissue. (B) Effect of DFMO pre-treatment or its vehicle on ODC activity in rats injected with CFA or PBS. Data expressed as the mean ± SEM from 6 rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the PBS group. $^{\#}P < 0.05$ compared to vehicle-treated groups injected with CFA or PBS (two-way ANOVA followed by Bonferroni's test).

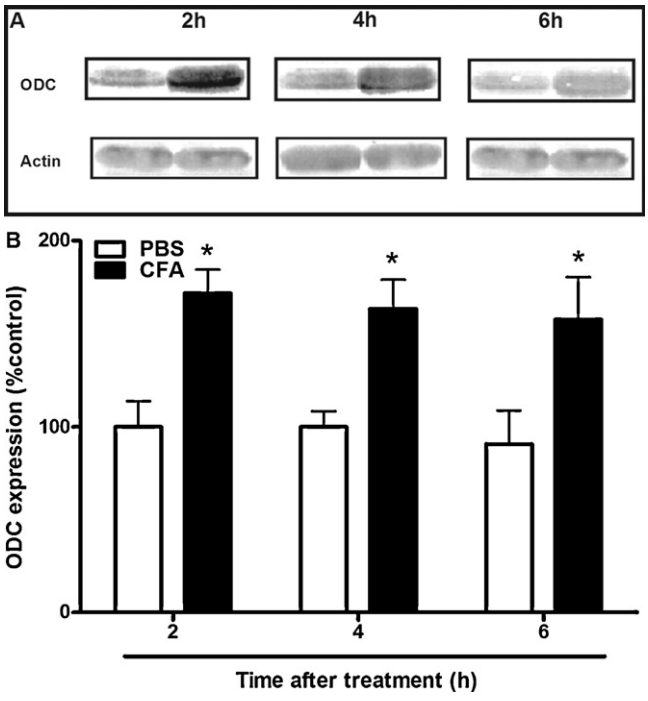

Fig. 5. Effect of CFA on ODC protein expression in injected paw tissue. (A) Western blot analysis of ODC expression in paw samples 2, 4 and 6 h after injection of CFA or PBS. To demonstrate equal loading, a representative actin-stained protein band is shown. (B) Quantification of ODC protein expression. Data expressed as the mean ± SEM from 4 to 6 rats. $^{*}P < 0.05$ compared to PBS control (one-way ANOVA followed by SNK test).

baseline activity of ODC by 33 ± 9% in vehicle-treated animals (Fig. 4B).

Because ODC activity could be altered by CFA, we also observed the effect of adjuvant on ODC expression. We observed that CFA injection increased ODC protein expression 2, 4 and 6 h after CFA injection, compared with that in the PBS group (Fig. 5A and B).

3.4. Effect of PKC activation on allodynia, edema and increased ODC activity induced by CFA injection

We first measured changes in PKC activation in the CFA-induced inflammatory state. CFA significantly increased the detection of the ratio between phosphorylated and total forms of PKC 4 h after its injection, and decreased the phospho-PKC form of PKCs after 6 h, without changes in the total form of PKC (Fig. 6A–E). Moreover, we assessed the effects of GF109203X, a selective PKC inhibitor, on mechanical allodynia, paw edema and increased ODC activity induced by CFA. The administration of GF109203X (1 nmol/paw) reduced the mechanical allodynia but not the paw edema induced by CFA in 4 h [$F(1,5) = 6.208$, $P < 0.05$], with an inhibition of 74 ± 4%, compared with the vehicle group (Fig. 7A and B). GF109203X also prevented an increase in ODC activity after 4 h the CFA injection without changing ODC activity in PBS-treated animals (Fig. 8).

4. Discussion

Polyamines are ubiquitous and aliphatic amines involved in several biological processes. Here, we show that peripheral polyamines are related to the induction of inflammatory pain, because ODC inhibition reduced endogenous polyamine production and decreased the nociception and edema related to inflammatory processes. Moreover, injection of exogenous polyamines caused nociception and edema, and the activity and expression of ODC were increased in the inflammatory tissue. We also demonstrated that the action of PKC is related to the increased activity of ODC and nociception and edema production.

It is known that CFA produces chronic inflammation in rodents [13]. In the present study, we showed that subcutaneous administration of the ODC inhibitor DFMO had a long-lasting, antinociceptive effect on the mechanical allodynia induced by CFA in rats. Our results are in accordance with previous findings that showed that a polyamine-deficient diet relieves the mechanical hyperalgesia induced by inflammation in rats [9,10]. Thus, both the restriction of polyamine ingestion and the inhibition of polyamine synthesis could be strategies for improving the treatment of inflammatory pain. Furthermore, we also observed that DFMO treatment did not alter CFA-induced thermal hyperalgesia. Since it has been described that mechanical and thermal stimuli are detected by different afferent fibers [26], the target to the nociceptive action of polyamines seems to be restricted to the subset of fibers that mediates mechanical pain.

Several clinical studies have demonstrated a critical role for polyamines in arthritis. Increased levels of polyamines are found in synovial fluid, synovial membrane, urine and lymphocytes of arthritic patients and seem to be related to the activity and progression of the disease [13,27,28]. Furthermore, the anti-arthritic effect of methotrexate seems to be associated with its ability to reduce polyamine levels [14,28]. There are also increased levels of polyamines in the paws of rats injected with CFA [29]. Confirming the idea that peripheral polyamines function as pro-nociceptive agents, we showed that exogenous polyamines injected into the rat paw induced mechanical allodynia. It has been previously demonstrated that intrathecally injected spermine produced nociceptive behaviors in rodents [11,12]. Thus, similar to the spinal cord injections, peripheral polyamines

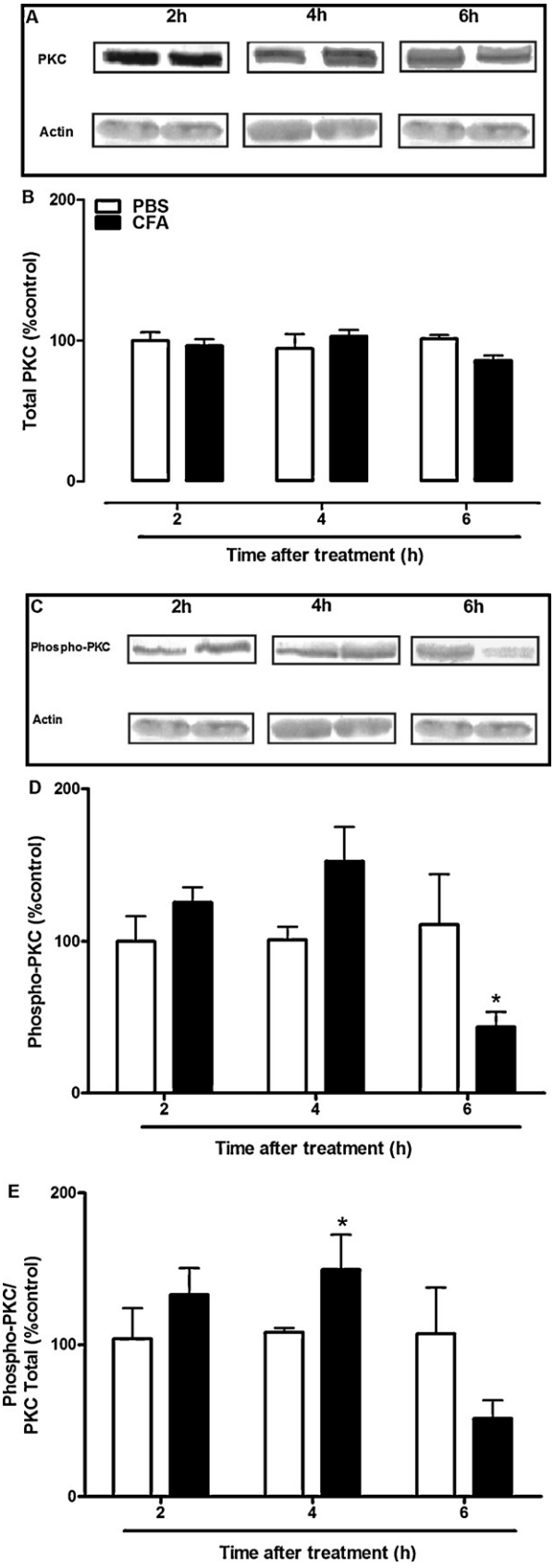

Fig. 6. Effect of CFA on PKC activation assessed by the expression of the total and phosphorylated forms of PKC and the ratio of these forms. (A) Western blot analysis of the total form and (C) analysis of the phosphorylated form of PKC expression in paw samples 2, 4 and 6 h after the injection of CFA or PBS. To demonstrate equal loading, a representative actin-stained protein band is show. (B) Quantifications of the total form, (D) phosphorylated form and (E) the ratio of the total and phosphorylated forms of PKC. Data expressed as the mean ± SEM from 4 rats. $^{*}P < 0.05$ compared to PBS-injected rats (Student's *t*-test).

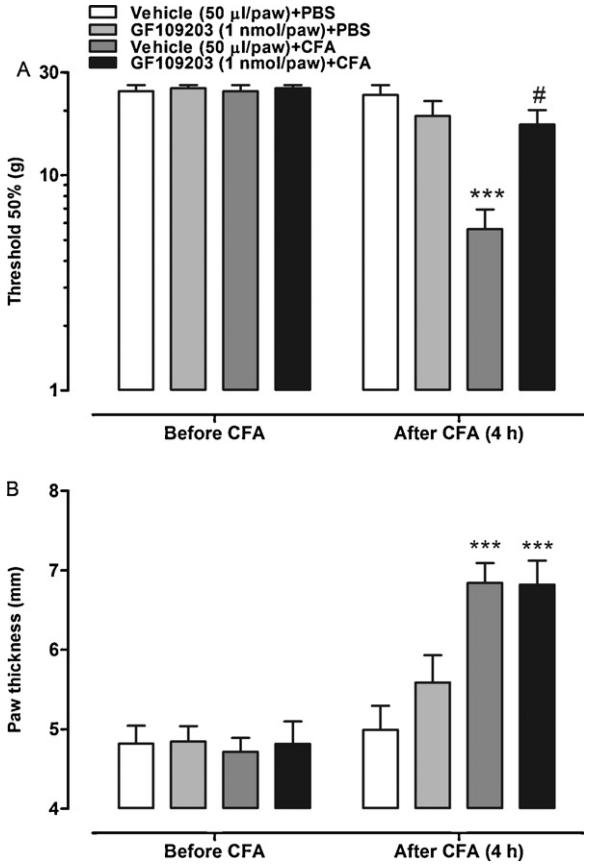

Fig. 7. Effect of pre-treatment with GF109203X on mechanical allodynia (A) and paw edema (B) assessed 4 h after CFA administration. Data expressed as the mean ± SEM from 5 rats. $^{**}P < 0.01$ compared to vehicle and PBS group. $^{\#\#}P < 0.01$ compared to vehicle and CFA group (one-way ANOVA).

produced in inflamed tissue also have pro-nociceptive actions. The spermine-solution concentrations injected (0.3–10 mM to generate doses of 0.03–1 μmol/paw) into the rat paw are in the same range of the spermine concentration in synovial tissues from arthritic patients (about 0.3 mM) and in paw tissue of CFA-injected rats (about 400 mM) [13,29]. This suggests that the doses of polyamines used in our study to cause nociception may be found in inflamed tissues.

We found that the potency, but not the efficacy, of polyamines to induce allodynia is dependent on their charge because spermine, which is the most charged polyamine, was more potent than putrescine in producing nociception. It has been described that some polyamine actions, such as interacting with the ion channel, are charge-dependent [30]. The exact target that peripheral polyamines interact with to produce nociception is still unknown; however, in the spinal cord, the glutamate NMDA receptor is an important target [11,12]. There are also NMDA receptors in the peripheral terminals of nociceptors that, when stimulated by glutamate, induce nociception in rodents [31]. Moreover, polyamines may also stimulate the vanilloid TRPV1 receptor, which could trigger nociceptor activation [30]. However, the exact mechanisms responsible for the nociceptive effect induced by polyamines needs to be determined. Besides NMDA and TRPV1 receptors, polyamines may interact with different affinities in

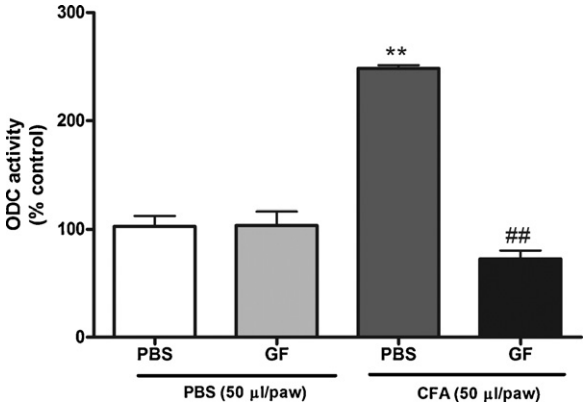

Fig. 8. Effect of GF109203X on ODC activity in CFA or PBS-treated rats. Data expressed as the mean ± SEM from 4 rats. $^{**}P < 0.01$ compared to vehicle and PBS group. $^{\#}P < 0.01$ compared to vehicle and CFA group (one-way ANOVA).

several other targets [32] that could modulate positively or negatively the nociceptive process. This plethora of targets may explain why we did not detect dose–response relationships for nociceptive and edematogenic actions of polyamines.

During inflammatory processes, events, such as edema and enhanced nociception, may occur. Similar to the nociceptive behavior, polyamine injection induced edema, and spermine remained the most potent polyamine tested (it produced edema with lower doses than the other polyamines), while spermidine was the most efficacious polyamine (it induced a greater edema – E_{max} value – when compared with the other polyamines). In addition, our study also showed that DFMO treatment reduced the paw edema induced by CFA. In accordance with our findings, it has been reported that DFMO treatment reduces edema produced by lung or brain injury [33,34].

We also observed that CFA increased ODC activity and expression, compared to that in the control group. Moreover, DFMO, administered at a dose that possessed anti-allodynic and anti-edematogenic effects, was able to reduce ODC activity in CFA-treated and control animals. ODC, the rate-limiting enzyme in polyamine biosynthesis, is very highly regulated, and alterations in its activity are through changes in the amount of ODC protein, which turns over very rapidly [6]. In our study, CFA increased the amount of ODC protein and the activity of ODC 2–6 h after its injection. Thus, the increased ODC activity observed after CFA injection is, at least in part, related to the increase in protein.

ODC activity varies in response to many stimuli. PKC activity influences polyamine metabolism through a critical regulation of ODC activation, at the levels of transcription, translocation and protein turnover [6,35]. For example, ODC activity may be decreased by PKC depletion and increased by PKC over-expression [5]. Similar to what is found in the spinal cord of CFA-treated rodents [36], we detected, for the first time, an increase in PKC activation, which was detected as an increase in the ratio of phosphorylated and total forms of PKC 4 h after CFA administration. This effect was not an unexpected finding, because an adjuvant injection increases the local level of several pro-nociceptive mediators that stimulate PKC, such as cytokines and the nerve growth factor [37,38]. We also detected a decrease in the phosphorylated form of PKC 6 h after CFA was given. This may be explained by the fact that phosphorylation of PKC renders it sensitive to degradation [24].

GF109203X, an inhibitor of PKC, significantly inhibited the ODC activity and mechanical allodynia increases observed in adjuvant-injected animals without altering ODC activity in control rats. This indicates that PKC is involved in polyamine-related inflammatory nociception, Furthermore, because the PKC inhibitor was more effective than the ODC inhibitor in reducing the mechanical allodynia caused by CFA, PKC seems to have other pro-nociceptive targets besides ODC. Several ion channels and receptors can be modulated by PKC in sensory neurons to induce pain [38]. PKC inhibition was not able to alter edema starting 4 h after CFA administration. This agrees with a previous study, which demonstrated that PKC is not involved in the first phase (1–9 days) of CFA-induced edema in rats [39].

Taken together, these results suggest that endogenously synthesized polyamines are involved in the development of nociception caused by an adjuvant. Moreover, polyamine production in inflammatory sites seems to be related to an increase in ODC activity stimulated by PKC activation. Thus, controlling polyamine synthesis and action could be a potential method for controlling inflammatory pain.

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References

[1] Kusano T, Berberich T, Tateda C, Takahashi Y. Polyamines: essential factors for growth and survival. Planta 2008;228:367–81.
[2] Casero RA, Marton LJ. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Nat Rev Drug Discov 2007;6:373–90.
[3] Schipper RG, Verhosfstad AAJ. Distribution patterns of ornithine decarboxylase in cells and tissues: facts, problems, and postulates. J Histochem Cytochem 2002;50:1143–60.
[4] Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. Biochem J 2003;376:1–14.
[5] Zhao YJ, Zhang WH, Xu CQ, Li HZ, Wang LN, Li H, et al. Involvement of the ornithine decarboxylase/polyamine system in precondition-induced cardioprotection through an interaction with PKC in rat hearts. Mol Cell Biochem 2009;332:135–44.
[6] Pegg AE. Regulation of ornithine decarboxylase. J Biol Chem 2006;281:14529–32.
[7] Larqué E, Sabater-Molina M, Zamora S. Biological significance of dietary polyamines. Nutrition 2007;23:87–95.
[8] Rubin MA, Stiegemeier AJ, Volkweis MA, Oliveira DM, Fenili AC, Rafael LB, et al. Intra-amygdala spermine administration improves inhibitory avoidance performance in rats. Eur J Pharmacol 2001;423:35–9.
[9] Estebe JP, Legay F, Gentili M, Wodey E, Leduc C, Ecoffey C, et al. An evaluation of a polyamine-deficient diet for the treatment of inflammatory pain. Anesth Analg 2006;102:1781–8.
[10] Rivat C, Richebé P, Laboureyras E, Laulin JP, Havouis R, Noble F, et al. Polyamine deficient diet to relieve pain hypersensitivity. Pain 2008;137:125–37.
[11] Kolhekar R, Meller ST, Gebhart GF. N-methyl-D-aspartate receptor-mediated changes in thermal nociception: allosteric modulation at glycine and polyamine recognition sites. J Neurosci 1994;63:925–36.
[12] Tan-No K, Taira A, Wako K, Niijima F, Nakagawasai O, Tadano T, et al. Intrathecally administered spermine produces the scratching, biting and licking behaviour in mice. Pain 2000;86:55–61.
[13] Yukioka K, Wakitani S, Yukioka M, Furumitsu Y, Shichikawa K, Ochi T, et al. Polyamine levels in synovial tissues and synovial fluids of patients with rheumatoid arthritis. J Rheumtol 1992;19:689–92.
[14] Nesher G, Osborn TG, Moore TL. In vitro effects of methotrexate on polyamine levels in lymphocytes from rheumatoid arthritis patients. Clin Exp Rheumatol 1996;14:395–9.
[15] Pillai RB, Tolia V, Rabah R, Simpson PM, Vijesurier R, Lin CH. Increased colonic ornithine decarboxylase activity in inflammatory bowel disease in children. Dig Dis Sci 1999;44:1565–70.
[16] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.
[17] Ferreira J, Trichés KM, Medeiros R, Calixto JB. Mechanisms involved in the nociception produced peripheral protein kinase C activation in mice. Pain 2005;117:171–81.
[18] Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Meth 1994;53:55–63.
[19] Dixon WJ. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 1980;20:441–62.

[20] Milano J, Rossato MF, Oliveira SM, Drewes C, Machado P, Beck P. Antinociceptive action of 4-methyl-5-trifluomethyl-5-hydroxy-4,5-dihydro-1 H-pyrazole methyl ester in models of inflammatory pain in mice. Life Sci 2008;83:739–46.
[21] Tabib A. Determination of ornithine decarboxylase activity using [$^{14}CO_2$] ornithine. In: Morgan DML, editor. Polyamines Protocols Methods in Molecular Biology, vol. 74. 1998. p. 33–40.
[22] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[23] Calvo IR, Ocón B, Moya PM, Suárez MD, Zarzuelo A, Augustin OM, et al. Reversible Ponceau staining as a loading control alternative to action in Western blots. Anal Biochem 2010;401:318–20.
[24] Ohno S, Konno Y, Akita Y. A point mutation at the putative ATP-binding site of protein kinase C alpha abolishes the kinase activity and renders it down-regulation-insensitive. A molecular link between autophosphorylation and down-regulation. J Biol Chem 1990;265:6296–300.
[25] Kassuya CAL, Ferreira J, Claudino RF, Calixto JB. Intraplantar PGE_2 causes nociceptive behaviour and mechanical allodynia: the role of prostanoid E receptors and protein kinases. Br J Pharmacol 2007;150:727–37.
[26] Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI. Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 2009;106:9075–80.
[27] Furumitsu Y, Yukioka K, Kojima A. Levels of urinary polyamines in patients with rheumatoid arthritis. J Rheumatol 1993;20:1661–5.
[28] Nesher G, Osborn TG, Moore TL. Effect of treatment with methotrexate, hydroxychloroquine, and prednisone on lymphocyte polyamine levels in rheumatoid arthritis: correlation with the clinical response and rheumatoid factor synthesis. Clin Exp Rheumatol 1997;15:343–7.
[29] Chakradhar LV, Naik SR. Polyamines in inflammation and their modulation by conventional anti-inflammatory drugs. Indian J Exp Biol 2007;45:649–53.
[30] Ahern GP, Wang X, Miyares RL. Polyamines are potent ligands for the capsaicin receptor TRPV1. J Biol Chem 2006;281:8991–5.
[31] Carlton SM. Peripheral excitatory amino acids. Curr Opin Pharmacol 2001;1:52–6.
[32] Bowie D, Mayer ML. Inward rectification of both AMPA and Kainate subtype glutamate receptors generated by polyamines-mediated ion channel block. Neuron 1995;15:453–62.
[33] Başkaya MK, Rao AM, Puckett L. Effect of difluoromethylornithine treatment on regional ornithine decarboxylase activity and edema formation after experimental brain injury. J Neurotrauma 1996;13:85–92.
[34] Olson JW, Atkinson JE, Hacker AD. Suppression of polyamine biosynthesis prevents monocrotaline-induced pulmonary edema and arterial medial thickening. Toxicol Appl Pharmacol 1985;81:91–9.
[35] Shantz LM, Levin VA. Regulation of ornithine decarboxylase during oncogenic transformation: mechanisms and therapeutic potential. Amino acids 2007;33:213–23.
[36] Cheng HT, Suzuki M, Hegarty DM. Inflammatory pain-induced signaling events following a conditional deletion of the N-methyl-D-aspartate receptor in spinal cord dorsal horn. Neuroscience 2008;155:948–58.
[37] Woolf CJ, Allchorne A, Safieh-Garabedian B. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol 1997;121:417–24.
[38] Velázquez KT, Mohammad H, Sweitzer SM. Protein kinase C in pain: involvement of multiple isoforms. Pharmacol Res 2007;55:578–89.
[39] Birchall AM, Bishop J, Bradshaw D. Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. J Pharmacol Exp Ther 1994;268:922–9.