

## The long-lasting sensitization of primary afferent nociceptors induced by inflammation involves prostanoid and dopaminergic systems in mice

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### ABSTRACT

In recent years, evidence that sensitization of primary afferent nociceptors is an important event associated with chronic pain has been accumulating. The present study aimed to evaluate the participation of the prostanoid and sympathetic components in the long-lasting sensitization of nociceptors induced by acute inflammation in mice. The intraplantar administration of carrageenan (100 µg) enhanced the nociceptive response to a small dose of PGE<sub>2</sub> (9 ng/paw) or dopamine (3 µg/paw) up to 30 days later. This long-lasting sensitization is dependent on dopaminergic and prostanoid systems, since the pre-treatment with chlorpromazine (3 µg/paw) or indomethacin (100 µg/paw), but not local (6 µg/paw) or systemic (6 mg/kg) treatment with morphine, prevented its development. In agreement with this idea, the previous intraplantar administration of hyperalgesic doses of PGE<sub>2</sub> or dopamine also induced long-lasting sensitization, which was fully prevented by pretreatment with EP<sub>4</sub> and D<sub>1</sub> antagonists, respectively. In summary, the present work described in mice a long-lasting sensitization of nociceptors, initiated by an acute inflammatory stimulation and dependent on dopaminergic and prostanoid systems. The present data represent new insights on the mechanisms of peripheral sensitization that could contribute to establish the basis of new therapeutic strategies for acute and chronic inflammatory pain.

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### 1. Introduction

While the physiological pain has an important protective function, chronic pain can take on a disease character in pathological states, such as inflammation and neuropathy (Kuner, 2010). When pain persists for years, its broader effects on psychological health and performance of social responsibilities in work and family life can be profound (Turk and Rudy, 1988). Chronic pain not only differs from acute pain in its onset and duration, but also in its underlying mechanisms, and thus it often responds poorly to conventional analgesics. Better treatment of chronic pain will require clear understanding of the mechanisms that contribute to the transition from an acute tissue insult to chronic pain, and testing of pharmacological agents in such settings.

Recently, it has been proposed that chronic pain can result from a persistent sensitization of primary afferent nociceptors, which typically

develops as a consequence of tissue insult (Ferreira et al., 1990; Gold and Gebhart, 2010). The clinical consequence of the sensitization of nociceptors is allodynia and hyperalgesia, which may correspond to a reduction in the nociceptive threshold and to an increase in the magnitude of the response to noxious stimulation, respectively (Bessou and Perl, 1969). Although the mechanisms that lead the acute injury to turn into chronic pain are poorly understood, clinical evidence suggests a relationship between the sensitization of nociceptors and pain maintenance (Gold and Gebhart, 2010). Usually, acute insults resolve without persisting pain, suggesting that brief nociceptor sensitizations are generally reversible. On the other hand, during chronic pain states initiated with tissue lesion, the sensitization tends to be irreversible. In addition, there are chronic pain states in which tissue pathology is not obvious, but the peripheral input and nociceptor sensitization are sufficient for the maintenance of pain (Gracely et al., 1992; Price et al., 2006, 2009; Staud et al., 2009; Verne et al., 2003). Considering this point of view, the duration of acute sensitization of nociceptors can be an important determinant to the development of chronic pain.

In fact, in some clinical chronic pain states, following the resolution of an episode of acute inflammation, an increased susceptibility to pain induced by subsequent stimuli is observed (Jacobson et al., 1989; MacIntyre et al., 1995; Melhorn, 1998). In line with this clinical

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observation, it has been demonstrated that acute inflammation, produced by carrageenan administration in the rat hind paw, induces an increased susceptibility to the hyperalgesia mediated by inflammatory mediators, such as prostaglandins (Aley et al., 2000). Indeed, prostaglandins are important inflammatory mediators that contribute to the sensitization of nociceptors (Ferreira, 1972; Ferreira and Nakamura, 1979). In addition to prostaglandins, the involvement of a sympathetic component has been described (Coderre et al., 1984; Cunha et al., 2005; Khasar et al., 1999; Nakamura and Ferreira, 1987; Safieh-Garabedian et al., 2002).

The present work aimed to evaluate the participation of prostaglandin and sympathetic components in the long-lasting sensitization of nociceptors induced by local inflammation in mice, by using the hyperalgesic priming model. This study intended to understand mechanisms of nociceptor sensitization and to identify new molecular targets for pharmacological intervention.

## 2. Methods

### 2.1. Animals

Experiments were performed using male Swiss Webster mice (20–25 g, University of São Paulo, Ribeirão Preto, Brazil). Animals were housed at  $24 \pm 1^\circ\text{C}$ , under a 12:12 h light-dark cycle (lights on at 07:00 AM), with free access to chow and tap water until the day of the experiment, when only water was made available to them. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983) and the Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and any discomfort. Each animal was used only once and all behavioral testing was performed between 8:00 a.m. and 4:00 p.m. Behavioral tests were done without knowing which experimental group each mouse belonged to.

### 2.2. Nociceptive mechanical test

Mechanical hyperalgesia was evaluated in mice using a Dynamic Plantar Aesthesiometer (Ugo Basile) test. This apparatus has a pressure transducer coupled to a digital force detector that records the applied force in grams. Mice were placed in acrylic cages with wire grid floors 15–30 min before starting the test to environment adaptation. The test consists of evoking a hind paw flexion reflex using a filament-containing Universal Tip (10  $\mu\text{L}$ , T-300, Axygen) that touches the plantar surface and exerts an upward force (maximal 50 g) on the plantar surface of the mouse hind paw. The end point was defined by the withdrawal of the paw followed by clear flinching movements. With paw withdrawal, the recorded force was automatically displayed. The results are expressed by the intensity of hyperalgesia (in grams) calculated by subtracting the force measured after the treatment from the basal value ( $\Delta$  force). The nociceptive threshold of mouse paws measured at the first experimental day and before any experimental procedure was considered the baseline value and was used throughout the experimental period.

### 2.3. Drug preparation and administration

The agents used in this study were: indomethacin (Prodome, Campinas, SP, Brazil); morphine sulfate (Cristália, Itapira, SP, Brazil); dopamine, chlorpromazine, L741626 ( $D_2$  antagonist), SCH-23390 ( $D_1$  antagonist), L-745870 ( $D_4$  antagonist), AH23848 ( $EP_4$  antagonist) and PGE<sub>2</sub> (Sigma, St. Louis, MO, USA); carrageenan (FMC, Philadelphia, USA). All the drugs were dissolved in 0.15 M NaCl (saline), except for indomethacin, which was dissolved in Tris buffer (Merck, Darmstadt, Germany) and for PGE<sub>2</sub>, which was stored in dimethyl sulfoxide 2% (Sigma, St. Louis, MO, USA) and dissolved in saline. All drugs were administrated locally (hind paw, intraplantar – i.p.l.)

with a 29 G hypodermic needle in a volume of 25  $\mu\text{L}$  per paw, with the exception of morphine, which was also delivered systemically by subcutaneous (s.c.) route.

### 2.4. Statistical analysis

Results are presented as means  $\pm$  SEM of measurements made on 6 animals in each group and represent the intensity of hyperalgesia. The curves were analyzed by two-way ANOVA to find interactions between time and treatment. To analyze distinct points of the curves we used one-way ANOVA followed by Bonferroni's pos-test. Differences were considered to be statistically significant at  $p < 0.05$ .

### 2.5. Experimental protocols

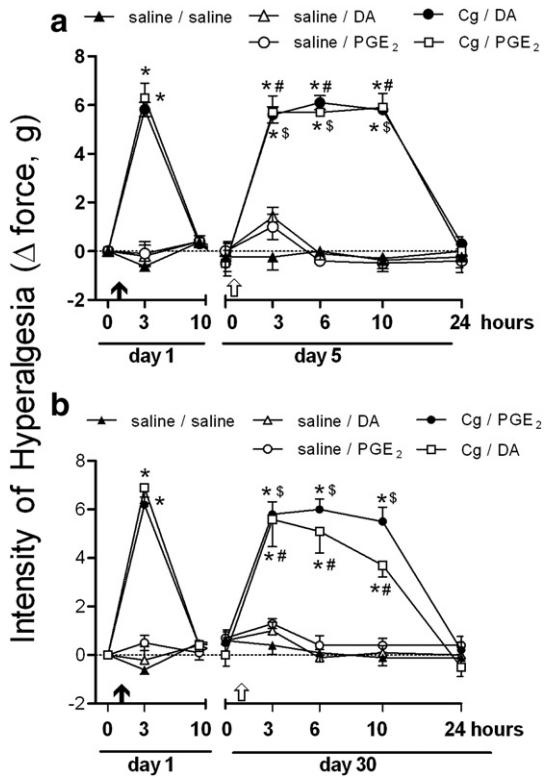
Induction of long-lasting sensitization of nociceptors: in this protocol the effects of a single administration of a pro-inflammatory agent (carrageenan) or two agents known to be released by carrageenan (PGE<sub>2</sub> and dopamine; Nakamura and Ferreira, 1987) were evaluated. In the first experimental day, the nociceptive threshold was evaluated before any treatment. Carrageenan (100  $\mu\text{g}$ ), PGE<sub>2</sub> (90 ng), dopamine (30  $\mu\text{g}$ ), or saline were administered by intraplantar route, and the intensity of hyperalgesia was evaluated 2–3 h later. Aiming to quantify the long-lasting sensitization, the response to an intraplantar injection of PGE<sub>2</sub> (9 ng or 90 ng), dopamine (3  $\mu\text{g}$ ) or saline was evaluated 5 or 30 days after the acute inflammation. The intensity of hyperalgesia was evaluated at different times, as indicated in the figures.

We also tested the effects of pre-treatment with the cyclooxygenase inhibitor indomethacin, the dopamine-receptor antagonist chlorpromazine, and the morphine against carrageenan-induced sensitization. Indomethacin (100  $\mu\text{g}/\text{paw}$ ), chlorpromazine (100  $\mu\text{g}/\text{paw}$ ), and morphine (6  $\mu\text{g}/\text{paw}$ ) were administered 30 min before the carrageenan injection at doses derived from previous studies (Cunha et al., 2005). Morphine (6 mg/kg) was also administered by subcutaneous route 30 min before and 12 h after the carrageenan administration. In addition, the effects of dopamine antagonists ( $D_2$ , L741626;  $D_1$ , SCH23390;  $D_4$ , L745870) and PGE<sub>2</sub> antagonist (EP<sub>4</sub>, AH23848) were evaluated in PGE<sub>2</sub>- or DA-induced sensitization.

## 3. Results

In the present study, we describe a long-lasting sensitization of nociceptors induced by local inflammation in mice, which is dependent on the dopaminergic and prostanoid systems. As shown in Fig. 1, the intraplantar treatment with carrageenan (100  $\mu\text{g}$ ) in the first experimental day produced a short-term hyperalgesia from which the animals fully recovered, with values similar to normal baseline at experimental days five (Fig. 1a) and thirty (Fig. 1b). Subcutaneous intraplantar injection of a small dose of PGE<sub>2</sub> (9 ng/paw) or dopamine (3  $\mu\text{g}/\text{paw}$ ), at the same site into which carrageenan had been injected 5 or 30 days earlier, resulted in an increased hyperalgesic response. During this sensitized state, the mechanical nociceptive threshold has returned to pre-carrageenan values (Fig. 1, day 5 and day 30). However, administration of PGE<sub>2</sub> or dopamine, at doses that only induce a slight and brief hyperalgesia in normal conditions, induced an improved hyperalgesic effect (Fig. 1).

Because the carrageenan-induced hyperalgesia in mice is dependent on prostaglandins and sympathetic amines, the contribution of these two components to inflammation-induced long-lasting sensitization was evaluated. The pre-treatment with a cyclooxygenase inhibitor, indomethacin (100  $\mu\text{g}/\text{paw}$ ), or a dopaminergic antagonist, chlorpromazine (3  $\mu\text{g}/\text{paw}$ ), partially inhibited the carrageenan-induced acute hyperalgesia, but completely prevented the development of the long-lasting sensitization (Fig. 2a). On the other hand, the systemic pre-treatment with morphine (6 mg/kg/s.c.; Fig. 2b) twice in the first experimental day, fully prevented the acute hyperalgesia induced by



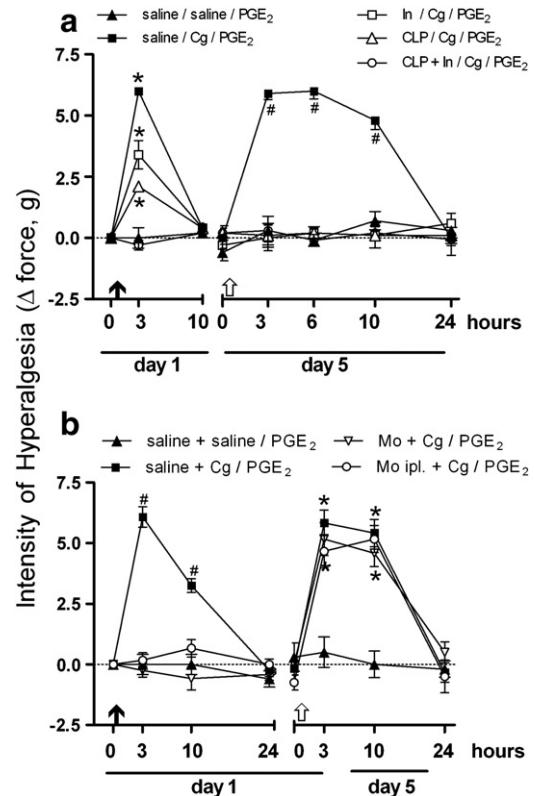
**Fig. 1.** Increased response to hyperalgesic mediators induced by previous local inflammation. Panels a and b represent acute hyperalgesia induced by i.pl. injection of carrageenan (Cg; 100 µg) in the first experimental day (day 1) and hyperalgesic response induced by i.pl. injection of a small dose of PGE<sub>2</sub> (9 ng) or dopamine (DA; 3 µg) 5 days (day 5; panel a) or 30 days (day 30; panel b) latter. Full and open arrows represent the moment of carrageenan and PGE<sub>2</sub> or dopamine administration, respectively. The nociceptive mechanical threshold was evaluated before and 3 h after drug administration. Data are reported as means ± SEM; n = 6 mice per group. (\*p < 0.001 vs. saline/saline group. #p < 0.001 vs. saline/DA group. \$p < 0.001 vs. saline/PGE<sub>2</sub> group; repeated measures of two-way ANOVA followed by the Bonferroni's test).

carrageenan, although it did not affect the induction of the long-lasting sensitization. Similar profile of activity was observed with the intraplantar injection of morphine (6 µg/paw). Intraplantar injection of a small dose of PGE<sub>2</sub> (9 ng/paw) or dopamine (3 µg/paw), at the same site where PGE<sub>2</sub> (90 ng/paw; Fig. 3a) or dopamine (30 µg/paw; Fig. 3b) had been injected 5 days earlier, also resulted in an increased hyperalgesic response.

To determine the pharmacological receptors involved with the long-lasting sensitization of nociceptors in mice, the effects of the pre-treatment with PGE<sub>2</sub> or dopamine antagonists were evaluated. The pre-treatment with a D<sub>1</sub> antagonist (SCH23390, 27–240 µg/paw; Fig. 4b), but not with D<sub>2</sub> and D<sub>4</sub> antagonists (L741626, 11–100 µg/paw and L745870, 11–100 µg/paw; Fig. 4a and c, respectively) fully prevented the acute hyperalgesia induced by dopamine and the induction of the long-lasting sensitization. Similarly, the pre-treatment with an EP<sub>4</sub> antagonist (AH23848, 10–1000 ng/paw; Fig. 5), fully prevented the acute hyperalgesia and the long-lasting sensitization induced by PGE<sub>2</sub>. The pre-treatment with a D<sub>1</sub> antagonist (SCH23390, 240 µg/paw) did not prevent the long-lasting sensitization induced by PGE<sub>2</sub>. Similarly, the pre-treatment with the EP<sub>4</sub> antagonist (AH23848, 1000 ng/paw) did not prevent the sensitization induced by dopamine (data not shown).

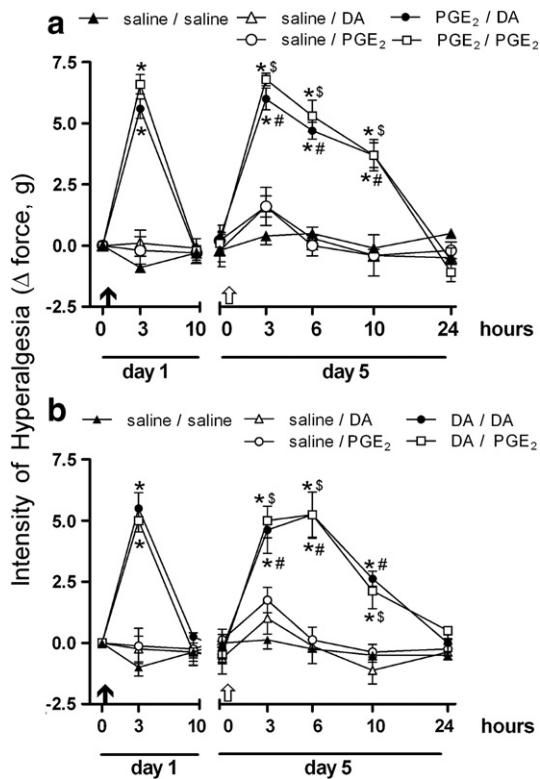
#### 4. Discussion and conclusions

Over the past 20 years, the most successful analgesic development activities have been confined to reformulation of conventional analgesic drugs, such as opioids and AINES (Kissin, 2010). Considering

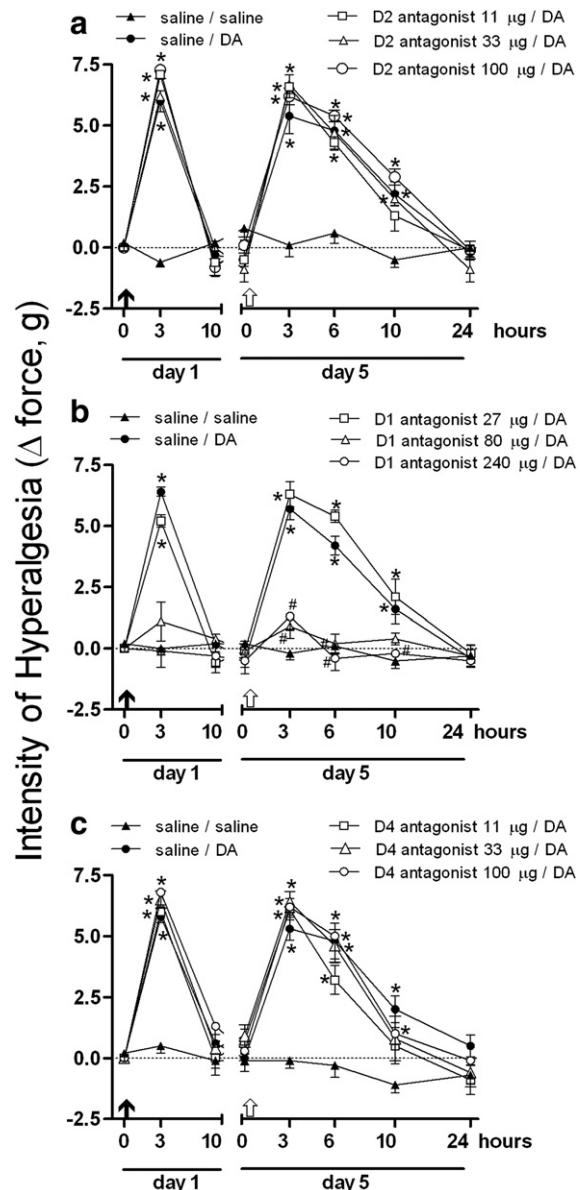


**Fig. 2.** Effects of pre-treatment with indomethacin, chlorpromazine and morphine in the increased response to hyperalgesic mediators induced by previous inflammation. Panel a represents acute hyperalgesia induced by i.pl. injection of carrageenan (Cg; 100 µg) in the first experimental day (day 1) and hyperalgesic response induced by a small dose of PGE<sub>2</sub> (9 ng/paw) 5 days after (day 5). Previously to carrageenan administration (30 min before), the animals received saline, indomethacin (In; 100 µg/paw), chlorpromazine (CLP; 3 µg/paw) or indomethacin + chlorpromazine (CLP+In; at the same doses). Five days later the hyperalgesic response to PGE<sub>2</sub> (9 ng/paw) was evaluated. Full and open arrows represent the moment of carrageenan and PGE<sub>2</sub> administration, respectively. Panel b shows the hyperalgesic response to PGE<sub>2</sub> (9 ng/paw) in mice pre-treated with morphine and carrageenan 5 days before. Previously to carrageenan administration (30 min before), the animals received an intraplantar injection of morphine (Mo i.pl.; 6 µg/paw). In another experimental group, morphine (Mo; 6 mg/kg) was administered systemically by subcutaneous route twice 24 h before carrageenan. The nociceptive mechanical threshold was evaluated before and 3 h after drug administration. Data are reported as means ± SEM; n = 6 mice per group. (\*p < 0.01 vs. saline/saline/PGE<sub>2</sub> group; #p < 0.001 vs. remaining groups; repeated measures of two-way ANOVA followed by the Bonferroni's test).

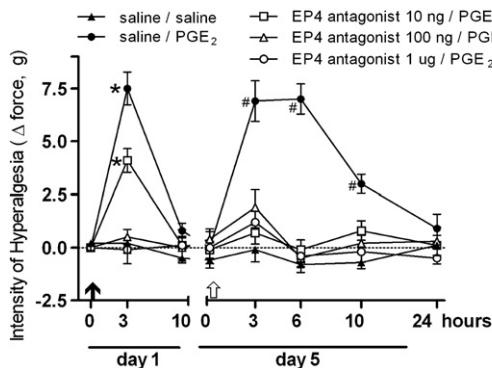
that the majority of the existing analgesics for chronic pain are relatively ineffective and do not reduce pain in all treated individuals, new strategies are needed. In this context, the elucidation of mechanisms that underlie the transition from acute inflammatory pain sensation to chronic pain is crucial to define new pharmacological approaches. The aim of the present study was to verify whether prostaglandins and sympathomimetic amines released during inflammation of peripheral tissue could increase the susceptibility of primary sensorial neurons to subsequent sensitizations. In fact, Aley et al. (2000) demonstrated that acute paw inflammation chronically increases the susceptibility of primary sensorial neurons to the inflammatory mediators induced hyperalgesia. Such an increased of the susceptibility of the primary afferent neurons could underlie the development of chronic pain. In the present study we describe, in a mouse model, a long-lasting sensitization of nociceptors induced by local inflammation that can represent the priming state previously described in rats (Aley et al., 2000). As shown here, a single episode of acute inflammation created a state of increased susceptibility to hyperalgesic mediators, such as PGE<sub>2</sub> and dopamine. Therefore, levels of inflammatory mediators that would be painless in the normal animal may cause significant pain in the sensitized state.



Peripheral sensitization commonly results from inflammation-associated changes in the chemical environment of the primary afferent fibers (McMahon et al., 2008). After the inflammatory stimuli, a wide array of endogenous factors, such as neurotransmitters, eicosanoids, and cytokines are released from activated nociceptors or non-neuronal cells. The interactions between cell-surface receptors of nociceptors and these factors enhance excitability of nociceptors, thereby raising its sensitivity to subsequent stimulus (Basbaum et al., 2009). These events can induce a temporary sensitization of the affected area or a persistent sensitization associated with unremitting pain. Carrageenan, which was used to induce the initial inflammation in the present work, is a classical agent for the induction of experimental inflammation, a model considered relevant due to its similarity to clinically important inflammatory pain states (Dawson et al., 1991; Di Rosa, 1972). The carrageenan-induced hyperalgesia in mice is dependent on prostaglandins and sympathetic amines (Cunha et al., 2005; Nakamura and Ferreira, 1987). These two mediators are involved in sensitization of nociceptors (Cunha et al., 2005; Ferreira and Nakamura, 1979; Khasar et al., 1999; Nakamura and Ferreira, 1987). Prostaglandins and sympathetic amines are present at sites of inflammation (Bley et al., 1998; Villena et al., 1999) and produce hyperalgesia by a direct action on primary afferent nociceptors (Ferreira et al., 1978; Gold et al., 1996). Sympathetic amines (norepinephrine and dopamine) have also been shown to functionally up-regulate nociceptors (Coderre et al., 1984; Duarte et al., 1988; Nakamura and Ferreira, 1987; Wall and Gutnick, 1974). In the present study, the pre-treatment with indomethacin or chlorpromazine completely prevented the development



of the long-lasting sensitization, suggesting that this phenomenon is dependent on the activation of dopaminergic and prostanoid systems. Corroborating with this idea, the preceding intraplantar administration of PGE<sub>2</sub> or dopamine, at hyperalgesic doses, also prolonged and enhanced the nociceptive response to subsequent administration of hyperalgesic mediators. These results suggest that similar messenger systems mediate both acute hyperalgesia and long-lasting peripheral sensitization during inflammatory conditions. To evaluate whether the induction of the nociceptor sensitization requires a hyperalgesic event, mice were pretreated with an analgesic dose of morphine. Morphine fully prevented the acute hyperalgesia, but did not affect the long-lasting



**Fig. 5.** Effects of pre-treatment with EP<sub>4</sub> receptor antagonist in the increased response to hyperalgesic mediators induced by PGE<sub>2</sub>. Figure represents acute hyperalgesia induced by i.pl. injection of PGE<sub>2</sub> (90 ng/paw) in the first experimental day (day 1) and hyperalgesic response induced by a small dose of PGE<sub>2</sub> (9 ng/paw) 5 days after (day 5). Previously to PGE<sub>2</sub> administration (30 min before), the animals received saline (25 µL/paw) or EP<sub>4</sub> antagonist (AH23848). Arrows represent the moment of the intraplantar injections of PGE<sub>2</sub>. The nociceptive mechanical threshold was evaluated before and 3 h after drug administration. Data are reported as means ± SEM; n=6 mice per group. (\*p<0.001 vs. saline/saline group; #p<0.001 vs. remaining groups; repeated measures of two-way ANOVA followed by the Bonferroni's test).

sensitization development. These results indicate that the development of nociceptor sensitization is not dependent on the nociceptive experience per se, but rather to an activation of specific receptors and intracellular signaling pathways.

Next, the pharmacological receptors involved with the sensitization of nociceptors were evaluated. The pharmacological antagonism of D<sub>1</sub> and EP<sub>4</sub> receptors fully prevented the acute hyperalgesia and the long-lasting sensitization induced by dopamine and PGE<sub>2</sub>, respectively. In line with the present results observed in mice, a key role of D<sub>1</sub> receptor subtype in the hyperalgesia induced by intraplantar injection of dopamine was previously demonstrated in rats (Ferreira et al., 1990). Similarly, the EP<sub>4</sub> receptors expressed on primary afferent sensory neurons have been associated with PGE<sub>2</sub>-mediated sensitization and hyperalgesia in models of acute and chronic inflammatory pain (Lin et al., 2006; Nakao et al., 2007). D<sub>1</sub> and EP<sub>4</sub> receptors stimulate the adenylyl cyclase/adenosine 30,50-cyclic monophosphate (cAMP) secondary messenger pathway (Coleman et al., 1994; Memo et al., 1986; Missale et al., 1998; Monsma et al., 1990; Sugimoto and Narumiya, 2007), which plays a pivotal role in the sensitization of nociceptors during inflammation (Cunha et al., 1999; Ferreira and Nakamura, 1979; Ouseph et al., 1995; Taiwo et al., 1989; Taiwo and Levine, 1991; Villarreal et al., 2009). This data suggest that the long-lasting sensitization induced by PGE<sub>2</sub> or dopamine may involve enhancement of intracellular cAMP.

In summary, the present work described in mice a long-lasting sensitization of nociceptors, initiated by an acute inflammatory stimulation which is dependent on the activation of dopaminergic and prostanoid systems. This long-lasting pronociceptive state or nociceptive memory may be considered a phenomenon of primary afferent nociceptors plasticity, and is probably present in chronic inflammatory pain. The concept of peripheral nociceptive memory will allow us to better understand chronic pain and its therapeutics. The present data bring new insights into mechanisms of peripheral sensitization that could contribute to form the basis of new therapeutic strategies for treating acute and chronic inflammatory pain.

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