15d-Prostaglandin J$_2$ inhibits inflammatory hypernociception: involvement of peripheral opioid receptor.


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Running Title page

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Nonstandard abbreviations:
15d-PGJ$_2$: 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$
PPAR-$\gamma$: peroxisome proliferator-activated receptors $\gamma$
TMJ: Temporomandibular Joint

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Abstract

The 15-deoxy-Δ^{12,14}-Prostaglandin J₂ (15d-PGJ₂) is an endogenous ligand of peroxisome proliferator-activated receptors γ (PPAR-γ), and is now recognized as a potent anti-inflammatory mediator. However, information regarding the influence of 15d-PGJ₂ on inflammatory pain is still unknown. In this study, we evaluated the effect of 15d-PGJ₂ upon inflammatory hypernociception and the mechanisms involved in this effect. It was observed that intraplantar administration of 15d-PGJ₂ (30-300 ng/paw) inhibits the mechanical hypernociception induced by carrageenan (100 µg/paw) and by the directly acting hypernociceptive mediator, PGE₂. Moreover, 15d-PGJ₂ (100 ng/TMJ) inhibits formalin induced temporomandibular joint hypernociception. On the other hand, the direct administration of 15d-PGJ₂ into the dorsal root ganglion, was ineffective to block PGE₂-induced hypernociception. In addition, the 15d-PGJ₂ antinociceptive effect was enhanced by the increase of macrophage population in paw due local injection of thioglycollate, suggesting the involvement of these cells on the 15d-PGJ₂-antinociceptive effect. Moreover, the antinociceptive effect of 15d-PGJ₂ was also blocked by naloxone and by the PPAR-γ antagonist GW9662, suggesting the involvement of peripheral opioids and PPAR-γ receptor in the process. Similarly to opioids, the 15d-PGJ₂ antinociceptive action depends on the nitric oxide/cGMP/PKG/K⁺ATP channel pathway since it was prevented by the pre-treatment with the inhibitors of nitric oxide synthase (L-NMMA), guanilate cyclase (ODQ), protein kinase G (KT5823) or with the ATP-sensitive potassium channel blocker (glibenclamide). Together, these results demonstrate for the first time that 15d-PGJ₂ inhibits inflammatory hypernociception via PPAR-γ activation. This effect seems to be dependent on endogenous opioids and local macrophages.
INTRODUCTION

Pain is one of the classical signals of the inflammatory process. It is now accepted that the sensitization of the primary sensory neurons is essential to inflammatory pain. In humans, this nociceptor sensitization usually leads to the clinical conditions known as hyperalgesia (an increased response to a stimulus that is normally painful) or allodynia (pain due to a stimulus that does not normally provoke pain). In the present study, we are using the term “hypernociception” to designate this decrease in the nociceptive threshold response in experimental animals.

The mechanisms involved in the sensitization of the primary sensory neurons and consequently, in the establishment of inflammatory hypernociception may be divided in two phases. Firstly, the non-neuronal events: the resident and migrated immune cells produce a sequence of hypernociceptive inflammatory mediators initiated by TNFα, which triggers the release of IL-1β, and chemokines that in turn stimulate the release of directly acting hypernociceptive mediators (Verri et al., 2006). The most well-known directly acting hypernociceptive mediators are prostaglandins. These mediators are considered directly acting, because they activate their specific receptors present on the nociceptive neurons membrane. The second phase includes the neuronal events: activation of receptors on primary nociceptive neurons that trigger intracellular signaling pathways, such as cyclic AMP (cAMP), protein kinase A (PKA) and protein kinase C (PKC) (Aley & Levine, 1999; Khasar et al., 1999). These signaling pathways result in subsequent phosphorylation of the voltage-dependent sodium channels (Gold, 1998) and inhibition of the voltage-dependent potassium channels (Evans et al., 1999). Consequently, the nociceptor threshold is lowered and ultimately leads to an enhancement of neurons excitability.

Experimentally, the peripheral pharmacologic control of inflammatory pain is based on two main strategies. The first is the use of drugs that prevent the nociceptor
sensitization, such as non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin and aspirin-like drugs), which inhibit prostaglandin synthesis (Ferreira, 1972), and therefore, prevent the development of hypernociception. The second strategy is the direct blockade of the ongoing nociceptor sensitization, which can be achieved by the use of peripheral morphine (opioids), dipyrone and diclofenac (Lorenzetti & Ferreira, 1985; Ferreira et al., 1991). In fact, these drugs reverse the already established hypernociception induced by prostaglandin E$_2$ (PGE$_2$) in the rat hind paws. In addition, several evidences support that their antinociceptive activities are due to the activation of the L-arginine/NO/cGMP/PKG/K$^+$$_{(ATP)}$ channels pathway (Ferreira et al., 1991; Sachs et al., 2004).

The 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) is one of the derivatives of PGD$_2$ metabolism pathway and is a natural ligand for peroxisome proliferator-activated receptors $\gamma$ (PPAR-$\gamma$) (Schoonjans et al., 1997; Ricote et al., 1998). It has been demonstrated that fluctuation of 15d-PGJ$_2$ levels is associated to the inflammatory process (Willoughby et al., 2000; Ricote et al., 1998), suggesting that it may play an important role in the regulation of the inflammatory reaction in vivo. Several in vitro studies demonstrated that pharmacological activation of PPAR-$\gamma$ by 15d-PGJ$_2$ produces anti-inflammatory effects, such as repression of the expression of several inflammatory response genes in activated macrophages, including the genes encoding TNF-$\alpha$, gelatinase B and cyclooxygenase (COX)-2 (Jiang et al., 1998; Ricote et al., 1998). In line with these findings, in vivo treatment with PPAR-$\gamma$ agonists have been reported to attenuate several experimental inflammatory diseases, such as colitis in mice (Su et al., 1999; Desreumax et al., 2001), adjuvant-induced arthritis in rats and reduction of systemic inflammation in polymicrobial sepsis (Kawahito et al., 2000; Zingarelli et al., 2003). Thus, PPAR-$\gamma$ activation may translate into beneficial effects to control inflammation. However, whether this treatment approach has beneficial effects on inflammatory pain remains unclear. Therefore, in the present study we investigated the
effect of 15d-PGJ$_2$ on the inflammatory hypernociception in rats and also the cellular mechanisms involved in this effect.
METHODS

Animals. The behavioral experiments were performed on male Wistar rats weighing 180-200 g housed in the animal care facility of the School of Medicine of Ribeirão Preto and taken to the testing room at least 1 hour before the experiments. Food and water were available ad libitum. All behavioral tests were performed between 9:00 AM and 5:00 PM, and the animals were only used once. Animal care and handling procedures were in accordance with the guidelines of the International Association for the Study of Pain (IASP) on the use of animals in pain research. All efforts were made to minimize the number of animals used and their discomfort.

Drugs. The drugs used in this study were 15-delta-prostaglandin J₂ (15d-PGJ₂), 2-chloro-5-nitro-N-phenylbenzamide (GW9662) and indolo[2,3-a]pyrrolo[3,4-c]carbazole aglycone (KT5823) (from Calbiochem, San Diego, CA); naloxone, glibenclamide, formalin solution were prepared from commercially stock formalin (an aqueous solution of 37% of formaldehyde) and further diluted in 0.9% NaCl and prostaglandin E₂ (PGE₂) (from Sigma, St. Louis, MO); N(G)-monomethyl-L-arginine acetate (L-NMMA; from Research Biochemicals, Natick, MA); thioglycollate (Fluid Thioglycollate Medium dehydrated – DIFCO laboratories, Detroit, USA), 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one (ODQ) (from Tocris Cookson, Ballwin, MO). The stock solution of PGE₂ (1 µg/µL) was prepared in 10% ethanol, and additional dilutions were made in 0.9% NaCl (saline); the final concentration of ethanol was 1%. The ATP-sensitive potassium channels blocker (Alves & Duarte 2002) glibenclamide was dissolved in 2% Tween 80 and re-suspended in saline. Naloxone and L-NMMA were dissolved in saline. GW9662, ODQ and KT5823 were dissolved in dimethyl sulfoxide (Sigma) and re-suspended in saline to minimize the final
concentration of DMSO (max 0.5%). Rat TNF-α was a gift from Dr. Stephen Poole (National Institute for Biological Standards and Control, UK).

**Intraplantar drug administration:** A hypodermic 26G needle was inserted subcutaneously into the plantar surface of the rat’s hind paw, between the five distal footpads. At the same place where mechanical stimulus was applied, the drugs were administered in a volume of 50 µL (Vivancos et al., 2003).

**Intraganglionar drug administration:** Briefly, after shaving the fur over the lower back, rats were lightly anesthetized and placed over a small cylinder to elevate the lumbar region. The intraganglionar injection was performed using a 30 cm PE-10 catheter (*Intramedic Clay Adams*, internal diameter 0.28 mm and external diameter 0.61 mm), calibrated in such way that 25 mm corresponded to an injected volume of 1 µL. The needle injection was 1.5 cm laterally to the vertebral column, about 0.5 cm caudal from a virtual line passing over the rostral borders of the iliac crests. Delicate movements of the needle were made until the bone resistance was diminished and a paw flinch reflex was observed. The paw flinch reflex was used as a sign that the needle tip penetrated the DRG of the fifth lumbar spinal nerve located underneath the transversal process of the fifth lumbar vertebra. After the needle reached the ganglion, 5 µL of solution was injected (Ferrari et al., 2007).

**Evaluation of Mechanical Hypernociception:** Hypernociceptive mechanical threshold was measured by the electronic von Frey method, as described: in a quiet room, rats were placed in acrylic cages (12 x 20 x 17 cm) with wire grid floors, 15–30 min before the start of testing. During this adaptation period, the paws were tested (probed) three times. The
test consisted of evoking a hind paw flexion reflex with a hand-held force transducer adapted with a 0.7 mm² polypropylene tip (electronic von Frey hair; IITC Life Science, Woodland Hills, CA). A tilted mirror placed under the grid provided a clear view of the rat hindpaw. The investigator was trained to apply the tip in between the five distal footpads with a gradual increase in pressure. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The maximum force applied was 80 g. The end point was characterized by the removal of the paw in a clear flinch response after the paw withdrawal. The animals were tested before and after treatments. A different investigator performed each test, as was the solution preparation and the intraplantar and intraganglionar injections. The results are expressed by the Δ withdraw threshold (in g) that was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before treatments.

**Temporomandibular Joint (TMJ) injections:** Animals were briefly anesthetized by inhalation of halothane to allow TMJ injection, which was performed with a 30-gauge needle introduced into the left TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µL). Injection volumes were 15 µL in all cases. Each animal regained consciousness approximately 30 s after discontinuing the anesthetic.

**Testing procedure for TMJ pain:** Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23º C. Each animal was manipulated for 7 days to be habituated to the experimental manipulation. After this period, the animal was placed in a test chamber (30x30x30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. Each animal
immediately recovered from anesthesia after TMJ injection and was returned to the test chamber for counting nociceptive responses during the following 45-min observation period. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period as previously described. Results are expressed as the duration time of nociceptive behavior (Clemente et al., 2004). At the conclusion of the experiment, animals were anesthetized by an intraperitoneal injection of a mixture of urethane (1 g/kg) and α-chloralose (50 mg/kg), followed by intravenous administration of Evans blue dye (1%, 5 mg/Kg), in order to visualize formalin-induced plasma extravasation upon post-mortem examination of injected TMJs. This procedure also allowed confirmation that the plasma extravasation induced by TMJ injection at the correct site was restricted to the immediate TMJ region (data not shown).

**Formalin paw test:** Rats were placed in an open Plexiglas observation chamber for 30 min to accommodate to their surroundings, and then removed for formalin administration. Rats were gently restrained while the dorsum of the hindpaw was subcutaneously administered with 50 µl of formalin 1% (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) using a 30 gauge needle. Following injection, the rat was returned to the observation chamber for a 60-min observation period. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. The recording time was divided into 12 blocks of 5 min and a pain score was determined for each block by measuring the number of lifts or flinches of the affected limb during the observation time. Such behavior could vary from a simple lift of the paw (not associated with locomotion) to a vigorous shaking of the limb, or it could be a rippling of
the back muscle associated with limb movement. Lifts or flinches were discrete and easily quantifiable.

**Cytokine measurements:** Two hours after carrageenan intraplantar injection, animals were terminally anaesthetized, and skin tissues of the plantar region were removed from the injected and control paws (saline). The samples were trituated and homogenized in 500 µL of the appropriate buffer containing protease inhibitors followed by a centrifugation of 10 min/2000 g. The supernatants were used to determined the levels of TNF-α as described previously (Cunha et al. 2005) by enzyme-linked immunosorbent assay (ELISA). The results are expressed as picograms (pg) of each cytokine per paw. As a control, the concentrations of these cytokines were determined in animals injected with saline.

**Experimental protocols**

1) **Effect of 15d-PGJ2 on carrageenan, formalin and TNF-α-induced mechanical hypernociception:** Rats were pre-treated with 15d-PGJ2 (30, 100 and 300 ng/50 µL/paw; i.pl) and, after 30 min, they received an i.pl. injection of carrageenan (100 µg/50 µL/paw; i.pl), formalin (1%/50 µL/paw; i.pl) or TNF-α (1 pg/50 µL/paw; i.pl). Mechanical hypernociception was evaluated after the carrageenan and TNF-α challenge by the electronic von Frey test.

2) **Effect of 15d-PGJ2 on formalin-induced TMJ nociception:** Rats were pre-treated with 15d-PGJ2 (100 ng/15 µL/TMJ) and, after 30 min, they received an intra-articular injection of 1.5% formalin. Behavioral nociception response was evaluated during 45 minutes.
3) **Effect of 15d-PGJ$_2$ on PGE$_2$-induced mechanical hypernociception:** Rats were pretreated with 15d-PGJ$_2$ (30, 100 and 300 ng/50 μL/paw; i.pl) and, after 30 min they received an i.pl. injection of PGE$_2$ (100 ng/50 μL/paw; i.pl). Mechanical hypernociception was evaluated 3 h after the PGE$_2$ challenge by the electronic von Frey test.

4) **Effect of PPAR-γ receptor antagonist on 15d-PGJ$_2$-induced antinociception:** Rats were pretreated (30 minutes) with the PPAR-γ receptor antagonist GW9662 (0.3, 1 and 3 ng/50 μL/paw; i.pl) followed by 15d-PGJ$_2$ (100 ng/50 μL/paw; i.pl) administration. After 30 minutes, PGE$_2$ (100 ng/50 μL/paw; i.pl) was injected. The mechanical hypernociception was evaluated 3 h after the PGE$_2$ challenge by the electronic von Frey test. All animals received a final volume of 150 μL of solutions.

5) **Effect of 15d-PGJ$_2$ administered directly into the DRG:** Rats were pretreated (30 min) with intraganglionic (100 ng/10 μL/DRG) or intraplantar (100 ng/50 μL/paw) injection of 15d-PGJ$_2$, followed by PGE$_2$ (100 ng/50 μL/paw; i.pl) injection. Mechanical hypernociception was evaluated 3 h after the PGE$_2$ challenge by the electronic von Frey test.

6) **Effect of the non-selective opioid receptor antagonist naloxone on 15d-PGJ$_2$-induced antinociception:** Rats were pretreated (30 min) with naloxone (1 μg/50 μL/paw; i.pl.) followed by 15d-PGJ$_2$ (100 ng/50 μL/paw; i.pl) administration. After 30 minutes, PGE$_2$ (100 ng/50 μL/paw; i.pl) was injected. Mechanical hypernociception was evaluated 3 h after the PGE$_2$ challenge by the electronic von Frey test. All animals received a final volume of 150 μL of solutions.
7) The role of macrophage for the antinociceptive effect of 15d-PGJ₂: Rats were pretreated with thioglycollate 1% (100 µL/paw; i.pl.). After 3 days, mechanical hypernociception were evaluated before animals receive the following treatments: pretreatment (30 min) with naloxone (1 µg/50 µL/paw; i.pl.) followed by 15d-PGJ₂ (30 ng/50 µL/paw; i.pl) administration. After 30 minutes, PGE₂ (100 ng/50 µL/paw; i.pl) was injected. Mechanical hypernociception was evaluated 3 h after the PGE₂ challenge by the electronic von Frey test. All animals received a final volume of 150 µL of solutions.

8) Quantification of macrophage tissue accumulation by N-acetylglucosaminidase (NAG) activity measurement: Plantar skin tissue of thioglycollate- or saline-injected rats were homogenated in 1.0 mL cooled (4°C) 0.9% saline containing 0.1% v/v Triton X-100, vortex-homogenized, and centrifuged at 4°C for 10 min at 1500 g. The supernatants were saved and used for NAG assay.

NAG assay reaction: The reaction was started at 37°C for 10 min in a 96-well microplate by the addition of 100 µL p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma), dissolved in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na₂HPO₄, pH 4.5) in a final concentration of 2.24 mM–100 µL supernatant from tissue sample processing, dissolved in citrate/phosphate buffer at appropriate dilutions. The reaction was terminated by the addition of 100 µL 0.2 M glycine buffer (pH 10.6) and was quantified at 405 nm in a spectrophotometer (Emax, Molecular Devices). The macrophage content was calculated from a standard curve based on NAG activity expressed as absorbance increase at 405 nm from 3% thioglycollate peritoneal-induced macrophages assayed in parallel. The results were expressed in relative number of macrophages per mg wet tissue (Belo et al., 2004).
9) **Role of NO/cGMP/PKG/K^+_{ATP} channels pathway on 15d-PGJ_2-induced antinociception:** Rats were divided in groups of five animals, and each group received different pretreatments with the following drugs: non-selective inhibitor of nitric oxide synthase L-NMMA (50 μg/50 μL/paw i.pl.; 30 min), inhibitor of soluble guanilate cyclase enzyme ODQ (8 μg/50 μL/paw; i.pl.; 30 min), inhibitor of protein kinase G KT5823 (1,5 μg/50 μL/paw; i.pl.; 10 min) or the ATP-potassium sensitive channel blocker glibenclamide (160 μg/50 μL/paw; i.pl.; 30 min). After that, all groups received 15d-PGJ_2 (100 ng/50 μL/paw; i.pl). After 30 minutes, PGE_2 (100 ng/50 μL/paw; i.pl) was injected. Mechanical hypernociception was evaluated 3 h after the PGE_2 challenge by the electronic von Frey test. All animals received a final volume of 150 μL of solutions. The selected doses of all other antagonists employed were obtained from previous studies (Sachs et al., 2004).

**Statistical analysis.** Two independent experiments were performed and the results were presented as the mean ± standard error mean (s.e.m.) of the results (n = 5 per experiment). The differences between the groups were compared using One-way analysis of variance (ANOVA) to obtain the degree of significance, followed by the Bonferroni multiple comparison test to compare the groups and doses (behavioral experiments). The established level of significance was *P < 0.05.*
RESULTS

1) 15d-PGJ$_2$ inhibits carrageenan-induced mechanical inflammatory hypernociception and formalin-induced nociception in the TMJ joint. Intraplantar administration of 15d-PGJ$_2$ (30, 100 and 300 ng/paw) inhibited the mechanical hypernociception induced by intraplantar administration of carrageenan (100 µg/paw – Figure 1A). This effect was significant 1, 3 and 5 h after carrageenan administration (Figure 1B). The administration of 15d-PGJ$_2$ (100 ng/paw) in the contralateral paw did not inhibit the mechanical hypernociception induced by the carrageenan indicating that it is acting locally (Figure 1A). In contrast with these results, it was observed that 15d-PGJ$_2$ was not able to inhibit carrageenan-induced thermal hypernociception (data not shown). We also observed that the administration of 15d-PGJ$_2$ did not inhibit the formalin induced nociception in the rat paw on both phases I and II pain behavior (data not shown). However, it inhibited formalin-induced nociception in the TMJ of rats (Figure 1C).

2) 15d-PGJ$_2$ did not inhibit carrageenan-induced TNF-α release but inhibited TNF-α-induced mechanical hypernociception. We have previously demonstrated that the carrageenan-induced hypernociception depends on TNF-α production (Cunha et al., 1992). Herein, 15d-PGJ$_2$ did not alter the release of TNF-α induced by carrageenan (Figure 1D), but TNF-α hypernociception was inhibited by 15d-PGJ$_2$ (Figure 1E). Therefore, the antinociceptive effect of 15d-PGJ$_2$ does not depend on the inhibition of the cytokine release, but rather on the inhibition of cytokine hypernociceptive action.

3) 15d-PGJ$_2$ inhibits PGE$_2$-induced mechanical hypernociception. Further addressing the 15d-PGJ$_2$ mechanism of action, we investigated its effect upon the mechanical hypernociception induced by a directly acting hypernociceptive mediator, PGE$_2$. This
approach is related to the demonstration that after carrageenan stimulus, there is the release of a cascade of cytokines, which include TNF-α. Then, cytokines are responsible for inducing PGE₂ production that directly sensitizes the nociceptors (Verri et al., 2006). Thus, considering that 15d-PGJ₂ inhibits the TNF-α-induced hypernociception (Figure 1E), but not carrageenan-induced production of TNF-α (Figure 1D), it is conceivable that if 15d-PGJ₂ inhibits PGE₂-induced mechanical hypernociception, its mechanism of action may be different from conventional NSAIDs and inhibitors of cytokine production, and therefore, opening the possibility of direct blockade of ongoing hypernociception by 15d-PGJ₂. In fact, it was detected that local treatment with 15d-PGJ₂ inhibited PGE₂-induced mechanical hypernociception (100 ng/paw, - Figure 2A). These results suggest that 15d-PGJ₂ presents a peripheral opioid-like mechanism because, as mentioned before, this type of peripheral antinociceptive drug is able to inhibit directly acting sensitizing mediator-induced hypernociception. Furthermore, intraplantar administration of 15d-PGJ₂ (100 ng/paw) did not alter the nociceptive threshold of the animals, disproving a possible hypoalgesic effect and suggesting an effect restricted to the inflammatory process. It is noteworthy to mention that the doses of carrageenan and PGE₂ used induce hypernociception only in the ipsilateral paw (Vivancos et al., 2004).

4) The antinociceptive action of 15d-PGJ₂ depends on PPAR-γ activation. In an attempt to investigate if the antinociceptive action of 15d-PGJ₂ depends on PPAR-γ activation, we tested the effect of the selective PPAR-γ antagonist GW9662 upon 15d-PGJ₂-induced antinociception. Local pre-treatment of rats with GW9662 (0.3, 1 and 3 ng/paw), 15 min before 15d-PGJ₂ (100 ng/paw) injection, reversed in a dose-dependent manner (P < 0.05) the antinociceptive activity of 15d-PGJ₂ on PGE₂-induced mechanical hypernociception (Figure 2B).
5) **The antinociceptive effect of 15d-PGJ$_2$ depends on peripheral resident cells.** In order to verify if 15d-PGJ$_2$ was exerting its antinociceptive effect by acting in the resident cells or directly in the peripheral sensitive neuron, animals were pre-treated with 15d-PGJ$_2$ by two different routes, intraplantar or intraganglionar. When intraplantarly administered, 15d-PGJ$_2$ (100 ng/paw) inhibited the PGE$_2$-induced hypernociception (100 ng/paw) as observed with morphine (6 µg/paw), which presents peripheral antinociceptive effects by acting directly on sensitive neurons. Importantly, when administrated alone, 15d-PGJ$_2$ did not show any alteration on nociceptive threshold (right column; intraplantar administration). However, the intraganglionar injection of 15d-PGJ$_2$ (100 ng/DRG) did not produce antinociceptive effect upon PGE$_2$-induced hypernociception (Figure 3) whereas, as expected, intraganglionar administration with morphine (6 µg/DRG) was effective. It is important to mention that intraganglionar administration of 15d-PGJ$_2$ (100 ng/DRG) did not modify the nociceptive threshold of rats (right column; intraganglionar administration).

6) **Activation of peripheral opioid receptors mediates 15d-PGJ$_2$-antinociception effects.** Figure 4, Panel A shows that locally administered naloxone (1 µg/paw) abolished the antinociceptive effect of 15d-PGJ$_2$ (100 ng/paw). This result suggests that the antinociceptive action of 15d-PGJ$_2$ depends on local opioids receptor activation. Considering that one of the characteristics of opioid agonists is their capacity to reverse already established hypernociception induced by prostaglandin E$_2$ (PGE$_2$) (Ferreira, 1979), we sought to verify the effect of post-treatment with 15d-PGJ$_2$ on mechanical hypernociception induced by PGE$_2$. Similar to pretreatment (30 min before PGE$_2$ injection),
local post-treatment with 15d-PGJ$_2$ (100 ng/paw), 1 hour after PGE$_2$ injection, significantly reduced ($P < 0.05$) PGE$_2$-induced mechanical hypernociception (Figure 4, Panel B).

7) Macrophages seem to be involved in the antinociceptive effect of 15d-PGJ$_2$: participation of opioid receptors. In an attempt to investigate whether the source of opioid-release induced by 15d-PGJ$_2$ could be the resident macrophages, we tested the antinociceptive effect of a sublimiar dose of 15d-PGJ$_2$ in a group of animals presenting an increased accumulation of macrophages in their paws induced by a previously administration of thioglycollate. Animals that received an intraplantar administration of thioglycollate did not show significant change in their nociceptive threshold 3 days later (data not shown). Although the intensity of hypernociception induced by PGE$_2$ was similar in naïve and thioglycollate injected rats, this latter group of animals (100 ng/paw) presented a markedly antinociception induced by the sublimiar dose of 15d-PGJ$_2$ (30 ng/paw). This effect was also reversed by the local pretreatment with naloxone (Figure 5, Panel A). Confirming that thioglycollate administration increased the number of macrophages in the rat paw, it was observed that NAG activity in the injected paw increased significantly (Figure 5, Panel B). The increase in paw tissue macrophage population by thioglycollate injection was also confirmed by histopathological sections (data not shown).

8) The NO/cGMP/PKG/K$^+$$_{ATP}$ pathway mediates the antinociceptive effect of 15d-PGJ$_2$. The pre-treatment with an inhibitor of the enzyme nitric oxide synthase (NOS) L-NMMA (50 µg/paw), the specific inhibitor of the soluble guanilate cyclase (sGC) ODQ (8 µg/paw), the protein kinase G (PKG) inhibitor KT5823 (1.5 µg/paw), or the K$^+$$_{ATP}$ channel blocker glibenclamide (160 µg/paw) 30 min before 15d-PGJ$_2$ injection, abolished the
antinociceptive effect of 15d-PGJ$_2$ upon PGE$_2$-induced hypernociception (Figure 6, Panels A to D, respectively).
Discussion

Several investigations have reported in the literature that 15d-PGJ\(_2\) presents anti-inflammatory effects (Jiang et al., 1998; Ricote et al., 1999). For instance, its systemic administration ameliorates experimental inflammatory diseases, including ischemia-reperfusion injury and arthritis (Kawahito et al., 2000; Chatterjee et al., 2004). In the present study, we demonstrated for the first time an antinociceptive action of 15d-PGJ\(_2\) upon mechanical inflammatory hypernociception. The local treatment of rats hindpaw with 15d-PGJ\(_2\) inhibited carrageenan- and PGE\(_2\)-induced mechanical hypernociception. It also inhibited formalin induced TMJ nociception. Furthermore, our results showed that such effects might depend on PPAR-\(\gamma\) activation, peripheral opioids receptor activation and, resident macrophages.

Previous studies demonstrated that inflammatory hypernociception induced by carrageenan results in release of a cascade of mediators initiated by the production of the hypernociceptive cytokines TNF-\(\alpha\), IL-1\(\beta\) and chemokines. These cytokines stimulate the release of the directly acting hypernociceptive mediators represented mainly by prostaglandins which directly act on nociceptive neurons (Cunha et al., 2005, Verri et al., 2006). Regarding the anti-inflammatory mechanism of 15d-PGJ\(_2\), it has been demonstrated that it inhibited the expression of several inflammatory molecules such as COX-2 and TNF-\(\alpha\) (Jiang et al., 1998; Ricote et al., 1998). However, in our experimental model, 15d-PGJ\(_2\) was not able to inhibit the production of TNF-\(\alpha\), but inhibited TNF-\(\alpha\) and PGE\(_2\)-induced mechanical hypernociception. In agreement, the hypernociceptive effect of TNF-\(\alpha\) is dependent on prostaglandin production (Cunha et al. 1992). It is important to mention that analgesics which act by inhibition of COX (NSAID), or cytokine production (thalidomide) are not able to affect PGE\(_2\)-induced hypernociception (Ribeiro et al., 2000). On the other hand, there are peripheral antinociceptive drugs such as peripheral opioids
that inhibit the ongoing hypernociception induced by PGE₂ (Ferreira, 1972; Sachs et al., 2004). Therefore, we are suggesting that 15d-PGJ₂ presents a peripheral opioid-like effect.

The fact that 15d-PGJ₂ did not inhibit carrageenan-induced thermal hypernociception suggests that different mechanisms are involved in the genesis of thermal and mechanical hypernociception. In fact, the thermal test detects mainly the superficial nociceptive fibers activation, whereas our mechanical test detects subcutaneous nociceptor activation. Recently, we demonstrated that different mechanisms are involved in the activation of these different nociceptive fibers (Vivancos et al., 2003). Further supporting the antinociceptive action of 15d-PGJ₂, although it not affected formalin induced-nociception in cutaneous tissues our results demonstrated an inhibition of formalin-induced nociception in TMJ. It is well demonstrated that this test has an inflammatory component (Clemente et al., 2004). It is clear that inflammatory conditions can result in hyperalgesia produced by peripheral sensitization of nociceptors and by central sensitization of the nociceptive neurons. The differences between the subcutaneous and TMJs nociceptors may result from the fact that both tissues are predominantly innervated by different subsets of primary nociceptive neurons. Since deep inputs may be more effective in inducing central neuronal excitation than cutaneous inputs, greater sensory disturbances may occur in pain conditions involving deep tissues than in those involving cutaneous tissues (Imbe et al., 2005). Otherwise, it has been demonstrated that the TMJ tissues are more sensitive to sympatomimethic amines and PGE₂ than cutaneous tissues (Rodrigues et al., 2006). Thus, we hypothesized that TMJ is more sensible to prostaglandins compared to paw tissue which could explain our results.

There are evidences that the activity of 15d-PGJ₂ depends either on PPAR-γ activation and/or on mechanisms independent of this nuclear receptor (Straus et al., 2000). The blockade of ongoing hypernociception by 15d-PGJ₂ was dependent on PPAR-γ activation. In line with our results, the antinociceptive role of PPAR-γ has already been
demonstrated in different models of inflammatory pain. Oliveira et al. (2007) observed that systemic administration of pioglitazone, a thiazolidinedione that selectively activates PPAR-\(\gamma\), reduces the second phase of formalin test (inflammatory phase). However, pioglitazone is ineffective upon the first phase of the formalin test, which is considered a non-inflammatory phase. Likewise, paw administration of 15d-PGJ\(_2\) did not alter the peripheral nociceptive threshold of naïve animals, confirming that the antinociceptive effect of PPAR-\(\gamma\) activation is restricted to ongoing inflammatory process. On the other hand, injection of PPAR-\(\gamma\) agonist in the mouse paw did not reduce the second phase of formalin-induced nociception (LoVerme et al., 2006). Corroborating, we observed that local administration of 15d-PGJ\(_2\) did not inhibit formalin-induced nociception in the rat paw (data not shown). Another contradiction between our study and the study conducted by Oliveira et al. (2007) is the fact that in this latter study, PPAR-\(\gamma\) agonist did not inhibit carrageenan-induced mechanical hypernociception. These contradictions could be a result of differences in PPAR-\(\gamma\) agonists which could have different pharmacological profiles, as well the routes of administration of PPAR-\(\gamma\) agonist used, or dose of carrageenan. Moreover, Oliveira et al. (2007) used a variation of the von Frey method in which only one filament is used and the frequency of the paw withdrawals is analyzed. In the present study, an electronic device detects the pressure necessary to induce a withdraw response, which is more sensible than the von Frey filaments to detect for instance differences between doses (Vivancos et al., 2004). Thus, all these differences may explain the discrepant results.

The antinociceptive effect of 15d-PGJ\(_2\) seems to be limited to the periphery. Actually, there is evidence that peripheral endogenous antinociceptive mechanisms may counteract inflammatory pain through the release of endogenous opioid peptides or anti-inflammatory cytokines (Stein et al., 2003; Verri et al., 2006). These findings, together with the fact that
15d-PGJ₂ post-treatment, similarly to peripheral opioids, directly blocked established hypernociception induced by PGE₂, raised the hypothesis that 15d-PGJ₂ is promoting peripheral analgesia by the stimulation of endogenous opioids release. Accordingly, naloxone, prevented the antinociceptive action of 15d-PGJ₂. Important that these results do not exclude the possibility that 15d-PGJ₂ is directly activating opioid receptors present in primary sensitive neurons. Nevertheless, the observation that differently of morphine (Ferrari et al., 2007), 15d-PGJ₂ was not able to cause antinociception when administrated intraganglionarly, supporting the suggestion that 15d-PGJ₂ is not acting directly on sensitive neurons, pointing to an action through endogenous opioids release by paw resident cells. The involvement of peripheral endogenous opioids release in the antinociceptive effect of other drugs has already been demonstrated (Ibrahim et al., 2005; Mousa et al., 1996). For instance, CB2 cannabinoid receptor activation produces peripheral antinociception by stimulating beta-endorphin release from resident keratinocytes, which acts at local neuronal μ-opioid receptors (Ibraim et al., 2005).

In an attempt to determine the cell source of peripheral opioids release stimulated by 15d-PGJ₂, it was observed that increasing the number of peripheral macrophages by previous administration of thioglycollate in the rat paw enhances the antinociceptive effect of 15d-PGJ₂. This result suggests that naloxone sensitive antinociceptive effect of 15d-PGJ₂ probably depends on paw skin macrophages. Indeed, there are evidences that opioid-containing macrophages are involved in the endogenous control of inflammatory pain (Brack et al., 2004). Furthermore, PPAR-γ is markedly expressed in activated macrophages, which also suggests the possibility of PPAR-γ activation by 15d-PGJ₂ resulting in the release of opioids by resident macrophages (Ricote et al., 1998). However, the supernatants of macrophage cultures stimulated with 15d-PGJ₂ were not able to inhibit PGE₂-induced hypernociception (data not shown). A possible explanation is that the amount released in vitro was not enough to produce antinociception in vivo. Alternatively,
the production of endogenous opioids depends on macrophages but also on other
surround resident cells present in the paw of the animals. Keratinocytes are a possibility,
which are known sources of endogenous opioids as mentioned before (Ibraim et al.,
2005).

Similarly to peripheral opioids, the antinociceptive mechanism of 15d-PGJ2 involves
activation of the L-arginine/NO/cGMP/PKG pathway. This conclusion is supported by the
observation that the peripheral antinociceptive activity of 15d-PGJ2 was prevented by
inhibitors of NOS, cGMP and PKG. The analgesic activity of the L-arginine/NO/cGMP/PKG
pathway is operated by opening ATPK+ channels (Sachs et al., 2004). This concept is
based on the observation that the peripheral antinociceptive activities of opioids, NO
donors and cGMP, are inhibited by K+ATP channels blockers (Sachs et al., 2004). Further
investigating peripheral opioids in the antinociceptive effect of 15d-PGJ2, it was observed
that glibenclamide prevented the peripheral antinociceptive effect of 15d-PGJ2. Although
the activation of L-arginine/NO/cGMP/PKG/K+ATP channels pathway seems to be involved
in peripheral analgesic activity of opioids, there is overwhelming evidence that this
analgesic effect of opioids also depends on inhibition of cAMP formation and of Ca2+
channels present in the peripheral nociceptive neurons (Levine & Taiwo, 1989; Stein et al.,
2003). Thus, although the activation of L-arginine/NO/cGMP/PKG/K+ATP channels pathway
is pharmacologically relevant for 15d-PGJ2 activity, it was not disproved the involvement
of inhibition of cAMP formation and of Ca2+ translocation also in 15d-PGJ2 analgesic effect.

Besides the opioids system, there are also evidences of interaction between PPAR-γ
and cannabinoids (Burstein et al., 2005). Indeed, it has been demonstrated that
cannabinoid receptor agonists present effect via activation of PPAR-γ receptor. Although we
did not investigate the participation of endogenous cannabinoids in the antinociceptive
action of 15d-PGJ2 there are evidences that PPAR-α synergizes with cannabinoids to
produce analgesia (Russo et al. 2007). Therefore, further studies are necessary to elucidate the contribution of endocannabinoids to the antinociceptive effect of 15d-PGJ2.

In summary, we demonstrated that 15d-PGJ2 presents a peripheral antinociceptive effect, which depends on activation of PPAR-γ and peripheral opioids receptors. It seems that 15d-PGJ2 promotes an endogenous opioids release, which may be related to macrophages. In conclusion, we are adding further data supporting the potential therapeutic use of 15d-PGJ2 in the control of inflammatory pain. Furthermore, our results may contribute to a better comprehension of experimental data in which 15d-PGJ2 is used as a pharmacological tool and may provide new insights for the development of novel analgesics.

Acknowledgements

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References:


Levine JD, Taiwo YO (1989) Involvement of the mu-opiate receptor in peripheral analgesia. *Neuroscience* **32**:571-575.


Footnotes

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M.H.N., G.R.S. and T.M.C. contributed equally to this work.
Legends for Figures

Figure 1: Peripheral antinociceptive effect of 15d-PGJ$_2$. (A) 15d-PGJ$_2$ was administrated in the rats hind paws at the doses 30, 100 and 300 ng (100 µl / i.pl.) 30 min before the intraplantar administration of carrageenan (Cg, 100 µg / 50 µl / i.pl.), and hypernociception was evaluated 3 hours after stimulus injection. (B) Time-dependence of 15d-PGJ$_2$ (100 ng/paw) in the Cg (100 µg)-induced hypernociception. (C) Effect of 15d-PGJ$_2$ (100 ng/paw) in the formalin 1.5%-induced nociception in the TMJ joint. (D) Effect of 15d-PGJ$_2$ (100 ng/paw) on Cg-induced TNF-α production in the paw skin. (E) Effect of 15d-PGJ$_2$ (100 ng/paw) on TNF-α (1 pg/paw)-induced hypernociception. The results are expressed as the mean ± SD of 5 animals per group. * indicates statistical significance compared to saline and ** compared to vehicle plus group Cg (A and B), Formalin (C), TNF-α (E). $P < 0.05$, one-way ANOVA followed by the Bonferroni test.

Figure 2: 15d-PGJ$_2$ inhibits PGE$_2$-induced hypernociception: Role of PPAR-γ. (A) Rats were treated with 15d-PGJ$_2$ (30, 100 or 300 ng/paw) before i.pl. stimulus with PGE$_2$ (100 ng/paw). (B) Animals were pre-treated (30 min) with GW9662 (0.3, 1 or 3 ng / 50 µl / i.pl.) or vehicle (saline), followed by 15d-PGJ$_2$ administration (100 ng / 50 µl / i.pl.). After 15 min, PGE$_2$ was administered intraplantarly (100 ng / 50 µl / i.pl.). Mechanical hypernociceptive threshold was evaluated 3 h after administration of PGE$_2$ by electronic von Frey test. The results are expressed as the mean ± SD of 10 animals per group. * indicates statistical significance compared to saline group, ** compared to PGE$_2$ control group, and # compared to vehicle plus 15d-PGJ$_2$ group. $P < 0.05$, one-way ANOVA followed by the Bonferroni test.
Figure 3: 15d-PGJ$_2$ does not inhibit PGE$_2$-induced hypernociception when administered into the dorsal root ganglion. Mechanical hypernociception induced by i.pl. injection of PGE$_2$ (100 ng / 100 µl / i.pl.) was inhibited by intraplantar injection of 15d-PGJ$_2$ (100 ng / 50 µl / i.pl.) but not by intraganglionar injection of 15d-PGJ$_2$ (100 ng / 10 µl / i.gl.) 15 min before. Morphine (6 µg / i.pl or i.gl.) was used as a control. Direct injection of 15d-PGJ$_2$ by intraplantar (black bars) or intraganglionar (open bars) administration did not show any threshold alteration (right column). Mechanical hypernociceptive threshold was evaluated 3 h after administration of PGE$_2$ by electronic von Frey test. The results are expressed as the mean ± SD of 10 animals per group. * indicates statistical significance compared to vehicle group. $P < 0.05$, one-way ANOVA followed by the Bonferroni test.

Figure 4: 15d-PGJ$_2$ induces antinociception by stimulating the release of endogenous opioids. (A) 15d-PGJ$_2$ (100 ng / 50 µl / i.pl.) or saline (50 µl / i.pl.) was administered 30 min before PGE$_2$ (100 ng / 50 µl / i.pl.) injection. Naloxone (Nlx; 1 µg / 50 µl / i.pl.) abolished the antinociceptive effect of 15d-PGJ$_2$. (B) The antinociceptive effect of post-treatment with 15d-PGJ$_2$ (1 hour after PGE$_2$ injection) on PGE$_2$-evoked hypernociception was evaluated (Panel B). Mechanical hypernociceptive threshold was evaluated 3 h after administration of PGE$_2$ by electronic von Frey test. The results are expressed as the mean ± SD of 10 animals per group. * indicates statistical significance compared to saline group and ** compared to vehicle plus PGE$_2$ group. $P < 0.05$, one-way ANOVA followed by the Bonferroni test.

Figure 5: Role of macrophages in the 15d-PGJ$_2$ antinociceptive effect: Participation of opioid receptors. (A) Control groups received an i.pl. injection of PGE$_2$ (100 ng / 50 µl - first bar) or pretreatment with a sub-limiar dose of 15d-PGJ$_2$ (30 ng / 50 µl / i.pl) and, after
15 min, PGE₂ (100 ng / 50 µl / i.pl - second bar). Rats were pre-treated with thioglycollate 1% (100 µl / i.pl.) and, after three days, it was administered the following treatments: PGE₂ (100 ng / 50 µl / i.pl. - third bar); pretreatment with 15d-PGJ₂ (30 ng / 50 µl / i.pl.) and, after 15 min, PGE₂ (100 ng / 50 µl / i.pl. - fourth bar); pretreatment (30 min) with naloxone (1 µg / 50 µl / i.pl.) followed by injection of PGE₂ (100 ng / 50 µl / i.pl. - fifth bar) or pretreatment (30 min) with naloxone (1 µg / 50 µl / i.pl.) followed by 15d-PGJ₂ (30 ng / 50 µl / i.pl) administration. After 15 min, PGE₂ (100 ng / 50 µl / i.pl) was injected (sixth bar).

Mechanical hypernociceptive threshold was evaluated 3 h after administration of PGE₂ by electronic von Frey test. The results are expressed as the mean ± SD of 10 animals per group. (B) The macrophage accumulation in rat hindpaw tissue was measured by indirect methods based on NAG (N-acetylglucosaminidase) activity. Results represent the relative number of macrophages per mg wet tissue and are the mean ± SD of 10 animals per group. * indicates statistical significance compared to PGE₂ group (A) or saline group (B). P < 0.05; one-way ANOVA followed by the Bonferroni test.

**Figure 6: Inhibition of 15d-PGJ₂ antinociception by antagonists of NO/cGMPc/PKG/\(K^{+}\)\_ATP pathway** - Rats were pre-treated with the antagonists of NO pathway 15 min before 15d-PGJ₂ and 30 min before PGE₂-induced hypernociception. Panel A: pre-treatment with L-NMMA (50 ng / 50 µl / i.pl.); Panel B: pre-treatment with ODQ (8 µg / 50 µl / i.pl.); Panel C: pre-treatment with KT5823 (1.5 µg / 50 µl / i.pl.) and Panel D: pre-treatment with glibenclamide (Gbl; 160 µg / 50 µl / i.pl.). The intensity of hypernociception was evaluated 3 h after administration of PGE₂ by electronic von Frey test. Data are expressed as the mean ± SD of 10 animals per group. * indicates statistical significance compared to saline group, ** compared to PGE₂ control group, and # compared to vehicle plus 15d-PGJ₂ group. P < 0.05, one-way ANOVA followed by the Bonferroni test.
Figure 2

(A) INTENSITY OF HYPERNOCESSION (Δ withdrawal threshold, g)

Sal  PGE\(_2\) (100 ng/paw)

*  

Vehicle  30  100  300  100

PGJ\(_2\) (ng/paw)

(B) PGE\(_2\) (100ng/paw)

Sal  PGJ\(_2\) (100ng/paw)

*  

Vehicle  0.3  1.0  3.0

GW9662 (ng/paw)
**Figure 3**

Intensity of hypernociception (Δ withdrawal threshold, g) vs. vehicle, morphine, PGJ$_2$, and PGJ$_2$ following intraplantar and intraganglionic application of PGE$_2$ (100ng/paw).
Figure 4

A

INTENSITY OF HYPERNOCESSION

(Δ withdrawal threshold, g)

Sal   PGE$_2$ (100ng/paw)

Vehicle  NLX

PGJ$_2$ (100ng/paw)

B

INTENSITY OF HYPERNOCESSION

(Δ withdrawal threshold, g)

Sal   PGE$_2$ (100ng/paw)

Vehicle  60 min  After  30 min  After

PGJ$_2$ (100ng/paw)
Figure 6

A

**INTENSITY OF HYPERNOCEPTION**

Δ withdrawal threshold, g

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**INTENSITY OF HYPERNOCEPTION**

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C

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Δ withdrawal threshold, g

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D

**INTENSITY OF HYPERNOCEPTION**

Δ withdrawal threshold, g

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