Treatment with a Heme Oxygenase 1 Inducer Enhances the Antinociceptive Effects of μ-Opioid, δ-Opioid, and Cannabinoid 2 Receptors during Inflammatory Pain

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Received April 21, 2014; accepted June 19, 2014

ABSTRACT

The administration of μ-opioid receptor (MOR), δ-opioid receptor (DOR), and cannabinoid 2 receptor (CB2R) agonists attenuates inflammatory pain. We investigated whether treatment with the heme oxygenase 1 (HO-1) inducer, cobalt protoporphyrin IX (CoPP), could modulate the local effects and expression of MOR, DOR, or CB2R during chronic inflammatory pain. In mice with inflammatory pain induced by the subplantar administration of complete Freund’s adjuvant, we evaluated the effects of the intraperitoneal administration of 10 mg/kg CoPP on the antiallodynic and antihyperalgesic actions of locally administered MOR (morphine), DOR (DPDPE [D-Pen(2),D-Pen(5)]-enkephalin), or CB2R [JWH-015 [(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethylone] agonists and its reversion with the HO-1 inhibitor, tin protoporphyrin IX (SnPP). The effect of CoPP treatment on the dorsal root ganglia expression of HO-1, MOR, DOR, and CB2R was also assessed. The results show that treatment with CoPP increased the local antinociceptive effects produced by morphine, DPDPE, or JWH-015 during chronic inflammatory pain, and these effects were blocked by the subplantar administration of SnPP, indicating the participation of HO-1 in the antinociceptive actions. CoPP treatment, apart from inducing the expression of HO-1, also enhanced the expression of MOR, did not alter CB2R, and avoided the decreased expression of DOR induced by inflammatory pain. This study shows that the HO-1 inducer (CoPP) increased the local antinociceptive effects of MOR, DOR, and CB2R agonists during inflammatory pain by altering the peripheral expression of MOR and DOR. Therefore, the coadministration of CoPP with local morphine, DPDPE, or JWH-015 may be a good strategy for the management of chronic inflammatory pain.

Introduction

It is well known that the local administration of μ-opioid receptor (MOR), δ-opioid receptor (DOR), or cannabinoid 2 receptor (CB2R) agonists elicits antiallodynic and antihyperalgesic effects during peripheral inflammation (Obara et al., 2009; Negrete et al., 2011; Hervera et al., 2013b). More interesting is the fact that under inflammatory pain conditions the local antinociceptive effects induced by MOR, DOR, and CB2R agonists are produced by the activation of the peripheral nitric oxide–cGMP–protein kinase G (PKG)–ATP-sensitive potassium (KATP) channels signaling pathway. Accordingly, the local antinociceptive effects of a MOR (morphine), DOR (DPDPE [D-Pen(2),D-Pen(5)]-enkephalin) or CB2R [JWH-015 [(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethylone] agonist were significantly reduced by their coadministration with selective neuronal or inducible nitric oxide synthases, L-guanylate cyclase, or PKG inhibitors, while the activation of this signaling pathway potentiated the peripheral antinociceptive effects produced by these agonists during inflammatory pain (Pacheco and Duarte, 2005; Hervera et al., 2009; Leánez et al., 2009; Cunha et al., 2010; Negrete et al., 2011). In addition, the expression of MOR, DOR, or CB2R after inflammatory diseases was also regulated by nitric oxide (Pol et al., 2005; Jiménez et al., 2006; Negrete et al., 2011).

It is also well known that, similar to nitric oxide, another gaseous neurotransmitter carbon monoxide synthesized by the heme oxygenase 1 (HO-1) enzyme also activates the cGMP-PKG pathway to modulate nociception (Steiner et al., 2001; Nascimento and Branco, 2007). Indeed, the administration of carbon monoxide–releasing molecules—a new class of chemical agents able to reproduce several biologic effects of HO-1–derived carbon monoxide, or the HO-1 inducer compound, cobalt protoporphyrin IX (CoPP)—inhibits inflammation, acute nociception, and neuropathic pain (Ferrándiz et al., 2008; Rosa et al., 2008; Egea et al., 2009; Maicas et al., 2010; Hervera et al., 2013a; Negrete et al., 2014). We have also

This work was supported by the Fondo de Investigación Sanitaria [Grant PS0900968], Madrid, Spain.

dx.doi.org/10.1124/jpet.114.215681

ABBREVIATIONS: AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM630, [6-iodo-2-methyl-1-[2-[(4-morpholinyl)[ethenyl]-1H-indol-3-yl][4-methoxyphenyl]-methanone; ANOVA, analysis of variance; CB1R, cannabinoid 1 receptor; CB2R, cannabinoid 2 receptor; CFA, complete Freund’s adjuvant; CoPP, cobalt protoporphyrin IX; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; DPDPE, [D-Pen(2),D-Pen(5)]-enkephalin; DOR, δ-opioid receptor; HO-1, heme oxygenase 1; JWH-015, (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethylone; KATP, ATP-sensitive potassium; MOR, μ-opioid receptor; NX-ME, naloxone methiodide; PKG, protein kinase G; SnPP, tin protoporphyrin IX.
recently demonstrated that carbon monoxide synthesized by HO-1 modulates the effects of morphine but not of DPDPE and JWH-015 under neuropathic pain conditions (Hervera et al., 2013b), although the role played by CoPP treatment on the effects and expression of MOR, DOR, and CB2R during chronic inflammatory pain has not been evaluated.

Therefore, in a mouse model of chronic inflammatory pain induced by the subplantar administration of complete Freund’s adjuvant (CFA), we evaluated 1) the antiallodynic and anti-hyperalgesic effects produced by the subplantar administration of specific MOR (morphine), DOR (DPDPE), or CB2R (JWH-015) agonists alone or combined with CoPP intraperitoneally administered; 2) the antinociceptive effects of morphine, DPDPE, and JWH-015 antinociceptive effects by their coadministration with specific antagonists; and 4) the effect of CoPP treatment on the expression of HO-1, MOR, DOR, and CB2R in the dorsal root ganglia from CFA-injected mice.

Materials and Methods
Experimental Animals. The experiments were performed in male C57BL/6 mice acquired from Harlan Laboratories (Barcelona, Spain). All mice weighing 21–25 g were housed under 12-hour light/dark conditions in a room with controlled temperature (22°C) and humidity (66%). Animals had free access to food and water and were used after a minimum of 7 days acclimatization to the housing conditions. All experiments were performed according with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Induction of Chronic Inflammatory Pain. Chronic inflammatory pain was induced by the subplantar injection of 30 μl CFA (Sigma-Aldrich, St. Louis, MO) into the right hind paw under brief anesthetic conditions with isoflurane, as described in our previous works (Hervera et al., 2009; Leánez et al., 2009). All experiments were performed 10 days after CFA injection. At this time point, all the animals developed a local inflammatory reaction, allodynia to mechanical stimuli, and hyperalgesia to noxious thermal stimuli, as previously reported by our group (Negrete et al., 2011).

The development of mechanical allodynia and thermal hyperalgesia was evaluated by using von Frey filaments and plantar tests, respectively. All animals were tested in each paradigm before and at 10 days after CFA injection.

Nociceptive Behavioral Tests. Mechanical allodynia was quantified by measuring the hind-paw withdrawal response to von Frey filament stimulation. In brief, animals were placed in methacrylate cylinders (20 cm high, 9 cm diameter; Servei Estació, Barcelona, Spain) with a wire-grid bottom through which the von Frey filaments (North Coast Medical, Inc., San Jose, CA) with a bending force in the range of 0.008–3.5 g were applied by using a modified version of the up–down paradigm, as previously reported by Chaplin et al. (1994). The 0.4-g filament was used first, and the 3.0-g filament was used as a cutoff. Then, the strength of the next filament was decreased or increased according to the response. The threshold of response was calculated from the sequence of filament strength used during the up–down procedure by using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that includes curve-fitting of the data. Clear paw withdrawal or shaking or licking of the paw was considered to be a nociceptive-like response. Both ipsilateral and contralateral hind paws were tested. Animals were allowed to habituate for 1 hour before testing to allow an appropriate behavioral immobility.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al. (1988). Paw withdrawal latency in response to radiant heat was measured using a plantar test apparatus (Ugo Basile, Varese, Italy). Briefly, mice were placed in methacrylate cylinders (20 cm high × 9 cm diameter) positioned on a glass surface. The heat source was positioned under the plantar surface of the hind paw and activated with a light beam intensity chosen in preliminary studies to give baseline latencies from 8–10 seconds in control mice. A cutoff time of 12 seconds was used to prevent tissue damage in the absence of a response. The mean paw withdrawal latencies from the ipsilateral and contralateral hind paws were determined from the average of three separate trials, taken at 5-minute intervals to prevent thermal sensitization and behavioral disturbances. Animals were habituated to the environment for 1 hour before the experiment to allow them to become quiet and to permit testing.

Western Blot Analysis. Animals were sacrificed at 0 days (naïve) and after CFA injection by cervical dislocation. Tissues from the ipsilateral section of the dorsal root ganglia (L3 to L5) were removed immediately after killing, frozen in liquid nitrogen, and stored at −80°C until assay. Samples from five animals were pooled into one experimental sample to obtain enough protein levels for performing Western blot analysis. The HO-1, MOR, DOR, and CB2R protein levels were analyzed by Western blot. Tissues were homogenized in ice-cold lysis buffer (50 mM Tris-Base, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% SDS, 1 mM Na3VO4, 25 mM NaF, 0.5% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail). All reagents were purchased at Sigma-Aldrich with the exception of NP-40 from Calbiochem (Darmstadt, Germany). The crude homogenate was solubilized for 1 hour at 4°C, sonicated for 10 seconds and centrifuged at 4°C for 15 minutes at 700g. The supernatant (60 μg of total protein) was mixed with 4× Laemmli loading buffer and then loaded onto 4% stacking/10% separating SDS polyacrylamide gels.

The proteins were electrophoretically transferred onto polyvinylidene fluoride membranes for 120 minutes, blocked with phosphate-buffered saline + 5% nonfat dry milk, and subsequently incubated overnight at 4°C with polyclonal rabbit anti-β-actin antibody (1:10,000; Sigma-Aldrich, St. Louis, MO) that includes curve-fitting of the data. Clear paw withdrawal or shaking or licking of the paw was considered to be a nociceptive-like response. Both ipsilateral and contralateral hind
DPDPE (50 μg), or JWH-015 (30 μg) in the contralateral and ipsilateral paw of CFA-injected animals (n = 6 animals per group).

In another set of experiments, we evaluated the mechanical antiallodynic and thermal antihyperalgesic effects produced by the subplantar administration of 290 μg SnPP alone or combined with the subplantar administration of a high dose of morphine (100 μg), DPDPE (150 μg), or JWH-015 (300 μg) in the contralateral and ipsilateral paw of CFA-injected animals (n = 6 animals per group).

The doses of CoPP and SnPP combined with morphine, DPDPE, or JWH-015 were selected in accordance to our previous studies (Hervera et al., 2013a,b). The doses of all tested opioid and cannabinoid receptor agonists subplantarly administered were chosen from the dose-response curves performed in this study, as the ones that produced a minimal or a maximal antinociceptive effect in CFA-injected mice.

The reversibility of the antinociceptive effects produced by the subplantar administration of morphine (100 μg), DPDPE (150 μg), or JWH-015 (300 μg), as doses that produce the maximal antiallodynic and antihyperalgesic effects after peripheral inflammation by their subplantar coadministration with specific (120 μg CTAP [H-D-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂]; 50 μg naltrindole; and 60 μg AM630 ([6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl][4-methoxyphenyl]-methanone]) and an unspecific peripheral opioid antagonist (50 μg nalorex methidide [NX-ME]) or a cannabinoid 1 receptor (CB1R) antagonist (150 μg AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]) (Hervera et al., 2010, 2011), was also evaluated (n = 6 animals per group). The doses of all tested opioid and cannabinoid receptor antagonists were selected according to our previous data obtained in animals with chronic pain (Hervera et al., 2009, 2013b; Negrete et al., 2011).

Finally, in another set of experiments we evaluated the effects of CoPP on the expression of HO-1, MOR, DOR, and CB2R in the ipsilateral site of the dorsal root ganglia from CFA-injected mice by use of Western blot assay. In these experiments, mice treated with vehicle were used as controls (n = 5 samples per group). The total number of animals used in this study was 291.

Drugs. CoPP and SnPP were purchased from Frontier Scientific (Livchem GmbH & Co, Frankfurt, Germany). Morphine hydrochloride was obtained from Alcaiber S.A. (Madrid, Spain), and DPDPE, CTAP, naltrindole, and NX-ME were acquired from Sigma-Aldrich. JWH-015, AM630, and AM251 were purchased from Tocris (Ellisville, MI). CoPP and SnPP were dissolved in dimethylsulfoxide (1% solution in saline). JWH-015, AM630, and AM251 were purchased from Tocris (Ellisville, MI).

Morphine hydrochloride, DPDPE, CoPP, and SnPP were dissolved in dimethylsulfoxide (1% solution in saline). JWH-015, AM630, and AM251 were purchased from Tocris (Ellisville, MI).

TABLE 1

Mechanical response (von Frey filaments strength, grams) and thermal response (withdrawal latency, seconds) in the contralateral and ipsilateral paw of mice after the subplantar administration of CFA. Results are shown as mean ± S.E.M.; n = 6 animals per experimental group.

<table>
<thead>
<tr>
<th>Paw</th>
<th>Mechanical Response (von Frey Filaments Strength)</th>
<th>Thermal Response (Withdrawal Latency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>s</td>
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<tr>
<td>Contralateral</td>
<td>2.4 ± 0.1*</td>
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</tr>
<tr>
<td>Ipsilateral</td>
<td>1.4 ± 0.1*</td>
<td>3.5 ± 0.2*</td>
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*P < 0.01 denotes statistically significant difference between ipsilateral and contralateral paw (paired Student’s t test) for each test.

Fig. 1. Effects of the subplantar administration of morphine, DPDPE, or JWH-015 on the mechanical allodynia and thermal hyperalgesia induced by peripheral inflammation. Mechanical antiallodynic and thermal antihyperalgesic effects produced by the subplantar administration of different doses of morphine (A and B), DPDPE (C and D), or JWH-015 (E and F) and their respective vehicles in the ipsilateral paw of CFA-injected mice. Data are expressed as mean value of maximal possible effect (%) ± S.E.M. (six animals for dose). *Statistically significant difference versus their respective vehicle treated animals (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each test, drug, and dose.
Effects of CoPP on the antiallodynic and antihyperalgesic responses to morphine. Mechanical antiallodynic (A) and thermal antihyperalgesic (B) effects produced by the subplantar administration of 50 μg of morphine or vehicle in the ipsilateral paw of CFA-injected mice pretreated with 10 mg/kg CoPP. The effect of the intraperitoneal administration of CoPP alone is also shown. Data are expressed as mean values of the maximal possible effect (% ± S.E.M. (6 animals per group). *Statistically significant difference versus control group treated with vehicle (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls test) for each behavioral test. **Statistically significant difference versus group treated with CoPP plus vehicle (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each behavioral test.

CTAP, NX-ME, and naltrindole were dissolved in saline solution (0.9% NaCl). All drugs were freshly prepared before use. CoPP was intraperitoneally administered 3–4 hours before testing in a final volume of 10 ml/kg. SnPP, morphine, DPDPE, JWH-015, CTAP, NX-ME, naltrindole, AM630, and AM251 were administered into the plantar side of the right paw 30 minutes before behavioral testing, in a final volume of 30 μl.

We used a paired Student's t test to compare the mechanical and thermal responses induced by peripheral inflammation in the ipsilateral paw of CFA-injected mice with the effects produced in the contralateral paws of mice. For each test and drug evaluated, the comparison of the effects produced by the subplantar administration of different doses of morphine, DPDPE, JWH-015, or their corresponding vehicle was evaluated by using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. For each behavioral test, the comparison of the effects produced by morphine, DPDPE, JWH-015, or their corresponding vehicle was evaluated by using one-way ANOVA followed by the Student-Newman-Keuls test for each behavioral test.

Changes on the expression of HO-1, MOR, DOR, and CB2R in the dorsal root ganglia from naïve and CFA-injected mice treated with vehicle or CoPP were also analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test.

Maximal possible effect(%) = [(drug – baseline)/(cutoff – baseline)] × 100

For each test, the reversal of the local antinociceptive effects produced by morphine, DPDPE, or JWH-015 with their respective antagonists and the effects produced by these antagonists administered alone were also analyzed by use of one-way ANOVA followed by the Student-Newman-Keuls test.

Changes on the expression of HO-1, MOR, DOR, and CB2R in the dorsal root ganglia from naive and CFA-injected mice treated with vehicle or CoPP were also analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

Results

Induction of Inflammatory Pain. In accordance with our previous findings, the subplantar administration of CFA produced unilateral mechanical allodynia and thermal hyperalgesia (Table 1). Indeed, peripheral inflammation led to a significant decrease of the threshold for evoking paw withdrawal to a mechanical stimulus and a decrease of paw withdrawal latency to thermal stimulus in the ipsilateral paw as compared with the contralateral paw (P < 0.01, paired Student's t test).

Effects of the Subplantar Administration of Morphine, DPDPE, and JWH-015 on the Mechanical Allodynia and Thermal Hyperalgesia Induced by Peripheral Inflammation in Mice. The subplantar administration of morphine...

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**Fig. 2.** Effects of CoPP on the antiallodynic and antihyperalgesic responses to morphine. Mechanical antiallodynic (A) and thermal antihyperalgesic (B) effects produced by the subplantar administration of 50 μg of morphine or vehicle in the ipsilateral paw of CFA-injected mice pretreated with 10 mg/kg CoPP. The effect of the intraperitoneal administration of CoPP alone is also shown. Data are expressed as mean values of the maximal possible effect (% ± S.E.M. (6 animals per group). *Statistically significant difference versus control group treated with vehicle (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls test) for each behavioral test. **Statistically significant difference versus group treated with CoPP plus vehicle (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each behavioral test.

**Fig. 3.** Effects of CoPP on the antiallodynic and antihyperalgesic responses to DPDPE. Mechanical antiallodynic (A) and thermal antihyperalgesic (B) effects produced by the subplantar administration of 50 μg DPDPE or vehicle in the ipsilateral paw of CFA-injected mice pretreated with 10 mg/kg CoPP. The effect of the intraperitoneal administration of CoPP alone is also shown. Data are expressed as mean values of the maximal possible effect (% ± S.E.M. (6 animals per group). *Statistically significant difference versus control group treated with vehicle (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls test) for each behavioral test. **Statistically significant difference versus group treated with DPDPE (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each behavioral test. ***Statistically significant difference versus group treated with CoPP plus vehicle (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each behavioral test.
by high doses of JWH-015 (75, 150, or 300 μg). The effects of the intraperitoneal administration of CoPP alone statistically significantly attenuated the mechanical allodynia (Fig. 2A) and thermal hyperalgesia (Fig. 2B) induced by peripheral inflammation (P < 0.001, one-way ANOVA versus control vehicle–treated mice). Our results also demonstrate that treatment with CoPP significantly increased the local antiallodynic (Fig. 2A) and antihyperalgesic (Fig. 2B) effects produced by the subplantar administration of morphine in the ipsilateral paw of CFA-injected mice (P < 0.001, one-way ANOVA versus control group treated with vehicle or morphine or CoPP plus vehicle).

The effects of the intraperitoneal administration of 10 mg/kg CoPP on the mechanical antiallodynic and thermal antihyperalgesic effects produced by the subplantar administration of CoPP alone is also shown. Data are expressed as mean values of the maximal possible effect (%) ± S.E.M. (6 animals per group). *Statistically significant difference versus control group treated with vehicle (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls test) for each behavioral test. #Statistically significant difference versus control group treated with JWH-015 (P < 0.05, one-way ANOVA followed by the Student-Newman-Keuls test) for each behavioral test.

(50–100 μg) dose-dependently inhibited the mechanical allodynia (Fig. 1A) and thermal hyperalgesia (Fig. 1B) induced by peripheral inflammation. Indeed, the mechanical antiallodynic and thermal antihyperalgesic effects produced by high doses of morphine (75 or 100 μg) in the ipsilateral paw of CFA-injected mice were statistically significantly higher than those produced by a low dose of the same drug or their corresponding vehicle-treated animals (P < 0.001, one-way ANOVA followed by the Student-Newman-Keuls test).

The subplantar administration of DPDPE (50–150 μg) dose-dependently inhibited the mechanical allodynia (Fig. 1C) and thermal hyperalgesia (Fig. 1D) induced by peripheral inflammation. Indeed, the mechanical antiallodynic and thermal antihyperalgesic effects produced by high doses of DPDPE (75, 100, or 150 μg) in the ipsilateral paw of CFA-injected mice were statistically significantly higher than those produced by a low dose of the same drug or their corresponding vehicle-treated animals (P < 0.001, one-way ANOVA followed by the Student-Newman-Keuls test).

In a similar way, the subplantar administration of JWH-015 (30–300 μg) also dose-dependently inhibited the mechanical allodynia (Fig. 1E) and thermal hyperalgesia (Fig. 1F) induced by peripheral inflammation. That is, the mechanical antiallodynic and thermal antihyperalgesic effects produced by high doses of JWH-015 (75, 150, or 300 μg) in the ipsilateral paw of CFA-injected mice were statistically significantly higher than those produced by 30 μg of the same drug or their corresponding vehicle-treated animals (P < 0.001, one-way ANOVA followed by the Student-Newman-Keuls test).

The subplantar administration of morphine, DPDPE, JWH-015, or vehicle did not elicit any antinociceptive effect in the contralateral paw of CFA-injected mice (data not shown).

**Effects of SnPP treatment on the antiallodynic and antihyperalgesic responses to morphine, DPDPE, and JWH-015 during Peripheral Inflammation.** The effects of the intraperitoneal administration of 10 mg/kg CoPP on the mechanical antiallodynic and thermal antihyperalgesic effects produced by the subplantar administration of a subanalgus dose of morphine (50 μg), DPDPE (50 μg), JWH-015 (30 μg), or vehicle in CFA-injected mice were investigated. For morphine, our results showed that the intraperitoneal administration of CoPP alone statistically significantly attenuated the mechanical allodynia (Fig. 2A) and thermal hyperalgesia (Fig. 2B) induced by peripheral inflammation (P < 0.001, one-way ANOVA versus control vehicle–treated mice). Our results also demonstrate that treatment with CoPP significantly increased the local antiallodynic (Fig. 2A) and antihyperalgesic (Fig. 2B) effects produced by the subplantar administration of morphine in the ipsilateral paw of CFA injected mice (P < 0.001, one-way ANOVA versus control group treated with vehicle or morphine or CoPP plus vehicle).
TABLE 2
Effects of the subplantar administration of morphine (100 μg), DPDPE (150 μg), or JWH-015 (300 μg) alone or combined with CTAP (120 μg) or NX-ME (50 μg), naltrindole (50 μg) or NX-ME (50 μg), or AM630 (50 μg) or AM251 (150 μg), respectively, on the mechanical allodynia and thermal hyperalgesia induced by the subplantar administration of CFA in the ipsilateral paw of mice.

Results are shown as mean ± S.E.M.; n = 6 animals per experimental group.

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*P < 0.05 denotes statistically significant differences versus their respective vehicle plus vehicle treated group (one-way ANOVA, followed by the Student-Newman-Keuls test) for each test and drug tested.

For DPDPE, treatment with CoPP also statistically significantly attenuated the mechanical allodynia (Fig. 3A) and thermal hyperalgesia (Fig. 3B) induced by peripheral inflammation in the ipsilateral paw of CFA-injected mice (P < 0.001, one-way ANOVA versus control vehicle–treated mice). Moreover, although treatment with CoPP did not alter the mechanical antiallodynic (Fig. 3A) effects of DPDPE, it statistically significantly increased the thermal antihyperalgesic (Fig. 3B) effects produced by the subplantar administration of DPDPE in the ipsilateral paw of CFA-injected mice (P < 0.001, one-way ANOVA versus control group treated with vehicle or DPDPE or CoPP plus vehicle).

Regarding JWH-015, similar to what occurred with morphine, treatment with CoPP statistically significantly enhanced the antiallodynic (Fig. 4A) and antihyperalgesic (Fig. 4B) effects produced by the subplantar administration of JWH-015 in the ipsilateral paw of CFA-injected mice (P < 0.001, one-way ANOVA versus control group treated with vehicle or JWH-015 or CoPP plus vehicle).

The subplantar administration of morphine, DPDPE, or JWH-015 alone or combined with SnPP had no statistically significant effect on the contralateral paw of CFA-injected animals (data not shown).

**Effects of the HO-1 Inhibitor, Tin Protoporphyrin IX, on the Antinociceptive Responses to Morphine, DPDPE, and JWH-015 in CFA-Injected Mice.** The effects of the subplantar administration of SnPP (290 μg) on the mechanical antiallodynic and thermal antihyperalgesic effects produced by the subplantar administration of morphine (100 μg), DPDPE (150 μg), or JWH-015 (300 μg) in CFA-injected mice were assessed.

For morphine, DPDPE, and JWH-015, and each test evaluated, our results show that although the subplantar administration of SnPP alone did not alter the mechanical allodynia (Fig. 5A) or thermal hyperalgesia (Fig. 5B) induced by peripheral inflammation, their local coadministration with a high dose of morphine, DPDPE, or JWH-015 statistically significantly decreased the local antiallodynic (Fig. 5A) and antihyperalgesic (Fig. 5B) effects produced by these drugs on the ipsilateral paw of CFA-injected mice (P < 0.001, one-way ANOVA versus group treated with morphine, DPDPE, or JWH-015 plus vehicle).

The subplantar administration of morphine, DPDPE, or JWH-015 alone or combined with SnPP had no statistically significant effect on the contralateral paw of CFA-injected animals (data not shown).

**Reversal of the Antinociceptive Effects of Morphine, DPDPE, and JWH-015 by Specific Antagonists after Peripheral Inflammation.** The antiallodynic and antihyperalgesic effects produced by the subplantar administration of 100 μg of morphine in the ipsilateral paw of CFA-injected mice were completely reversed by its subplantar coadministration with selective MOR (CTAP, 120 μg) or peripheral opioid receptor (NX-ME, 50 μg) antagonists (P < 0.001; one-way ANOVA, followed by Student-Newman-Keuls test) (Table 2). In a similar way, the antiallodynic and antihyperalgesic effects produced by 150 μg of DPDPE in the ipsilateral paw of CFA-injected mice were completely reversed by its subplantar coadministration with a selective DOR (naltrindole, 50 μg) or a peripheral opioid receptor (NX-ME, 50 μg) antagonist (P < 0.001, one-way ANOVA, followed by Student-Newman-Keuls test). In addition, the antinoceptive effects produced by 300 μg of JWH-015 in the ipsilateral paw of CFA-injected mice were also completely reversed by its subplantar coadministration with a selective CB2R antagonist (AM630, 60 μg; P < 0.001, one-way ANOVA, followed by Student-Newman-Keuls test). The subplantar administration of AM251 (a selective...
CB1R antagonist, 150 \( \mu g \) was unable to revert the local antiallodynic and antihyperalgesic effects produced by JWH-015.

The subplantar administration of the different agonists alone in the ipsilateral paw of CFA-injected mice (Table 2) as well as in the contralateral paw of these animals (data not shown) had no statistically significant effect on the different nociceptive responses evaluated in this study. In addition, the subplantar administration of all tested agonists alone or combined with their respective antagonists produced no statistically significant effect in the contralateral paw of CFA-injected mice (data not shown).

**Effect of CoPP Treatment on HO-1, MOR, DOR, and CB2R Protein Expression in the Dorsal Root Ganglia from CFA-Injected Mice.** The protein levels of HO-1, MOR, DOR, and CB2R in the dorsal root ganglia from CFA-injected mice treated with vehicle or CoPP as well as from control mice treated with vehicle are shown in Figs. 6 and 7. Our results show that the dorsal root ganglia expression of HO-1 (Fig. 6A) was statistically significantly increased by CoPP treatment \( (P < 0.001, \text{one-way ANOVA versus control vehicle and CFA-injected mice treated with vehicle}) \). Similarly, the expression of MOR (Fig. 6B) was also statistically significantly increased by CoPP treatment \( (P < 0.049, \text{one-way ANOVA versus control mice}) \). Moreover, the decreased protein levels of DOR in the dorsal root ganglia from CFA-injected mice did not occur with CoPP treatment \( (P < 0.001, \text{one-way ANOVA versus control mice and CFA-injected mice treated with CoPP}) \) (Fig. 7A), and the unchanged peripheral expression of CB2R in the dorsal root ganglia from CFA-injected mice remained unaltered with CoPP treatment (Fig. 7B).

**Discussion**

In this study, we demonstrated that treatment with an HO-1 inducer compound (CoPP) increased the local antinociceptive effects produced by a MOR, DOR, or CB2R agonist by enhancing the peripheral expression of MOR, leaving CB2R unaltered, and avoiding the decreased protein levels of DOR induced by peripheral inflammation.

The antinociceptive and anti-inflammatory effects produced by substances that can liberate carbon monoxide during inflammatory diseases have previously been shown (Guillén et al., 2008; Negrete et al., 2014). In accordance, our results further demonstrated that the administration of CoPP (an HO-1 inducer) also inhibited the mechanical and thermal hypersensitivity induced by chronic peripheral inflammation in mice. As expected, treatment with CoPP increased the expression of HO-1, indicating that carbon monoxide synthesized by HO-1 is principally responsible for the antinociceptive actions produced by CoPP during chronic inflammatory pain (Rosa et al., 2008).

Our study also revealed, for first time, that treatment with CoPP significantly enhanced the local antinociceptive effects produced by morphine, DPDPE, and JWH-015 after chronic peripheral inflammation. Moreover, the peripheral antiallodynic and antihyperalgesic effects produced by these drugs were
significantly decreased by the subplantar administration of the HO-1 inhibitor SnPP, indicating that HO-1 participates in the local antinociceptive effects produced by a MOR, DOR, or CB2R agonist during chronic inflammatory pain. These results expand our and others’ previous data revealing that the peripheral antinociceptive effects of morphine, DPDPE, or JWH-015 during peripheral inflammation are produced by the activation of the cGMP-PKG-KATP channels signaling pathway triggered by nitric oxide (Pacheco and Duarte, 2005; Leánez et al., 2009; Hervera et al., 2009; Cunha et al., 2010; Negrete et al., 2011), further demonstrating that the activation of this pathway triggered by carbon monoxide synthesized by HO-1 also participates in this action.

Curiously, under neuropathic pain conditions, CoPP treatment likewise improved the local antinociceptive effects of morphine but decreased those produced by DPDPE and JWH-015 (Hervera et al., 2013a). These results suggest that although MOR agonists use the same mechanism of action to produce peripheral antinociception during inflammatory and neuropathic pain—that is, the activation of the peripheral carbon monoxide/nitric oxide-cGMP-PKG-KATP channels signaling pathway—DOR and CB2R agonists did not activate the same way to produce peripheral antinociception in both types of pain. Therefore, because the activation of the carbon monoxide/nitric oxide-cGMP-PKG-KATP channels signaling pathway enhanced the local antinociceptive effects of DOR and CB2R agonists during inflammation, the activation of this pathway limits their effects under sciatic nerve injury conditions. These data revealed the different mechanisms implicated in the inhibitory effects produced by DOR and CB2R agonists under inflammatory or neuropathic pain conditions.

The specificity of the antiallodynic and antihyperalgesic effects produced by the local administration of morphine and DPDPE after chronic peripheral inflammation was demonstrated by the complete reversion of their effects with their coadministration with selective antagonists (CTAP and naltindole). These effects are produced by interaction with peripheral opioid receptors as demonstrated by the reversal of their effects with the coadministration with a nonselective peripherally-acting opioid receptor antagonist (NX-ME). Finally, the specificity of the local antinociceptive effects of JWH-015 after chronic peripheral inflammation was also proven by the complete reversion of its actions with their coadministration with a selective CB2R (AM630), but not with a CB1R (AM251), antagonist.

In accordance with other studies, our results demonstrated that the dorsal root ganglia expression of MOR increased, the expression of DOR decreased, and those of CB2R did not change after chronic peripheral inflammation (Puehler et al., 2004; Obara et al., 2009; Gavériaux-Ruff and Kieffer, 2011; Negrete et al., 2011). Moreover, the present study also revealed that although treatment with CoPP enhanced the peripheral expression of MOR, it avoided the decreased expression of DOR and did not modify the protein levels of CB2R in the dorsal root ganglia from animals with peripheral inflammation. These data indicate that although the enhanced peripheral expression of MOR induced by CoPP treatment...
might be responsible for the increased local antiallodynic and antihyperalgesic effects produced by morphine combined with CoPP during chronic inflammation, the underexpressed expression of DOR observed in CoPP-treated mice could also explain the enhanced antihyperalgesic effects produced by DPDPE in these animals.

Regarding CB2R, the fact that CoPP treatment enhanced their antinociceptive actions without altering their expression could be related to the fact that during chronic inflammatory pain the peripheral and antihyperalgesic effects produced by JWH-015 are completed by the activation of the peripheral nitric oxide-cGMP-PKG-KATP channels signaling pathway mediated by local endogenous opioids (Negrete et al., 2011). As a consequence, the increased protein levels of MOR and/or for the underexpressed expression of DOR induced by CoPP treatment could be involved in the enhanced effects produced by a CB2R agonist during inflammatory pain although nonchanges in the expression of their receptors could be demonstrated in CFA-injected mice treated with CoPP. Nonetheless, a reduction of the overall inflammation produced by CoPP (Megías et al., 2009; Fan et al., 2011) could be also implicated in the enhanced local antinociceptive effects produced by morphine, DPDPE, and JWH-015 in CoPP-treated mice.

In summary, this study suggests, for first time, that treatment with an HO-1 inducer enhances the local antinociceptive effects of morphine, DPDPE, and JWH-015 through regulating the peripheral opioid receptor expression. We propose the coadministration of CoPP with MOR, DOR, or CB2R agonists as a promising strategy to improve their antinociceptive effects during chronic inflammatory pain.

Authorship Contributions

Participated in research design: Pol.

Conducted experiments: Carcolé, Castany, Leánez.

Performed data analysis: Carcolé, Pol.

Wrote or contributed to the writing of the manuscript: Pol.

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