Neuroscience Letters 614 (2016) 49–54

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research paper

The antinociceptive effects of a δ-opioid receptor agonist in mice with painful diabetic neuropathy: Involvement of heme oxygenase 1

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HIGHLIGHTS

- STZ-induced type 1 diabetic mice develop allodynia and hyperalgesia.
- DPDPE inhibited painful diabetic neuropathy.
- CORM-2 and CoPP treatments enhance the antinociceptive effects of DOR agonists.
- Heme oxygenase 1 participates in the analgesic effects of DPDPE during diabetes.

ARTICLE INFO

Article history:
Received 26 November 2015
Received in revised form
20 December 2015
Accepted 29 December 2015
Available online 4 January 2016

Keywords:
Analgesia
Diabetes
Pain
Opioid receptors
Heme oxygenases
Carbon monoxide

ABSTRACT

Diabetic neuropathy is poorly controlled by classical analgesics and the research of new therapeutic alternatives is indispensable. Our aim is to investigate if treatment with a carbon monoxide-releasing molecule (tricarbonyldichlororuthenium(II) dimer; CORM-2) or an inducible heme oxygenase (HO-1) inducer (cobalt protoporphyrin IX; CoPP) could enhance the antinociceptive effects produced by a δ-opioid receptor (DOR) agonist in mice with painful diabetic neuropathy.

In diabetic mice induced by streptozotocin (STZ) injection, the antiallodynic and antihyperalgesic effects produced by the subcutaneous administration of a DOR agonist ([D-Pen(2),D-Pen(5)]-Enkephalin; DPDPE) and the reversion of its effects with the administration of an HO-1 inhibitor (tin protoporphyrin IX; SnPP) were evaluated. Moreover, the antinociceptive effects produced by the intraperitoneal administration of 10 mg/kg of CORM-2 or CoPP, alone or combined, with a subanalgesic dose of DPDPE were also assessed.

Our results demonstrated that the subcutaneous administration of DPDPE inhibited the mechanical and thermal allodynia as well as the thermal hyperalgesia induced by diabetes in a dose-dependent manner. Moreover, while the antinociceptive effects produced by a low dose of DPDPE were enhanced by CORM-2 or CoPP co-treatments, the inhibitory effects produced by a high dose of DPDPE were completely reversed by the administration of an HO-1 inhibitor, SnPP, indicating the involvement of HO-1 in the antinociceptive effects produced by this DOR agonist during diabetic neuropathic pain in mice. In conclusion, this study shows that the administration of CORM-2 or CoPP combined with a DOR agonist could be an interesting strategy for the treatment of painful diabetic neuropathy.

1. Introduction

Painful neuropathy is one of the most common complications of diabetes mellitus, occurring in nearly 40% of people with type 1 diabetes, and remains an important clinical problem due to its resistance to classical analgesic drugs, such us morphine [1,2]. This loss in morphine antinociceptive efficacy was described in diabetic animals following the systemic, spinal and supraspinal administration of this μ-opioid receptor (MOR) agonist, accompanied by numerous undesirable effects that severely limit its effectiveness [3–6]. There-

Abbreviations: CoPP, cobalt protoporphyrin IX; CORM-2, tricarbonyldichlororuthenium(II) dimer; DOR, δ-opioid receptor; DPDPE, [D-Pen(2),D-Pen(5)]-Enkephalin; HO-1, inducible heme oxygenase; MOR, μ-opioid receptor; SnPP, tin protoporphyrin IX; STZ, streptozotocin.

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http://dx.doi.org/10.1016/j.neulet.2015.12.059
0304-3940/

fore, the investigation of new alternatives for alleviating painful diabetic neuropathy is indispensable.

In the few studies concerning the role played by δ-opioid receptors (DOR) on the development of type 1 diabetes in animals, everyone agrees that there are no changes in the expression of DOR and/or it could even increase [7,8]. These studies also revealed that the intracerebroventricular or intrathecal administration of DOR agonists as well as the continuous dorsal root ganglia activation of DOR inhibited the nociceptive responses induced by diabetes in rodents [7–10]. Nonetheless the role played by the systemic administration of DOR agonists in these animals has not been assessed. Therefore, our objective is to evaluate the antinociceptive effects produced by the subcutaneous administration of a specific DOR agonist ([D-Pen(2),D-Pen(5)]-Enkephalin; DPDPE) in diabetic mice.

Previous studies demonstrated that treatment with a carbon monoxide-releasing molecule (tricarbonyldichlororuthenium(II) dimer; CORM-2) or the heme oxygenase 1 (HO-1)-inducer compound, cobalt protoporphyrin IX (CoPP), enhanced the analgesic effects of morphine during acute and chronic pain [11–13] as well as those produced by DOR agonists in inflammatory pain [14]. But the effect produced by these treatments on the antinociceptive actions of DPDPE during painful diabetic neuropathy remains unknown. Therefore, in a model of streptozotocin (STZ)-induced diabetic neuropathy, we evaluated: (1) the antiallodynic and antihyperalgesic effects produced by the subcutaneous administration of different doses of DPDPE; (2) the antinociceptive effects of DPDPE in CORM-2 or CoPP diabetic treated mice and (3) the reversion of DPDPE antinociceptive effects with the HO-1 inhibitor, tin protoporphyrin IX (SnPP).

2. Material and methods

2.1. Animals

Experiments were performed in six to eight weeks old male C57BL/6 mice acquired from Harlan Laboratories (Barcelona, Spain). Mice weighing 22–25 g were housed under 12-h/12-h 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 6 days of acclimatization to housing conditions. All experiments were conducted between 9:00 AM and 5:00 PM and carried out according to the animals guidelines of the European Communities Council (86/609/ECC, 90/679/ECC; 98/81/CEE, 2003/65/EC, and Commission Recommendation 2007/526/EC) and approved by the local Ethical Committee of our Institution (Comissió d'Etica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Induction of diabetic neuropathy

Diabetes was induced by the intraperitoneal administration of five consecutive daily injections of 55 mg/kg of STZ (Sigma–Aldrich, St. Louis, MO) freshly prepared in citrate buffer (0.1 M, pH 4.5) [15]. Control animals receive an equal volume of citrate buffer alone (naïve). Animals were fasted prior to first administration of STZ and were allowed to feed again after injection. At 21 days after the injection of STZ, the tail vein blood glucose levels were measured to confirm hyperglycemia by using a glucometer (OneTouch® UltraMini®). The development of mechanical allodynia, thermal hyperalgesia and thermal allodynia was evaluated by using the von Frey filaments, plantar and cold plate tests, respectively. All animals were tested in each paradigm before and at 21 days after STZ injection.

2.3. 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) 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 reported by Chaplan et al. [16]. The filament of 3.0 g was used as a cut-off and 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. Both hind paws were tested. Animals were allowed to habituate for 1 h before testing to allow an appropriate behavioral immobility.

Thermal hyperalgesia was assessed as reported by Hargreaves et al. [17]. Paw withdrawal latency in response to radiant heat was measured using the 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 paw and activated with a light beam intensity. A cut-off time of 12 s was used to prevent tissue damage. The mean paw withdrawal latencies from both hind paws were determined from the average of three separate trials, taken at 5 min intervals to prevent thermal sensitization and behavioral disturbances. Animals were habituated to the environment for 1 h before the experiment to become quiet and to allow testing.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile), previously described by Bennett and Xie [18]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (4 ± 0.5 °C) for 5 min.

2.4. Experimental procedure

At first, we assessed the painful diabetic neuropathy induced by STZ. After baseline measurements established in the following sequence: von Frey filaments, plantar and cold plate tests, diabetes was induced and animals were again tested in each paradigm at day 21 after STZ injection ($n = 8$ animals per group). Basal glucose levels from the tail blood were measured. Mice treated with an equal volume of citrate buffer (naïve) were used as controls ($n = 8$ animals per group). All the following experiments were performed at 21 days after STZ injection when diabetic neuropathy was confirmed.

In a second set of experiments, we evaluated the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects of the subcutaneous administration of different doses of DPDPE (0.5, 1, 3 and 5 mg/kg) or vehicle in STZ-injected animals ($n = 6$ animals per dose).

In a third set of experiments, the antiallodynic and antihyperalgesic effects produced by the intraperitoneal administration of 10 mg/kg of CORM-2 or CoPP alone or combined with the subcutaneous administration of 0.5 mg/kg of DPDPE in STZ-injected mice were evaluated ($n = 6$ animals per group). The antinociceptive effects produced by the intraperitoneal administration of 10 mg/kg of SnPP alone or combined with the subcutaneous administration of 5 mg/kg of DPDPE in STZ-injected animals ($n = 6$ animals per group) we also assessed.

The doses of CORM-2, CoPP and SnPP were selected in accordance to previous pilot studies performed in this model and those used in other works [11,12]. The doses of DPDPE were chosen from the dose-response curves performed in this study, as the ones that produced a minimal or maximal antinociceptive effect in STZ-injected mice.

Table 1
Mechanical response (von Frey filaments strength, g), thermal heat response (withdrawal latency, s) and thermal cold response (paw lifts, number) on the hind paws of mice treated with citrate buffer (naïve) or STZ.

Treatment	Mechanical response Von Frey filaments strength (g)	Thermal heat response withdrawal latency (s)	Thermal cold response paw lifts (number)
Naïve	2.8 ± 0.1	9.5 ± 0.3	0.3 ± 0.2
STZ	1.2 ± 0.1*	6.5 ± 0.4*	2.7 ± 0.2*

Results are shown as mean values ± SEM; $n = 8$ animals per experimental group.
* $P < 0.001$ denotes significant differences between naïve and STZ-injected animals (unpaired Student t test) for each test.

2.5. Drugs

Streptozotocin, CORM-2 and DPDPE were purchased from Sigma. CoPP and SnPP were acquired from Frontier scientific (Livchem GmbH & Co., Frankfurt, Germany). CORM-2, CoPP and SnPP were dissolved in DMSO (1% solution in saline) and intraperitoneally administered at 3–4 h and 30 min before testing. DPDPE was dissolved in saline solution (0.9% NaCl) and administered subcutaneously 30 min before behavioral testing. All drugs were freshly prepared before use and administered in a final volume of 10 ml/kg. For each group treated with a drug, the respective control group received the same volume of vehicle.

2.6. Statistical analysis

Data are expressed as mean ± SEM. The statistical analysis was performed by using the SPSS (version 17 for Windows, IBM España, Madrid, Spain). All comparisons were run as two-tailed testing.

The comparison of the glucose levels, the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by STZ vs. their respective citrate buffer treated mice (naïve) was evaluated by an unpaired Student t test.

For each test, the comparison of the effects produced by the subcutaneous administration of different doses of DPDPE or vehicle was evaluated by using a one way ANOVA followed by the Student Newman Keuls test. For each behavioral test, the comparison of the effects produced by the administration of CORM-2, CoPP or SnPP on the antinociceptive effects of DPDPE was also assessed by using a one way ANOVA followed by the Student Newman Keuls test. In these experiments, antinociception in von Frey filaments and plantar tests is expressed as the percentage of maximal possible effect, where the test latencies pre (baseline) and postdrug administration are compared and calculated according to the following equation:

$$\text{Maximal possible effect (\%)} = \left[\frac{(\text{drug} - \text{baseline})}{(\text{cut-off} - \text{baseline})}\right] \times 100$$

In the cold plate test, the inhibitory effects were calculated according to the following equation:

$$\text{Inhibition (\%)} = \left[\frac{(\text{paw elevations number at baseline} - \text{paw elevations number after drug})}{\text{paw elevations number at baseline}}\right] \times 100$$

A value of $P < 0.05$ was considered as a significant.

3. Results

3.1. Induction of Neuropathic Pain

In accordance to other reports, the administration of STZ produced a significant increase in plasma glucose levels (21.8 ± 0.4 mmol/L in STZ injected animals vs. 8.1 ± 0.3 mmol/L in citrate buffer treated mice; $P < 0.002$; unpaired Student t test; $n = 8$ animals/group) and induced mechanical allodynia, thermal hyperalgesia and thermal allodynia (Table 1). Indeed, the administration of STZ led to a significant decrease of the threshold for evoking paw withdrawal to a mechanical stimulus, a decrease of paw withdrawal latency to thermal stimulus and an increase in the number of paw elevations to cold thermal stimulus in the hind paws of these animals compared with naïve mice ($P < 0.001$; Student t test; $n = 8$ animals/group).

3.2. Effects of the subcutaneous administration of DPDPE on the allodynia and hyperalgesia induced by STZ

The subcutaneous administration of DPDPE (0.5–5 mg/kg) dose-dependently inhibited the mechanical allodynia (Fig. 1 A), thermal hyperalgesia (Fig. 1B) and thermal allodynia (Fig. 1C) induced by STZ injection. Indeed, the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by 1, 3 and 5 mg/kg of DPDPE were significantly higher than those produced by their corresponding vehicle treated animals ($P < 0.001$, one way ANOVA followed by the Student Newman Keuls test). The administration of vehicle (0.9% NaCl) did not produce any significant antinociceptive effect.

3.3. Effects of CORM-2 and CoPP treatments on the antiallodynic and antihyperalgesic responses to DPDPE in STZ-injected mice

The effects of the intraperitoneal administration of 10 mg/kg of CORM-2, CoPP or vehicle (DMSO 1%) on the inhibition of the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by the subcutaneous administration of a subanalgesic dose of DPDPE (0.5 mg/kg) or vehicle in STZ-injected mice were investigated. The intraperitoneal administration of CORM-2 or CoPP significantly attenuated the mechanical allodynia (Fig. 2A), thermal hyperalgesia (Fig. 2B) and thermal allodynia (Fig. 2C) induced by STZ injection as compared to control group treated with vehicle ($P < 0.001$; one way ANOVA followed by the Student Newman Keuls test). Our results also revealed that treatments with CORM-2 or CoPP combined with the subcutaneous administration of a low dose of DPDPE significantly enhanced the mechanical antiallodynic (Fig. 2A), thermal antihyperalgesic (Fig. 2B) and thermal antiallodynic effects (Fig. 2C) produced by this drug as compared to their respective control group treated with vehicle, DPDPE, CORM-2 or CoPP plus vehicle ($P < 0.001$, one way ANOVA followed by the Student Newman Keuls test).

3.4. Reversal of the antinociceptive responses produced by DPDPE in STZ injected mice with the administration of the HO-1 inhibitor, SnPP

The effects of the intraperitoneal administration of 10 mg/kg of SnPP or vehicle (DMSO 1%) on the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by the subcutaneous administration of a high dose of DPDPE (5 mg/kg) in STZ-injected mice were also assessed. Our results showed that the coadministration of DPDPE with SnPP completely reversed the mechanical antiallodynic (Fig. 3A), thermal antihyperalgesic (Fig. 3B) and thermal antiallodynic (Fig. 3C) effects produced by

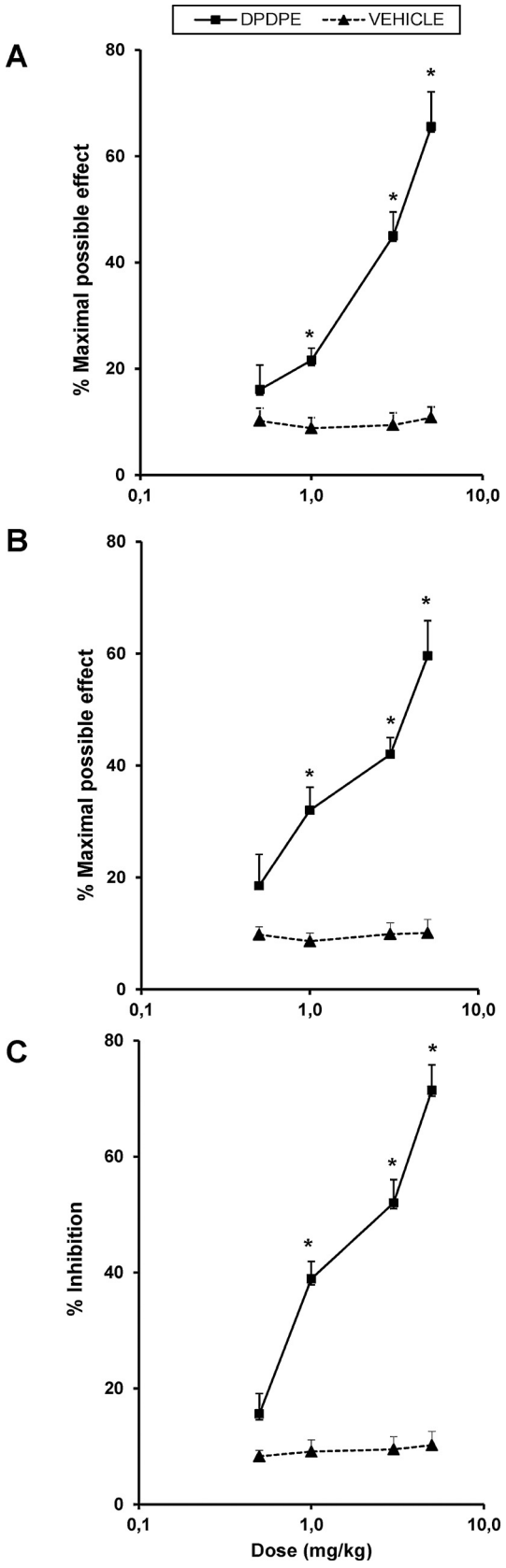

Fig. 1. Effects of the subcutaneous administration of [D-Pen(2), D-Pen(5)]-Enkephalin (DPDPE) on the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by STZ. Mechanical antiallodynic (A), thermal antihyperalgesic (B) and thermal antiallodynic effects (C) of the subcutaneous administration of different doses (logarithmic axis) of DPDPE (continuous lines) or their respective vehicle (discontinuous lines) in STZ injected mice. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia or inhibition (%) for thermal allodynia ± SEM (six animals for dose). For each test, * denotes significant differences versus their respective vehicle treated mice ($P < 0.05$; one way ANOVA followed by the Student Newman Keuls test).

this drug administered alone ($P < 0.001$, one way ANOVA followed by the Student Newman Keuls). Furthermore, the intraperitoneal administration of 10 mg/kg of SnPP plus vehicle did not produce any mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effect as compared with control-vehicle treated mice.

4. Discussion

In this study, we demonstrated that the subcutaneous administration of a specific DOR agonist inhibited the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by diabetes in mice. Moreover, the intraperitoneal administration of a carbon monoxide releasing molecule (CORM-2) or an HO-1 inducer compound (CoPP) both enhanced the antiallodynic and antihyperalgesic effects produced by DPDPE in diabetic mice. In addition, the antinociceptive effects produced by this DOR agonist were completely blocked by SnPP, an HO-1 inhibitor.

As mentioned before, painful diabetic neuropathy is difficult to treat due to its resistance to classical opioid analgesic drugs, such as morphine [19–21], subsequently high doses of this drug are required to inhibit neuropathic pain resulting in the induction of several side effects such as sedation, respiratory depression, constipation and tolerance, among others [6]. In contrast to MOR agonists, the activation of DOR has a reduced respiratory depression and minimal potential for the development of physical dependence and tolerance [6,22]. However and despite several authors have demonstrated the effectiveness of DOR agonists on the inhibition of inflammatory and neuropathic pain induced by sciatic nerve injury [11,14,23], only few studies have investigated the possible antinociceptive effects produced by these agonists in animals with painful diabetic neuropathy. In this study, we demonstrated that the subcutaneous administration of a DOR agonist (DPDPE) inhibited the allodynia and hyperalgesia induced by hyperglycemia in a dose dependent manner. These results are in accordance to previously obtained by Chattopadhyay et al. [10], demonstrating that the continuous dorsal root ganglia activation of DOR reduced the behavioral manifestations of painful diabetic neuropathy in rats as well as to those obtained by other authors showing that the spinal or intracerebroventricular application of DPDPE also inhibited the response to a mechanical or thermal stimuli in diabetic animals in a concentration-dependent manner [7,9]. Our findings supported these data and taking account the peptide structure of DPDPE with limited access to the central nervous system [24], we further demonstrated the peripheral inhibitory effects produced by this agonist in diabetic mice, although a central effect cannot be discarded. Nevertheless, our data confirmed the potential use of DOR agonists as an alternative for the treatment of painful diabetic neuropathy.

In this study, we also demonstrated that the intraperitoneal administration of CORM-2 and CoPP reduced the mechanical and thermal nociceptive responses induced by diabetes, as previously shown in other acute, inflammatory and nerve-injury induced neuropathic pain animal models [11,13,25]. Given that during neuropathic pain the systemic administration of CORM-2 and CoPP enhanced the spinal cord and dorsal root ganglia expression of HO-1 [11,26], we expected that both the peripheral and spinal cord induction of HO-1 might be responsible for the antinociceptive effects produced by these compounds in diabetic mice. Moreover, both treatments also enhanced the antinociceptive effects pro-

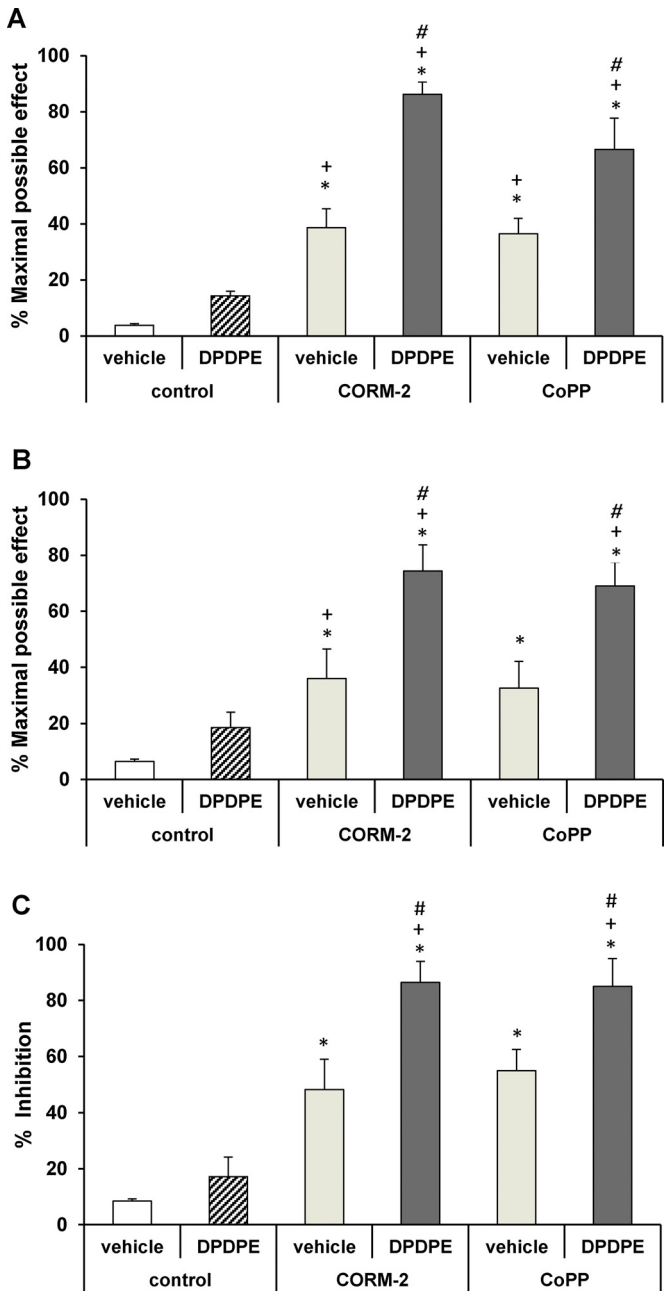

Fig. 2. Effects of tricarbonyldichloro ruthenium (II) dimer (CORM-2) and cobalt protoporphyrin IX (CoPP) on the antiallodynic and antihyperalgesic responses to D-Pen(2),D-Pen(5)]-Enkephalin (DPDPE). Mechanical antiallodynic (A), thermal antihyperalgesic (B), and thermal antiallodynic (C) effects of the subcutaneous administration of 0.5 mg/kg of DPDPE or vehicle in STZ injected mice pretreated with 10 mg/kg of CORM-2 or CoPP. The effects of the intraperitoneal administration of CORM-2 or CoPP alone are also shown. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia ± SEM (six animals per group). For each test, * denotes significant differences versus control group treated with vehicle ($P < 0.05$, one way ANOVA followed by Student Newman Keuls test), + denotes significant differences versus control group treated with DPDPE ($P < 0.05$, one way ANOVA followed by the Student Newman Keuls test) and # denotes significant differences versus group treated with CORM-2 or CoPP plus vehicle ($P < 0.05$; one way ANOVA followed by the Student Newman Keuls test).

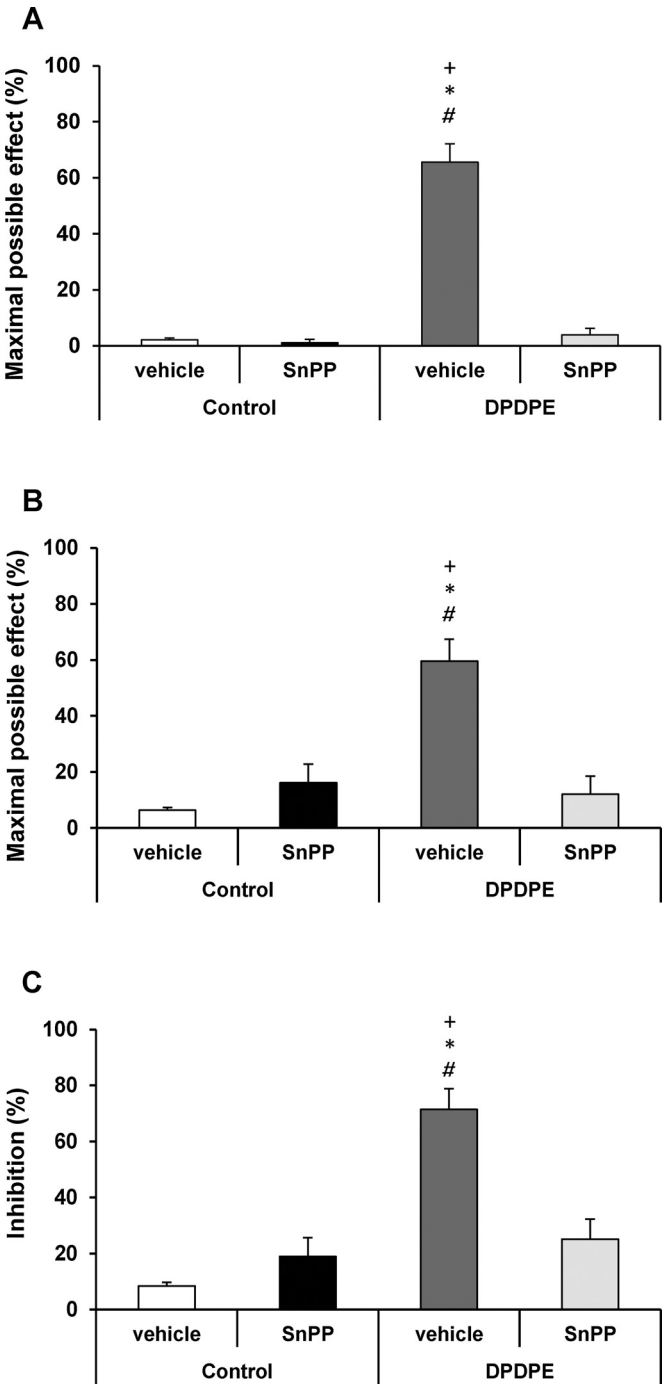

Fig. 3. Effects of tin protoporphyrin IX (SnPP) treatment on the antiallodynic and antihyperalgesic responses to [D-Pen(2),D-Pen(5)]-Enkephalin (DPDPE) in diabetic mice. Mechanical antiallodynic (A), thermal antihyperalgesic, (B) and thermal antiallodynic (C) effects of the subcutaneous administration of DPDPE (5 mg/kg) combined with SnPP (10 mg/kg) in STZ injected mice are shown. The effects of the subcutaneous administration of DPDPE, SnPP or vehicle alone are also represented. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia ± SEM (six animals per group). For each test, * denotes significant differences vs. control group treated with vehicle ($P < 0.05$, one way ANOVA followed by Student Newman Keuls test), + denotes significant differences vs. control group treated with SnPP ($P < 0.05$, one way ANOVA followed by the Student Newman Keuls test), and # denotes significant differences vs. group treated with DPDPE plus SnPP ($P < 0.05$, one way ANOVA followed by the Student Newman Keuls test).

duced by a subanalgesic dose of DPDPE in diabetic mice. These results are in accordance to other previous data obtained in animals with inflammatory pain wherein the induction of HO-1 also improved the peripheral antinociceptive actions of DOR agonists as well as those produced by other drugs [14,27,28] and supported

the essential role played by nitric oxide, other gaseous neurotransmitter, in mediating the inhibitory effects of DOR agonists in diabetic animals [7]. Considering that both CORM-2 and CoPP similarly enhanced the inhibitory effects of DPDPE, we hypothesized that carbon monoxide synthetized by HO-1 might be the principal responsible for the enhanced antinociceptive effects of this DOR agonist in diabetic mice by the activation of cGMP-PKG-ATP-sensitive K+ channels signaling pathway which culminates in the hyperpolarization of nociceptive neurons inducing analgesia [29]. The fact that the inhibitory effects of DPDPE in diabetic animals were completely reversed by the administration of an HO-1 inhibitor (SnPP) further supported this hypothesis. Nevertheless, an increased expression and/or a sensitization of peripheral DOR activated by HO-1 in CORM-2 and CoPP diabetic treated mice cannot be excluded. This possibility is under investigation in our laboratory.

In summary, we found that the DOR agonist DPDPE inhibited the nociceptive responses induced by hyperglycemia in a mice model of diabetic neuropathic pain. We also demonstrated that co-treatment with a carbon monoxide releasing molecule or an HO-1 inducer compound, both improved the inhibitory effects of DPDPE in diabetic mice. Furthermore, our data suggest that HO-1 plays an important role in the systemic antinociceptive actions of DOR agonists under neuropathic pain conditions. Finally, this study provides the first evidence that the co-administration of CORM-2 or CoPP with a DOR agonist (DPDPE) may provide an interesting alternative for treating diabetic neuropathic pain.

Acknowledgements

This work was supported by Ministerio de Economía y Competitividad, Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea [Grants: PS0900968 and PI1400927].

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