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ORIGINAL INVESTIGATION

The role of carbon monoxide on the anti-nociceptive effects and expression of cannabinoid 2 receptors during painful diabetic neuropathy in mice

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Abstract
Rationale The activation of cannabinoid 2 receptors (CB2R) attenuates chronic pain, but the role played by carbon monoxide synthesized by the inducible heme oxygenase 1 (HO-1) on the anti-nociceptive effects produced by a selective CB2R agonist, JWH-015, during painful diabetic neuropathy remains unknown.
Objectives and methods In streptozotocin (STZ)-induced diabetic mice, the anti-allodynic and anti-hyperalgesic effects of the subcutaneous administration of JWH-015 alone or combined with the intraperitoneal administration of a carbon monoxide-releasing molecule (tricarbonyldichlororuthenium(II) dimer (CORM-2)) or an HO-1 inducer compound (cobalt protoporphyrin IX (CoPP)) at 10 mg/kg were evaluated. Reversion of JWH-015 anti-nociceptive effects by the administration of an HO-1 inhibitor (tin protoporphyrin IX (SnPP)) and a CB2R antagonist (AM630) was also evaluated. Furthermore, the protein levels of HO-1, neuronal nitric oxide synthase (NOS1), and CB2R in diabetic mice treated with CORM-2 and CoPP alone or combined with JWH-015 were also assessed.
Results The administration of JWH-015 dose dependently inhibited hypersensitivity induced by diabetes. The effects of JWH-015 were enhanced by their coadministration with CORM-2 or CoPP and reversed by SnPP or AM630. The increased protein levels of HO-1 induced by CORM-2 and CoPP treatments were further enhanced in JWH-015-treated mice. All treatments similarly enhanced the peripheral expression of CB2R and avoided the spinal cord over-expression of NOS1 induced by diabetes.
Conclusions The activation of HO-1 enhanced the anti-nociceptive effects of JWH-015 in diabetic mice, suggesting that coadministration of JWH-015 with CORM-2 or CoPP might be an interesting approach for the treatment of painful diabetic neuropathy in mice.

Keywords Analgesia · Cannabinoid receptors · Carbon monoxide · Diabetes · Heme oxygenases · Painful diabetic neuropathy · Nitric oxide synthases

Introduction

It is well known that one of the most common complications of diabetes mellitus is the development of painful neuropathy, occurring in nearly 40 % of people with type 1 diabetes, which remains an important clinical problem (Dyck et al. 1993; Bril et al. 2011). Therefore, the investigation of new alternatives for alleviating painful neuropathy is indispensable.

The activation of cannabinoid system is known to play an important role in the modulation of acute and chronic pain. That is, the administration of both cannabinoid receptor 1 (CB1R) and 2 (CB2R) agonists reduced nociception in numerous animal pain models (Hervera et al. 2010; Hama and Sagen 2011; Negrete et al. 2011; Ikeda et al. 2013). However, while the analgesic potential derived from the stimulation of CB1R is accompanied with several central site effects, the administration of selective CB2R agonists inhibited nociception with little adverse effects (Rahn and Hohmann

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2009; Hsieh et al. 2011; Hervera et al. 2012a), suggesting that the administration of selective CB2R agonists might be an interesting target to explore for the treatment of painful diabetic neuropathy. Nevertheless, in the few existing studies related to the evaluation of the anti-nociceptive effects produced by cannabinoids in painful diabetic neuropathy, most of them evaluated the effects produced by the administration of mixed CB1R/CB2R agonists, such us WIN 55,212-2 (Ulugol et al. 2004; Doğrul et al. 2004; Vera et al. 2012; Toth et al. 2010; Ikeda et al. 2013). There are only few works examining the anti-nociceptive effects produced by selective CB2R agonists, such as MT178 and AM1241, in diabetic mice (Vincenzi et al. 2013; Bujalska-Zadrożny et al. 2015). In this study, we evaluated the anti-allodynic and anti-hyperalgesic effects produced by the subcutaneous administration of the selective CB2R agonist, JWH-015, in STZ-induced diabetic mice.

Carbon monoxide is a gaseous neurotransmitter synthesized by inducible (HO-1) and constitutive (HO-2) heme oxygenases. The enhanced expression of HO-1 produces potent anti-inflammatory and anti-nociceptive effects (Fan et al. 2011; Hervera et al. 2012b; Negrete et al. 2014; Carcolé et al. 2014). Indeed, the administration of HO-1-inducing compounds, such as cobalt protoporphyrin IX (CoPP) or carbon monoxide- releasing molecules, a new class of chemical agents able to reproduce several biological effects of HO-1-derived carbon monoxide, inhibits acute and chronic inflammatory or neuropathic pain induced by sciatic nerve injury (Hervera et al. 2012b; Negrete et al. 2014; Gou et al. 2014). Nevertheless, the exact contribution of carbon monoxide synthesized by HO-1 in the modulation of main symptoms of painful diabetic neuropathy remains unknown.

Several studies have reported a cross talk between carbon monoxide synthesized by HO-1 and nitric oxide synthesized by the neuronal nitric oxide synthase (NOS1) under chronic pain conditions (Hervera et al. 2012b; Negrete et al. 2014), but the possible interaction between both enzymes HO-1 and NOS1 in the spinal cord from animals with painful diabetic neuropathy has not been evaluated.

In previous studies, we have demonstrated that treatment with carbon monoxide-releasing molecule (tricarbonyldichlororuthenium(II) dimer (CORM-2)) or the HO-1 inducer, CoPP, enhanced the analgesic effects of morphine during acute and chronic pain (Hervera et al. 2013a, b; Gou et al. 2014), as well as those produced by CBR2 agonists in inflammatory pain (Carcolé et al. 2014), but the effects produced by these treatments on the anti-nociceptive actions of a CB2R agonist during painful diabetic neuropathy were poorly studied.

Therefore, in a mice model of streptozotocin (STZ)-induced diabetic neuropathy, we evaluated (1) the anti-nociceptive effects of a CB2R agonist ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015)) administered alone or combined with CORM-2 or CoPP; (2) the reversion of anti-nociceptive effects of JWH-015 with the HO-1 inhibitor, tin protoporphyrin IX (SnPP), and the CB2R antagonist, AM630; and (3) the effects of CORM-2 and CoPP treatments alone or combined with JWH-015 on the protein levels of HO-1, NOS1, and CB2R in diabetic mice.

Material and methods

Animals

Experiments were performed in 6–8-week-old male C57BL/6J mice acquired from Harlan Laboratories (Barcelona, Spain). Mice weighing 21–25 g were housed under 12/12-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 animal 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.

Drugs

STZ and CORM-2 were purchased from Sigma-Aldrich (St. Louis, MO). CoPP and SnPP were acquired from Frontier Scientific (Livchem GmbH & Co., Frankfurt, Germany). JWH-015 and AM630 were purchased from Tocris (Ellisville, MI). CORM-2, CoPP, and SnPP were dissolved in DMSO (1 % solution in saline) and intraperitoneally administered at 3–4 h (CORM-2 and CoPP) and 30 min (SnPP) before testing. JWH-015 and AM630 were dissolved in DMSO (50 % solution in saline) and administered subcutaneously 30 min before behavioral testing. The time of administration of all tested drugs was selected according to preliminary pilot studies and previous work (Hervera et al. 2013a). 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 their respective vehicle.

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). 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.

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. (1994). The filament of 3.0 g was used as a cutoff, 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. (1988). 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 cutoff 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 (1988). 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.

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 anti-allodynic and thermal anti-hyperalgesic effects of the subcutaneous administration of different doses of a selective CB2R agonist (JWH-015; 0.05–1 mg/kg) or vehicle in STZ-injected animals ($n = 6$ animals per dose).

In a third set of experiments, the anti-allodynic and anti-hyperalgesic effects produced by the intraperitoneal administration of 10 mg/kg of CORM-2 or CoPP alone or combined with the subcutaneous administration of 0.05 mg/kg of JWH-015 in STZ-injected mice were evaluated ($n = 6$ animals per group). The anti-nociceptive effects produced by the intraperitoneal administration of 10 mg/kg of SnPP and 1 mg/kg of AM630 alone or combined with the subcutaneous administration of 1 mg/kg of JWH-015 in STZ-injected animals ($n = 6$ animals per group) were also assessed.

The doses of CORM-2, CoPP, SnPP, and AM630 were selected in accordance to previous pilot studies and other works (Hervera et al. 2013a, b). The doses of JWH-015 were chosen from the dose–response curves performed in this study, as the ones that produced a minimal or maximal anti-nociceptive effect in STZ-injected mice.

In another set of experiments, we evaluated the effects of CORM-2 and CoPP treatments alone or combined with JWH-015 on the protein levels of HO-1 and NOS1 in the spinal cord as well as on the protein levels of CB2R in the spinal cord and dorsal root ganglia from diabetic mice by using Western blot assay. In these experiments, naïve mice treated with citrate buffer (vehicle) have been used as controls ($n = 4$–5 samples per group).

Western blot analysis

Naïve and STZ-induced diabetes mice treated with vehicle, CORM-2, or CoPP alone or combined with JWH-015 were killed by cervical dislocation, and tissues from the lumbar section of spinal cord and dorsal root ganglia (L3 to L5) were removed after sacrifice, frozen in liquid nitrogen, and stored at −80 °C until assay. Samples from the spinal cord and dorsal root ganglia of two animals were pooled into one experimental

sample to obtain enough protein levels for performing the Western blot analysis. The HO-1, NOS1, and CB2R protein levels were analyzed by Western blot. Tissues were homogenized in ice-cold lysis buffer (50 mM Tris·Base, 150 nM NaCl, 1 % NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1 % sodium dodecyl sulfate, 1 mM Na_3VO_4, 25 mM NaF, 0.5 % protease inhibitor cocktail, and 1 % phosphatase inhibitor cocktail). All reagents were purchased at Sigma (St. Louis, MO) with the exception of NP-40 from Calbiochem (Darmstadt, Germany). The crude homogenate was solubilized for 1 h at 4 °C, sonicated for 10 s, and centrifugated at 4 °C for 15 min at 700 g. The supernatant (60 μg of total protein) was mixed with 4× laemmli loading buffer and then loaded onto 4 % stacking/10 % separating sodium dodecyl sulfate polyacrylamide gels.

The proteins were electrophoretically transferred onto PVDF membrane for 120 min; blocked with PBST + 5 % nonfat dry milk; and subsequently incubated overnight at 4 °C with polyclonal rabbit anti-HO-1 (1:300, Stressgen, Ann Arbor, MI), anti-NOS1 (1:100, BD Transduction Laboratories, San Diego, CA), or anti-CB2R (1:500, Abcam, Cambridge, UK) antibodies. The proteins were detected by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized with chemiluminescence reagents (ECL Kit; GE Healthcare) and by exposure onto hyperfilm (GE Healthcare). The intensity of blots was quantified by densitometry. The membranes were stripped and reproved with a monoclonal rabbit anti-β-actin antibody (1:10.000, Sigma) used as a loading control.

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 versus their respective citrate buffer-treated mice (naïve) was evaluated by an unpaired Student's t test.

For each test, the comparison of the effects produced by the subcutaneous administration of different doses of JWH-015 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, SnPP, or AM630 on the anti-nociceptive effects of JWH-015 was also evaluated by using a one-way ANOVA followed by the Student-Newman-Keuls test. In these experiments, anti-nociception in von Frey filaments and plantar tests is expressed as the percentage of maximal possible effect, where the test latencies predrug (baseline) and postdrug administration are compared and calculated according to the following equation:

$$\text{Maximal possible effect}(\%) = [(\text{drug}-\text{baseline})/(\text{cutoff}-\text{baseline})] \times 100$$

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

$$\text{Inhibition}(\%) = \left[\left(\begin{array}{l}\text{paw elevations number at baseline}-\\ \text{paw elevations number after drug}\end{array}\right)\Big/\text{paw elevations number at baseline}\right] \times 100$$

Changes on the protein levels of HO-1, NOS1, and CB2R from naïve and STZ-injected mice treated with vehicle, CORM-2, or CoPP alone or combined with JWH-015 were analyzed by using a two-way ANOVA followed by the corresponding one-way ANOVA followed by the Student-Newman-Keuls test or Student's t test whenever applicable. A value of $P < 0.05$ was considered as a significant.

Results

Induction of neuropathic pain

In accordance to other reports, the administration of STZ produced a significant increase in plasma glucose levels (22.9 ± 0.3 mmol/L in STZ-injected animals versus 8.4 ± 0.2 mmol/L in citrate buffer-treated mice; $P < 0.001$; unpaired Student's 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

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	2.7 ± 0.1	9.4 ± 0.4	0.5 ± 0.3
STZ	1.1 ± 0.1*	6.8 ± 0.4*	2.8 ± 0.2*

Results are shown as mean values ± SEM; $n = 8$ animals per experimental group

*For each test, $P < 0.001$ denotes significant differences between naïve and STZ-injected animals (unpaired Student's t test)

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 those of vehicle-treated mice ($P < 0.001$; Student's t test; $n = 8$ animals/group).

Effects of the subcutaneous administration of JWH-015 on the allodynia and hyperalgesia induced by STZ

The subcutaneous administration of JWH-015 (0.05–1 mg/kg) dose dependently inhibited the mechanical allodynia (Fig. 1a), thermal hyperalgesia (Fig. 1b), and thermal allodynia (Fig. 1c) induced by STZ injection. Indeed, the mechanical anti-allodynic, thermal anti-hyperalgesic, and thermal anti-allodynic effects produced by 0.15, 0.5, and 1 mg/kg of JWH-015 in STZ-injected mice were significantly higher than those produced by their corresponding vehicle (DMSO 50 %)-treated animals ($P < 0.001$, one-way ANOVA followed by the Student-Newman-Keuls test). In addition, the effects produced by the administration of higher doses (0.5, or 1 mg/kg) of JWH-015 were significantly higher than those produced by lower doses (0.05 and 0.15 mg/kg) of this CB2R agonist.

Effects of CORM-2 and CoPP treatments on the anti-allodynic and anti-hyperalgesic responses to JWH-015 in STZ-injected mice

The effects of the intraperitoneal administration of 10 mg/kg of CORM-2, CoPP, or vehicle (DMSO 1 %) on the mechanical anti-allodynic, thermal anti-hyperalgesic, and thermal anti-allodynic effects produced by the subcutaneous administration of a subanalgesic dose of JWH-015 (0.05 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 with the 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 JWH-015 significantly enhanced the mechanical anti-allodynic (Fig. 2a), thermal anti-hyperalgesic (Fig. 2b), and thermal anti-allodynic effects (Fig. 2c) produced by this drug ($P < 0.001$; one-way ANOVA followed by the Student-

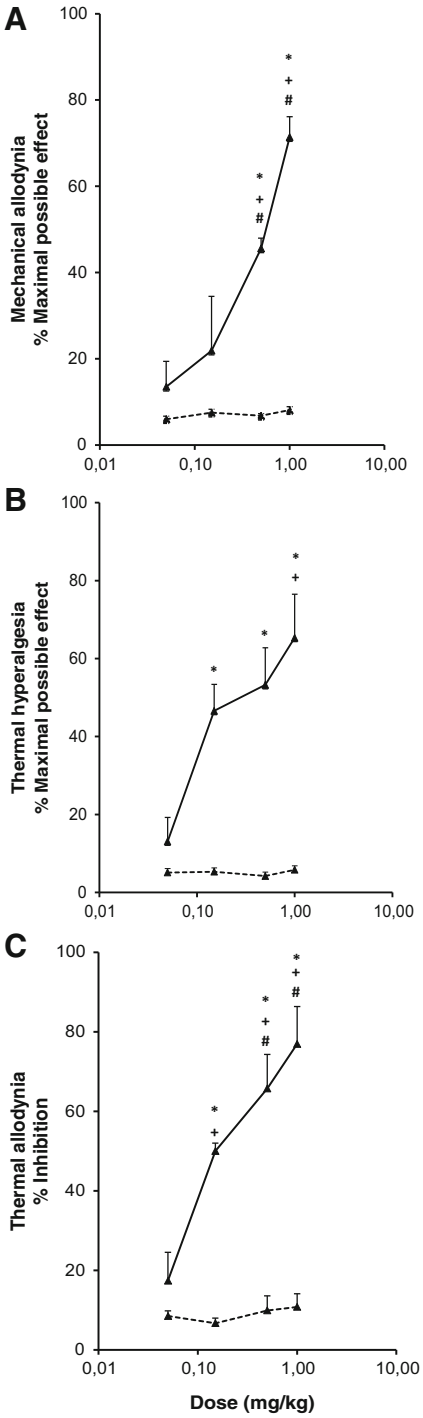

Fig. 1 Effects of the subcutaneous administration of JWH-015 on the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by the administration of STZ. Mechanical anti-allodynic (**a**), thermal anti-hyperalgesic (**b**), and thermal anti-allodynic (**c**) effects produced by the subcutaneous administration of different doses (logarithmic axis) of JWH-015 (*continuous lines*) or 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, *asterisk* denotes significant differences versus vehicle-treated mice, *plus sign* denotes significant differences versus the effects produced by a dose of 0.05 mg/kg of JWH-015, and *number sign* denotes significant differences versus the effects produced by a dose of 0.15 mg/kg of JWH-015 ($P < 0.05$; one-way ANOVA followed by the Student-Newman-Keuls test)

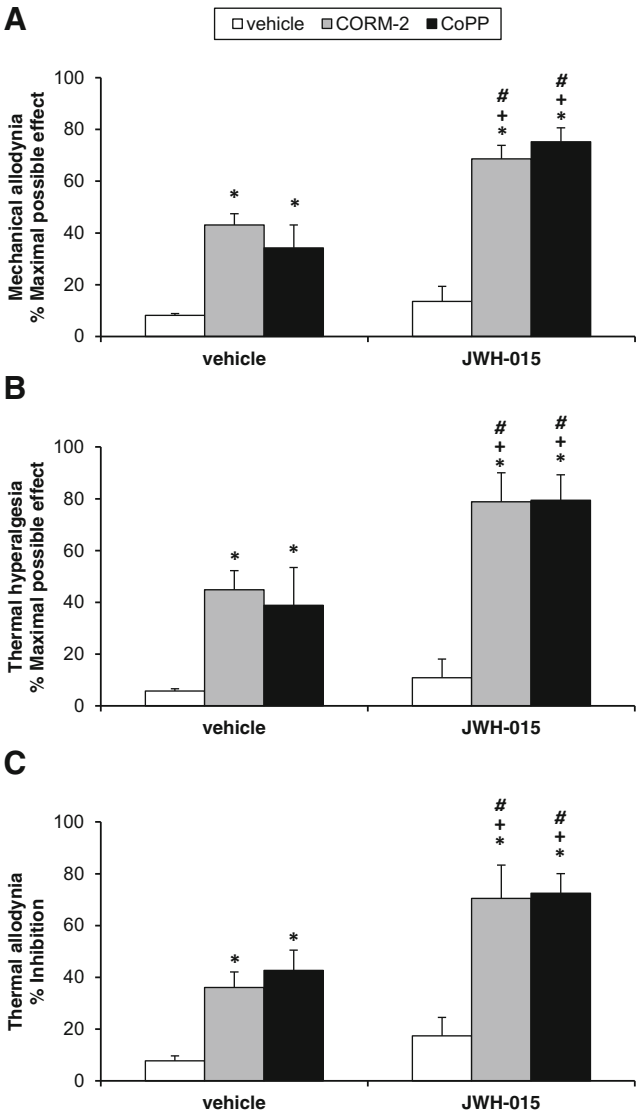

Fig. 2 Effects of CORM-2 and CoPP treatments on the anti-allodynic and anti-hyperalgesic responses to JWH-015 in diabetic mice. The mechanical anti-allodynic (**a**), thermal anti-hyperalgesic (**b**), and thermal anti-allodynic (**c**) effects produced by the subcutaneous administration of 0.05 mg/kg of JWH-015 or vehicle in STZ-injected mice pretreated with 10 mg/kg of CORM-2, CoPP, or vehicle are shown. The effects of the intraperitoneal administration of CORM-2 or CoPP 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 behavioral test, *asterisk* denotes significant differences versus control group treated with vehicle plus vehicle ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test), *plus sign* denotes significant differences versus group treated with JWH-015 plus vehicle ($P < 0.05$, one-way ANOVA followed by the Student-Newman-Keuls test), and *number sign* 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)

Newman-Keuls test) as compared versus their respective control group treated with vehicle, JWH-015, or CORM-2 or CoPP plus vehicle.

Reversal of the anti-nociceptive responses produced by JWH-015 in STZ-injected mice with the administration of the HO-1 inhibitor, SnPP, and the CB2R antagonist, AM630

The effects of the intraperitoneal administration of 10 mg/kg of SnPP, 1 mg/kg of AM630, or vehicle (DMSO 1 %) on the mechanical anti-allodynic, thermal anti-hyperalgesic, and thermal anti-allodynic effects produced by the subcutaneous administration of a high dose of JWH-015 (1 mg/kg) in STZ-injected mice were also assessed. Our results showed that the coadministration of JWH-015 with SnPP or AM630 completely reversed the mechanical anti-allodynic (Fig. 3a), thermal anti-hyperalgesic (Fig. 3b), and thermal anti-allodynic (Fig. 3c) effects produced by JWH-015 administered alone ($P < 0.001$; one-way ANOVA followed by the Student-Newman-Keuls test). Furthermore, the intraperitoneal administration of 10 mg/kg of SnPP or 1 mg/kg of AM630 plus vehicle did not produce any mechanical anti-allodynic, thermal anti-hyperalgesic, and thermal anti-allodynic effect as compared with vehicle-treated mice.

Effects of CORM-2 and CoPP treatments alone or combined with JWH-015 on the protein levels of HO-1, NOS1, and CB2R in the spinal cord and/or dorsal root ganglia from diabetic mice

The protein levels of HO-1 and NOS1 in the spinal cord from STZ-injected mice treated with vehicle, CORM-2, or CoPP alone or combined with JWH-015 as well as from naïve mice treated with vehicle are shown in Fig. 4. The two-way ANOVA showed a significant effect of JWH-015 ($P < 0.018$) as well as of CORM-2 and CoPP treatments ($P < 0.001$) without interaction between them on the expression of HO-1 in the spinal cord from diabetic mice. Indeed, while treatment with CORM-2 and CoPP, administered alone or combined with JWH-015, significantly enhanced the protein levels of HO-1 ($P < 0.005$; one-way ANOVA versus their corresponding naïve and STZ-treated mice; Fig. 4a), the expression of HO-1 in CORM-2- or CoPP plus JWH-015-treated mice was higher than those observed in CORM-2- or CoPP plus vehicle-treated animals ($P < 0.025$; Student's t test).

Regarding NOS1, the two-way ANOVA only revealed a significant effect of CORM-2 and CoPP treatments ($P < 0.001$). Therefore, the enhanced protein levels of NOS1 (Fig. 4b) induced by diabetes ($P < 0.012$; one-way ANOVA versus naïve mice) were similarly reduced by the intraperitoneal administration of CORM-2 and CoPP plus vehicle or combined with JWH-015 ($P < 0.001$; one-way ANOVA versus their respective STZ-treated mice).

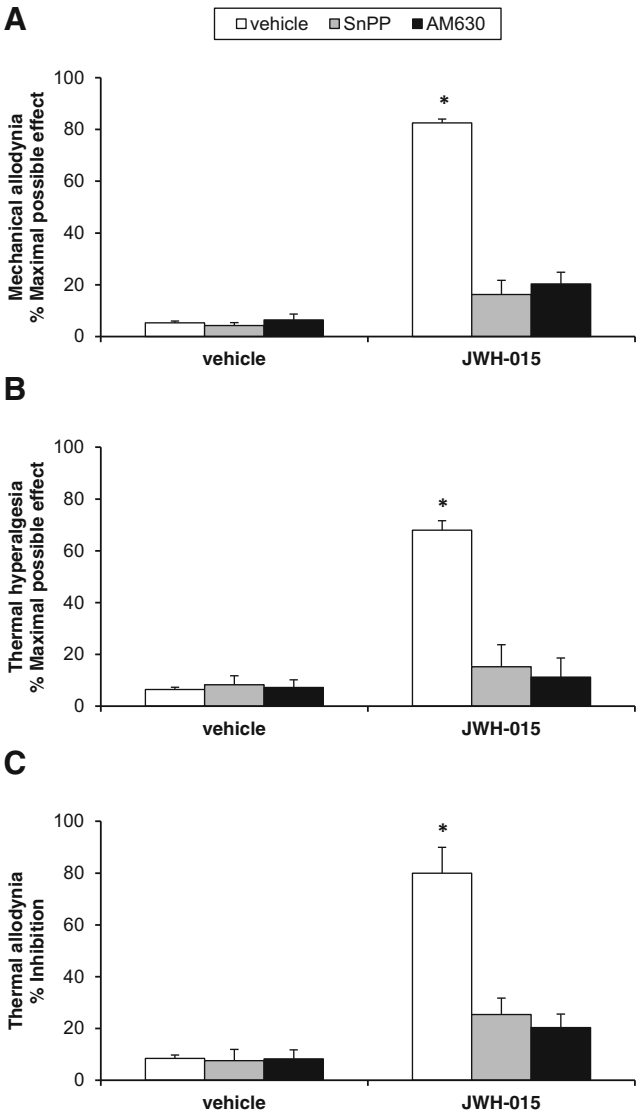

Fig. 3 Effects of SnPP and AM630 treatments on the anti-allodynic and anti-hyperalgesic responses to JWH-015 in diabetic mice. The mechanical anti-allodynic (**a**), thermal anti-hyperalgesic (**b**), and thermal anti-allodynic (**c**) effects produced by the subcutaneous administration of JWH-015 (1 mg/kg) combined with SnPP (10 mg/kg) or AM630 (1 mg/kg) in STZ-injected mice are shown. The effects of the subcutaneous administration of JWH-015, SnPP, AM630, 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 behavioral test, *asterisk* denotes significant differences versus all other groups ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test)

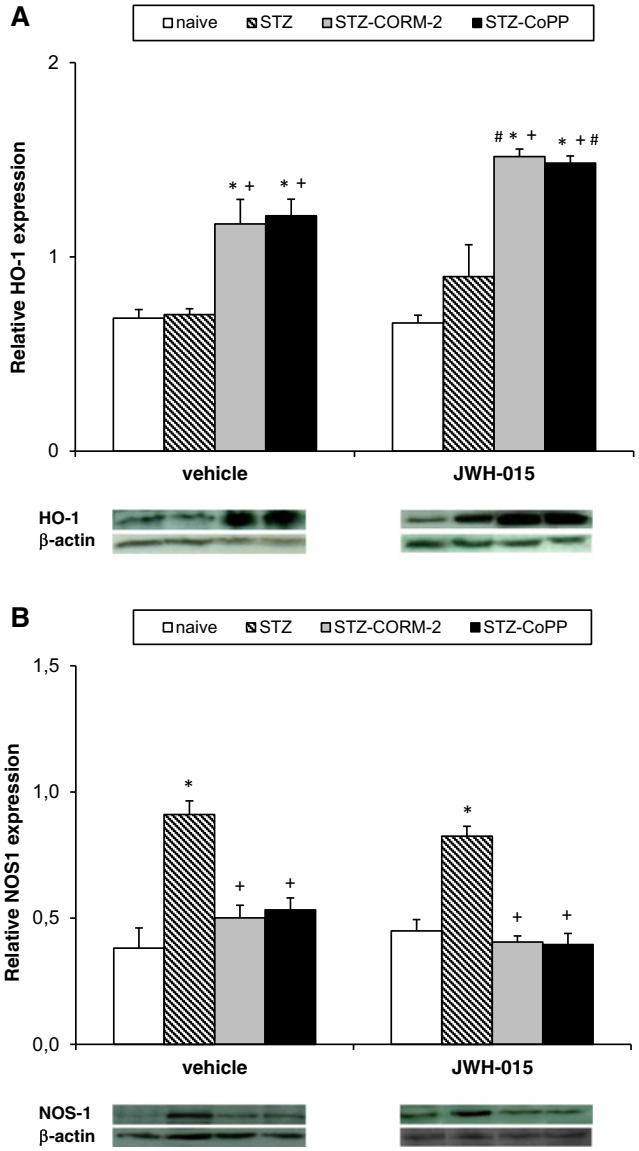

Fig. 4 Effects of CORM-2 and CoPP treatments alone or combined with JWH-015 on the HO-1 and NOS1 protein levels in the spinal cord from STZ-injected mice. The protein levels of HO-1 (**a**) and NOS1 (**b**) in the lumbar section of the spinal cord from STZ-injected mice treated with vehicle, CORM-2, or CoPP alone or combined with JWH-015 are represented. The expressions of these proteins from naïve mice have been also represented as controls (naïve). For each enzyme, *asterisk* indicates significant differences when compared versus naïve mice ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test), *plus sign* indicates significant differences when compared versus STZ-injected mice treated with their respective vehicles ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test), and *number sign* indicates significant differences when compared versus CORM-2- or CoPP-treated mice plus vehicle ($P < 0.02$, Student's t test). Representative blots normalized to β-actin content are shown. Data are expressed as mean values ± SEM; $n = 4$–5 samples per group

The protein levels of CBR2 in the spinal cord and dorsal root ganglia from STZ-injected mice treated with vehicle, CORM-2, or CoPP, administered alone or combined with JWH-015, as well as from naïve mice are also shown in Fig. 5. In both tissues, the two-way ANOVA only revealed a significant effect of CORM-2 and CoPP treatments ($P < 0.001$). Indeed, while the spinal cord levels of CB2R were significantly enhanced in STZ-injected mice treated with vehicle as well as with CORM-2 or CoPP plus vehicle or JWH-015 ($P < 0.015$; one-way ANOVA versus their respective

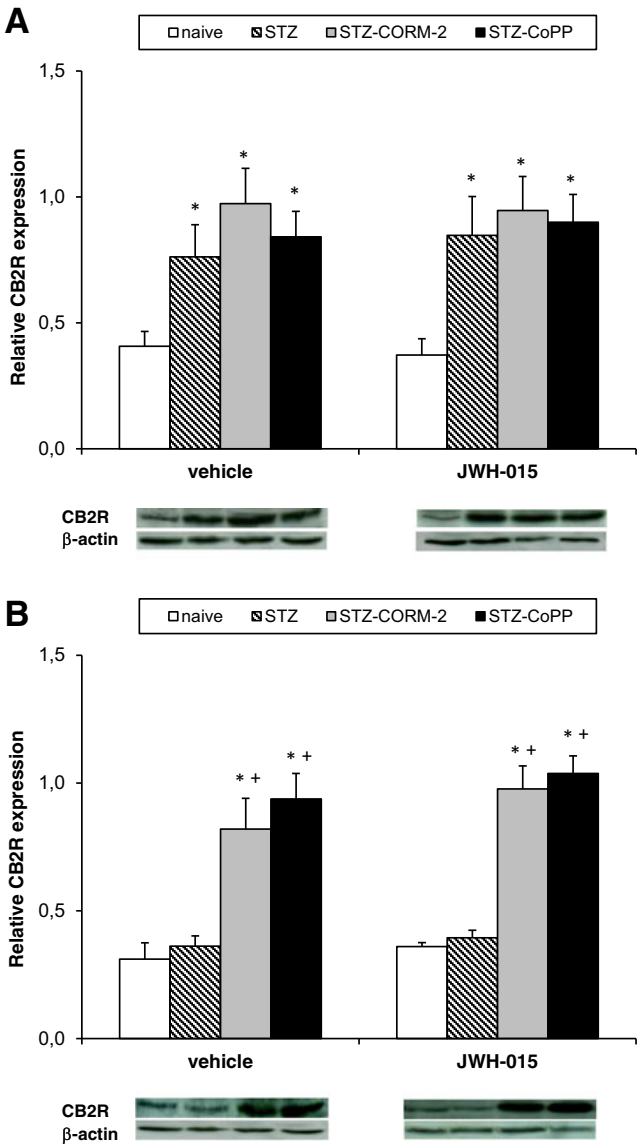

Fig. 5 Effect of CORM-2 and CoPP treatments alone or combined with JWH-015 on the protein levels of CB2R in the spinal cord and dorsal root ganglia from STZ-injected mice. The protein levels of CB2R in the spinal cord (**a**) and dorsal root ganglia (**b**) from naïve and STZ-injected mice treated with vehicle, CORM-2, or CoPP, alone or combined with JWH-015, are represented. The expression of this receptor in the spinal cord and dorsal rood ganglia from naïve mice has been also represented as controls (naïve). For each tissue, *asterisk* indicates significant differences when compared versus naïve mice ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test) and *plus sign* indicates significant differences when compared versus STZ-injected mice treated with their respective vehicles ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Representative blots normalized to β-actin content are shown. Data are expressed as mean values ± SEM; $n = 4–5$ samples per group

naïve animals; Fig. 5a), the dorsal root ganglia protein levels of CB2R were only increased in STZ-injected mice treated with CORM-2 or CoPP plus vehicle or JWH-015 ($P < 0.02$; one-way ANOVA versus their respective naïve and STZ-injected mice treated with vehicle; Fig. 5b).

Discussion

We demonstrated, for the first time, that the intraperitoneal administration of CORM-2 or CoPP enhanced the anti-allodynic and anti-hyperalgesic effects produced by JWH-015 in diabetic mice.

Moreover, the administration of CORM-2 or CoPP alone and combined with JWH-015 increased the expressions of HO-1 and CB2R and avoided the NOS1 over-expression induced by diabetes. Nevertheless, a further increase on the spinal cord over-expression of HO-1 induced by CORM-2 or CoPP treatments was observed in JWH-015-treated mice.

There is currently no effective treatment for forwarding painful diabetic neuropathy induced by diabetes. Furthermore, it is well known that drugs used in the clinical practice for their treatment (tricyclic anti-depressants, anti-epileptic drugs, and agonists of μ opioid receptors) have many undesirable effects which severely limit their effectiveness. Concerning μ opioid receptor agonists, their limited anti-nociceptive effects under painful diabetic neuropathy have been amply demonstrated (Chen and Pan 2003; Mousa et al. 2013). In contrast to opioids, cannabinoids represent an interesting alternative pharmacological option in the management of painful diabetic neuropathy. Indeed, several authors have shown the potent anti-nociceptive effects produced by the administration of selective CB1R and mixed CB1R/CB2R agonists in diabetic animals (Ulugol et al. 2004; Vera et al. 2012; Bujalska-Zadrożny et al. 2015). However, the fact that CB1R are widely distributed in the central nervous system cause that its activation triggered many physiological functions such as depression, anxiety, and addiction (Rahn and Hohmann 2009). In contrast, CB2R are mainly located in the spinal cord and dorsal root ganglia, and its stimulation by selective CB2R agonists have lesser effects on the psychological functions being an interesting alternative for the treatment of painful diabetic neuropathy (Wotherspoon et al. 2005). In accordance, several authors have reported the analgesic effects produced by the spinal or intraperitoneal administration of diverse selective CB2R agonists, such as L-759, 656, MT178, and AM 1241, in diabetic animals (Ikeda et al. 2013; Vincenzi et al. 2013; Bujalska-Zadrożny et al. 2015), but the effects produced by the subcutaneous administration of JWH-015, another selective CB2R agonist, have not been evaluated. Our results demonstrated that the subcutaneous administration of JWH-015 also inhibited the mechanical and thermal allodynia as well as the thermal hyperalgesia caused by STZ-induced diabetes in mice in a dose-dependent manner. The specificity of the anti-allodynic and anti-hyperalgesic effects produced by the systemic administration of this agonist was demonstrated by the complete reversal of its effects with the administration of a selective CB2R antagonist (AM630). These data supported the use of CB2R agonists as an

interesting alternative for the treatment of painful diabetic neuropathy.

In the present study, we further demonstrated that the administration of a carbon monoxide-releasing molecule (CORM-2) or an HO-1 inducer compound (CoPP) besides inhibiting painful diabetic neuropathy also enhanced the anti-allodynic and anti-hyperalgesic effects produced by JWH-015 in diabetic mice. The inhibitory effects of CORM-2 and CoPP treatments in diabetic mice further supported and amplified the knowledge of the anti-nociceptive effects produced by these compounds in several acute and chronic inflammatory pain as well as in nerve injury-induced neuropathic pain (Negrete et al. 2011; Hervera et al. 2012b; Gou et al. 2014). In accordance to other pain models, the increased expression of HO-1 induced by CORM-2 and CoPP treatments in diabetic mice revealed that the up-regulation of this enzyme may modulate the anti-nociceptive effects produced by both compounds during painful diabetic neuropathy.

Several studies have also shown the differential role played by nitric oxide synthesized by selective nitric oxide synthases on the modulation of diabetic neuropathy. That is, while the expression of the endothelial nitric oxide synthase decreased in the spinal cord of diabetic mice (Ohsawa et al. 2011), an increased spinal cord expression of neuronal and inducible nitric oxide synthases was demonstrated in these animals (Zochodne et al. 2000). As a consequence, the prevention of NOS1 gene deficiency mice to develop sensory neuropathy confirmed the participation of this enzyme in the induction of nociception induced by hyperglycemia, as clearly demonstrated by Vareniuk et al. (2009). In accordance to these data, our results demonstrated an increased protein level of NOS1 in the spinal cord of STZ-induced diabetic mice. Furthermore, this NOS1 over-expression was abolished in CORM-2 and CoPP diabetic treated mice revealing that nitric oxide, synthesized by NOS1, might be involved in the inhibitory effects produced by a carbon monoxide-releasing molecule or an HO-1 inducer compound under diabetic pain conditions. Our results also indicated that an interaction between nitric oxide and carbon monoxide systems takes place during painful diabetic neuropathy.

More interestingly is the improved anti-nociceptive actions of JWH-015 observed in CORM-2- or CoPP-treated mice, where the subanalgesic effects produced by a low dose of a CB2R agonist were significantly increased after their combination with a carbon monoxide-releasing or an HO-1 inducer compound. Moreover, the fact that the anti-allodynic and anti-hyperalgesic effects produced by a high dose of JWH-015 were completely blocked by the administration of an HO-1 inhibitor supported the hypothesis that HO-1 system may modulate CB2R mechanism in painful diabetic neuropathy processing. In agreement to these data, other in vitro and in vivo studies also demonstrated that the anti-inflammatory and anti-nociceptive effects produced by CB2R agonists during alcoholic liver diseases and peripheral inflammation are mediated through an HO-1-dependent mechanism (Louvet et al. 2011; Carcolé et al. 2014).

In order to evaluate the possible mechanisms implicated in the enhanced effects produced by JWH-015 in CORM-2 and CoPP diabetic treated mice, the effects of both treatments combined with JWH-015 on the protein levels of HO-1, NOS1, and CB2R were assessed. Our results showed that while the administration of JWH-015 alone did not alter the expression of HO-1, a significant increase in its expression was observed in CORM-2 or CoPP plus JWH-015-treated mice. Therefore and although several works have shown that treatment with a CB2R agonist administered alone enhanced the expression of HO-1 in the liver from alcoholic (Louvet et al. 2011) or hypertensive animals (Steib et al. 2013), these authors evaluated the effects produced by the continuous administration of a small dose or those produced by the acute administration of a high dose of a CB2R agonist. In this study, we evaluated the effects produced by the single administration of a small dose of JWH-015 in CORM-2- or CoPP-treated mice, which might explain the possible differences between our and these other results. Nonetheless, a significant increase in the expression of HO-1 was observed in CORM-2- or CoPP plus JWH-015-treated mice, indicating that HO-1 may modulate the enhanced anti-nociceptive effects produced by these drug combinations. Regarding NOS1, the administration of JWH-015 did not alter the inhibitory effects produced by CORM-2 or CoPP treatments in their expression. Finally and accordance to other works, our results showed that while the protein levels of CB2R were significantly increased in the spinal cord of diabetic animals (Ikeda et al. 2013), no changes in their expression were observed in the dorsal root ganglia of these animals. Moreover, while CORM-2 or CoPP administered alone or combined with JWH-015 did not alter the spinal cord expression of CB2R, all of these treatments similarly enhanced the protein levels of CB2R in the dorsal root ganglia of diabetic mice, which may explain the improvement on the anti-nociceptive effects of JWH-015 in diabetic mice. Nevertheless, the further increased expression of HO-1 triggered by CORM-2 or CoPP in JWH-015-treated mice and the reversion of their anti-nociceptive effects by SnPP indicated that the HO-1 system modulates the inhibition of painful diabetic neuropathy induced by JWH-015 in mice. Although further experiments are needed to identify the possible mechanisms implicated in this process, we can postulate that the up-regulated expression of HO-1 played a critical role in the enhanced anti-nociceptive effects produced by JWH-015 in CORM-2 or CoPP diabetic treated animals.

In conclusion, this study suggests that CORM-2 or CoPP treatments combined with a CB2R agonist could be an

interesting strategy for the treatment of painful diabetic neuropathy in mice.

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The experiments performed in this study comply with the current laws of Spain.

Compliance with ethical standards All experiments were conducted between 9:00 AM and 5:00 PM and carried out according to the animal 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.

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