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Adelmidrol, a palmitoylethanolamide analogue, as a new pharmacological treatment for the management of acute and chronic inflammation

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ABSTRACT

The aim of study was to examine the anti-inflammatory and analgesic effects of adelmidrol, an analogue of palmitoylethanolamide (PEA), in animal models of acute and chronic inflammation [carrageenan-induced paw edema (CAR) and collagen-induced arthritis (CIA)].

In order to elucidate whether the action of adelmidrol is related to activation of peroxisome proliferator-activated receptors (PPAR-α or PPAR-γ), we investigated the effects of PPAR-γ antagonist, GW9662 on adelmidrol mechanism. CAR induced paw edema, hyperalgesia and the activation of pro-inflammatory NF-κB pathway were markedly reduced by treatment with adelmidrol. GW9662, (administered prior to adelmidrol treatment), antagonized the effect of adelmidrol abolishing its positive action. On the contrary, the genetic absence of PPAR-α receptor did not modify the beneficial results of adelmidrol treatment in the acute model of inflammation.

In addition, for the first time, we demonstrated that adelmidrol was able to ameliorate both the clinical signs and the histopathology of the joint and the hind paw during chronic inflammation. In particular, the degree of oxidative damage and proinflammatory cytokines and chemokines production were significantly reduced in adelmidrol-treated mice. Moreover, in CIA model, the effect of GW9662 pre-treatment on adelmidrol mechanism was also confirmed. Thus, in this study, we report that adelmidrol reduces the development of acute and chronic inflammation and could represent a novel therapeutic approach for inflammation and pain.

1. Introduction

Inflammation and pain have been attributed in large part to elevated levels of prostaglandins, TNF-α and interleukins [1] and for this reason most anti-inflammatory agents possess analgesic activity [1]. CAR-induced paw edema is a common experimental model to study the acute phase of inflammation. The acute inflammation is characterized by increased vascular permeability, leukocyte infiltration, neutrophil-derived active oxygen species nitric oxide, cytokines and prostaglandins [2].

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation, chronic pain, destruction of joint cartilage and bone erosion. Type II collagen-induced arthritis (CIA) is a useful animal model of RA [3]. The pathogenesis of CIA is characterized by the host's response to type II collagen and production of auto-antibodies that recognize collagen [3]. Moreover, the activation of neutrophils, macrophages, lymphocytes into joint tissues and the formation of the pannus are important events in the pathogenesis of both CIA and human RA. Several laboratories have reported that mast cells (MCs) also have a role in the pathogenesis of inflammatory conditions and joint disorders [4].

In that regard, palmitoylethanolamide (PEA) and some of its analogues showed great efficacy in the treatment of pain and inflammation. Several of our works demonstrated the beneficial

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effects of PEA alone and in combination in different models of inflammation and pain [5,6] and in a mouse model of CIA [7]. The exact mechanism of action of PEA is not well known although PEA could interact with peroxisome proliferator-activated receptor (PPAR)-α, which are involved in its anti-inflammatory effects and with the orphan G-protein-coupled receptor, GPR55 [8]. Interestingly, a previous study of ours also indicated that PPAR-δ and PPAR-γ can contribute to the anti-inflammatory activity of PEA [9]. The genetic absence of the PPAR-α receptor significantly blocked the effect of PEA treatment in a model of SCI [9]. Pretreatment with GW9662 a potent PPAR-γ antagonist, or GSK0660 a PPAR-δ antagonist, counteracted the actions of PEA [9]. In the same time, mutant PPAR-α mice failed to respond to GW7647 (a synthetic PPAR-α agonist) or to PEA treatments in CAR induced paw inflammation model [10]. Moreover, the anti-inflammatory and analgesic effects of PEA have been reported and may be due to the ability to down-modulate MCs activation and MCs mediators release [8].

Adelmidrol, (International Nonproprietary Name INN) of the diamide derivative of azelaic acid is one of PEA analogues that belongs to ALIAmides family (Autacoid Local Injury Antagonist Amides) and the effects of adelmidrol may depend on the control of mast cell activation [8]. Several studies showed that topical treatment with adelmidrol increased MCs granular density, suggesting a decrease in their degranulation [11,12]. In addition, this compound showed some beneficial effects in a pilot study on mild atopic dermatitis [13].

Thus, the purpose of our study was to examine the anti-inflammatory effects of adelmidrol treatment during acute and chronic inflammation such as in a classical model of inflammation and hyperalgesia carrageenan-induced paw edema (CAR) [6] and in an animal model of autoimmune disease CIA. Thus, to better understand whether the mechanism of action of adelmidrol treatment could be related to PPAR-α/γ pathways as PEA analogue, we investigated the effect of PPAR-γ antagonist, GW9662 on the protective effects of adelmidrol and whether the genetic absence of PPAR-α receptor could modify its action during acute inflammation.

2. Materials and methods

2.1. Animals

Sprague–Dawley male rats (200–230 g, Envigo, Italy) and male mice (6–7 weeks old, 20–27 g) with a targeted disruption of the PPAR-α gene (PPAR-α KO) and littermate wildtype controls (PPAR-α WT) purchased from Jackson Laboratories (Envigo, Italy) and DBA/1 J male mice (9 weeks; Envigo, Italy), were used for studies.

Mice homozygous for the Pparat^{nij}Gonz targeted mutation mice are viable, fertile and appear normal in appearance and behavior. Exon eight, encoding the ligand-binding domain, was disrupted by the insertion of a 1.14 kb neomycin resistance gene in the opposite transcriptional direction. After electroporation of the targeting construct into J1 ES cells, the ES cells were injected into C57BL/6 N blastocysts. This stain was created on B6, 129S4 background and is maintained as a homozygote on a 129S4/SvJae background by brother sister mating.

Water and food were available *ad libitum*. This study was authorized by the University of Messina Review Board for the care of animals. Animal care was in conformity with the new legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU).

2.2. Experimental groups

The study was divided into three steps. First, we studied the analgesic and anti-inflammatory effects of adelmidrol in a model of acute inflammation such as CAR induced paw edema in rats.

Rats were randomly distributed to the following groups:

CAR+saline group*:* rats were subjected to carrageenan-induced paw edema ($n = 20$);
CAR+GW9662 (1 mg/kg)+adelmidrol (10 mg/kg) dissolved in saline: same as the CAR+saline group but GW9662 (1 mg/kg), a potent antagonist of PPAR-γ receptor, was administered intraperitoneally 30 min before CAR injection and adelmidrol was administered intraperitoneally (10 mg/kg) at the same time of CAR injection ($n = 20$);
CAR+adelmidrol (10 mg/kg) dissolved in saline: same as the CAR+saline group but adelmidrol was administered intraperitoneally (10 mg/kg i.p) at the same time of CAR injection ($n = 20$);

Then, to confirm whether the mechanism of action of adelmidrol is related to activation of PPAR-α receptors, we performed new experiments specifically in PPAR-α WT and KO mice.

PPAR-α WT and KO mice were randomly allocated to the following groups:

CAR+saline group: PPAR-α WT and KO mice were subjected to CAR induced paw edema ($n = 20$);
CAR+adelmidrol (10 mg/kg) dissolved in saline: PPAR-α WT and KO mice were subjected to CAR-induced paw edema and adelmidrol was administered intraperitoneally (10 mg/kg i.p) at the same time of CAR injection ($n = 20$);

The doses of adelmidrol were chosen based on a dose–response study carried out in our lab. The sham-operated group was subjected to the same surgical procedures as the CAR group, except that saline or drugs were administered instead of CAR ($n = 20$).

Finally, we tested the analgesic and anti-inflammatory effects of adelmidrol and the relation to PPAR-α and PPAR-γ pathways during chronic inflammation in a mouse model of autoimmune disease (CIA).

Mice were divided into five experimental groups:

CIA-control: mice were subjected to collagen-induced arthritis and administered intraperitoneally with vehicle (saline) every 24 h, starting from day 25 to day 35 ($n = 20$).
CIA+adelmidrol: mice subjected to CIA were administered intraperitoneally with adelmidrol 10 mg/kg dissolved in saline every 24 h, starting from day 25 to day 35 ($n = 20$).
CIA+GW9662+adelmidrol: mice subjected to CIA were administered with the PPAR-γ receptor antagonist GW9662 (1 mg/kg i.p.) 10 min before adelmidrol administration 10 mg/kg dissolved in saline every 24 h, starting from day 25 to day 35 ($n = 20$).
CIA+GW6471+adelmidrol: mice subjected to CIA were administered with the PPAR-α receptor antagonist GW6471 (1 mg/kg i.p.) 10 min before adelmidrol administration 10 mg/kg dissolved in saline every 24 h, starting from day 25 to day 35 ($n = 20$).
CFA-control: mice were injected at the base of the tail with 100 μl of CFA instead of the emulsion containing 100 μg of CII and were treated with saline, every 24 h from day 25 to day 35 ($n = 20$).
Sham-control: mice were injected at the base of the tail with 100 μl of 0.01 M acetic acid instead of the emulsion containing

100 μg of CII and were treated with saline (vehicle for adelmidrol), every 24 h from day 25 to day 35 (n = 20).

Sham-adelmidrol: Mice were injected at the base of the tail of 100 μl of 0.01 M acetic acid instead of the emulsion containing 100 μg of CII and were administered intraperitoneally with adelmidrol 10 mg/kg dissolved in saline every 24 h, starting from day 25 to day 35 (n = 20).

Although CFA is another component of the emulsion that could produce inflammation, no severe damage was observed in the group that received only CFA (data not shown) compared to CIA mice immunized with the emulsion of collagen with CFA containing Mycobacterium Tb. H37RA (CIA-control). For this reason, only data regarding CIA-control group were shown.

2.3. CAR-induced paw edema induction

Paw edema volume was measured as previously described in rats [2] and mice [10]. They received a subplantar injection of carrageenan (0.05 ml in mice and 0.1 ml in rats of a 1% suspension in 0.85% saline) (Sigma–Aldrich Milan, Italy) into the right hindpaw. The volume of paw edema was measured by a plethysmometer (Ugo Basile, Comerio, Varese, Italy) prior to car injection and every hour for 6 h. Edema was expressed as the increase in paw volume (mL) after carrageenan injection relative to the pre-injection value for each animal. Results are expressed as paw volume change (mL).

2.4. Behavioral analysis of CAR injected rats

Hyperalgesic responses to heat were determined by the Hargreaves' Method using a Basile Plantar Test [14] with a cut-off latency of 20 s to prevent tissue damage. Mechanical allodynia was also assessed using an electronic von Frey Anesthesimeter (IITC-Life Science Instruments, Woodland Hill, CA, USA) as described in a recent our study [15].

2.5. Histological analysis of CAR model

Paw biopsies were taken 6 h following intraplantar injection of CAR. Histological analysis in paw tissue was performed as previously indicated [6]. The degree of inflammation was evaluated according to [16] with a score from 0 to 5, defined as follows: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation and 5 = severe inflammation.

2.6. Measurement of cytokine levels in paw exudates

Tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β levels in the paw exudates were measured by ELISA, as described previously [2].

2.7. Western blots for IκB-α, COX-2, iNOS and NF-κB p65

Western blot analysis was performed as previously described [15]. The following primary antibodies were used: anti-IκB-α (Santa Cruz Biotechnology, 1:1000; D.B.A. s.r.l. Milan Italy), anti-NF-kB p65 (1:1000; Santa Cruz Biotechnology; D.B.A. s.r.l. Milan Italy), anti-COX-2 (1:500; Santa Cruz Biotechnology), anti-iNOS (1:500; Santa Cruz Biotechnology; D.B.A. s.r.l. Milan Italy) in 1 × PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 overnight at 4 °C. anti-β-actin or anti-lamin A/C antibody (1:1000; Santa Cruz Biotechnology; D.B.A. s.r.l. Milan Italy) was used as internal control. The relative expression of the protein bands of IkB-α (37 kDa), NF-κB p65 (65 kDa), iNOS (130 kDa), COX-2 (50 kDa) was detected with an enhanced chemiluminescence (ECL) system (Thermo, USA) and visualized with the Chemi Doc XRS (Bio-Rad, USA) and analyzed by using Image Lab 3.0 software (Bio-Rad, USA).

2.8. Induction of CIA

The induction of CIA was performed as described in our previous study [17]. Briefly, chicken type II collagen (CII) was suspended in 0.01 M acetic acid at a concentration of 2 mg/ml overnight at 4 °C and stored at −70 °C. Complete Freund adjuvant (CFA) was made with *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. CII was emulsified with an equal volume of CFA before injection. DBA/1 J mice were injected intradermally at the base of the tail with 100 μl of the emulsion (containing 100 μg of CII) on day 1. A second injection of CII in CFA was administered in mice on day 21.

2.9. Clinical assessment of CIA

The development of arthritis in all groups was evaluated every day starting from day 20 after the first injection by using a macroscopic scoring system as previously described [18]. Arthritic index for each animal was measured by adding the four scores of each paws. Clinical severity was determined by measuring the paw volume using a plethysmometry (model 7140; Ugo Basile).

2.10. Behavioral assays of CIA

Rotarod: Locomotor activities were assessed with a protocol previously used [19].

Thermal hyperalgesia: Hyperalgesic response to heat was determined by the Hargreaves Method using a plantar test (Ugo Basile, Comeria, Italy) [14] with a cut-off latency of 20 s used to prevent tissue injury. Data obtained were converted to percent maximal possible antinociceptive effect (%MPE) as follows: (response laten cy-baseline latency)/(cut-off latency-baseline latency) × 100.

Mechanical allodynia: Mechanical allodynia was also assessed using an electronic von Frey Anesthesimeter (IITC-Life Science Instruments, Woodland Hill, CA, USA). Animals were put in boxes of wire mesh, and an increasing force was applied to the hindpaw. The force able to cause lifting of the paw was considered as the withdrawal threshold. Allodynia was assessed before immunization (baseline level), prior to the onset of arthritis and up to 35 days after CIA induction [20].

2.11. Histologic examination of CIA

Histological analysis was performed as described on a previous our study [7].

2.12. Staining of mast cells

Identification of mast cells was performed in paw sections as described in previous studies [21].

2.13. Immunohistochemical localization of chymase, tryptase, and nitrotyrosine of CIA mice

Immunohistologic analysis was performed in paw sections as described in previous studies [7]. Sections were incubated overnight with (a) anti-chymase antibody (1:100 in PBS, vol/vol) (DBA, Milan, Italy), (b) anti-tryptase antibody (1:500 in PBS, vol/vol; D.B.A s.r.l, Milan, Italy), or (c) anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS, vol/vol; D.B.A s.r.l, Milan, Italy). Controls included buffer alone or nonspecific purified rabbit IgG. Sections were washed with PBS, incubated with secondary antibody. Specific labeling was detected with a biotin conjugated

goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vector) (D.B.A s.r.l, Milan, Italy). Immunocytochemistry photographs (n = 5) were assessed with densitometry by using computer-assisted color image analysis (Leica QWin V3, UK) and expressed as percent of total tissue area.

2.14. Radiography of CIA

Radiography was performed as previously described [7].

2.15. Measurement of cytokines in CIA mice

Tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β levels were measured in the plasma from CIA and sham mice, as previously described [22].

2.16. Measurement of chemokines in CIA mice

Levels of chemokines MIP-1α and MIP-2 were measured in the aqueous joint extracts as described previously [7].

2.17. Thiobarbituric acid-reactant substances measurement (MDA levels)

Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, was determined, as previously described [23].

2.18. Myeloperoxidase (MPO) assay

Neutrophil infiltration into the inflamed tissues was determinate by using an MPO assay, as previously described [24].

2.19. Materials

Adelmidrol was obtained by Epitech group S.P.A (Saccolongo Italy). GW6471 and GW9662 were purchased from Sigma–Aldrich (Milan, Italy). All chemicals were obtained from Sigma–Aldrich Company (Milan, Italy). All stock solutions were made in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK) or 10% ethanol (Sigma–Aldrich).

2.20. Data analysis

All values are expressed as mean ± standard deviation (SD) of the mean of n observations. For the *in vivo* studies, n represents the number of animals. For histology and immunohistochemistry analyses, the figures shown are representative of at least three experiments (histologic or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. Results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. For the arthritis studies, Mann–Whitney U test (two-tailed, independent) was used to compare medians of the arthritic indices [18]. A P value of less than 0.05 was considered significant.

3. Results

3.1. Role of PPAR receptors on the anti-inflammatory effects of adelmidrol in CAR induced paw inflammation and pain

In order to investigate the mechanism of action of adelmidrol, we performed a model of CAR induced paw edema in rats pretreated with a potent antagonist of PPAR-γ receptor (GW9662) and in PPAR-α WT and KO mice. In particular, to evaluate histologically the anti-inflammatory effects of administration of adelmidrol, paw tissues were examined by H&E staining. No histological damage was found in sham animals (Fig. 1A, A1 and see histological score E). In contrast, CAR injection led to a marked infiltration of inflammatory cells in rats (Fig. 1B, B1 and see histological score *E*) and in PPAR-α WT and KO mice (Fig. 2B, B1 and E, E1 and see histological score G) compared to control. Paw damage was more severe in PPAR-α KO than WT mice (E, E1 and see histological score G). Adelmidrol treatment was able to decrease the histological alteration in all experimental groups (Fig. 1D, D1 and see histological score E) including WT and KO mice (Fig. 2C, C1, F, F1 and see histological score E). However, CAR injected rats pretreated with GW9662 did not show a reduction of histological damage suggesting a possible antagonist effect of GW9662 pretreatment on adelmidrol action (Fig. 1C, C1 and see histological score E). Moreover, intraplantar injection of CAR caused a time dependent rise in paw volume that was highest after 5 h in rats (Fig. 1F) as well as in PPAR-α KO and WT mice (Fig. 2H). Paw edema was more evident in PPAR-α KO than WT mice (Fig 2H). Paw edema was significantly reduced by treatment with adelmidrol in all experimental groups including WT and KO mice (Figs. 1F and 2H). However, CAR injected rats pretreated with GW9662 did not show a reduction of paw edema (Fig. 1F).

In addition, injection of CAR also caused a time-dependent progress of thermal hyperalgesia that peaked at 3 h and was continued through 5 h (Fig. 3A). In the same time, an increased mechanical allodynia associated to central sensitization [15] was also detected in CAR injected rats (Fig. 3B). The administration of adelmidrol produced a clear and significant inhibition in the development of the CAR-induced thermal hyperalgesia and mechanical allodynia (Fig. 3A and B). In contrast, adelmidrol treatment did not reduce thermal hyperalgesia and allodynia in CAR injected rats pretreated with GW9662 (Fig. 3A and B).

3.2. The effects of adelmidrol on cytokines production and neutrophil infiltration in CAR injected rats

The development of histological alteration was correlated with neutrophil infiltration [25]. The adelmidrol administration produced a significant reduction in neutrophil infiltration (Fig. 1G). In that regard, a significant presence of mast cells (Fig. 3E, E1 and see H) were observed in the paw tissues from CAR injected rats. In contrast, less mast cell infiltration (Fig. 3G, G1 and see H) was found in the paw tissues from CAR injected rats treated by intraperitoneal administration of adelmidrol. Accordingly, a significant reduction of pro-inflammatory cytokines (TNF-α and IL-1β) was also observed in rats treated with adelmidrol (Fig. 3C and D). Pretreatment with GW9662 was able to inhibit adelmidrol action.

3.3. Effect of adelmidrol on expression of IκB-α and nuclear translocation of NF-κB p65

To better investigate the cellular mechanisms behind adelmidrol action we evaluated the inflammatory NF-κB pathway by western blot analysis. The basal expression of IκB-α was found in paw tissue from sham animals (Fig. 4A; see densitometry analysis A1); this expression was decreased in CAR-treated rats. Treatment with adelmidrol was able to increase IκB-α levels (Fig. 4A; see densitometry analysis A1). Moreover, nuclear p65 subunit translocation was increased after CAR induction (Fig. 4B; see densitometry analysis B1), and treatment with adelmidrol reduced translocation of p65 subunit (Fig. 4B; see densitometry analysis B1). Pretreatment with GW9662 was not able to reduce IκB-α degradation

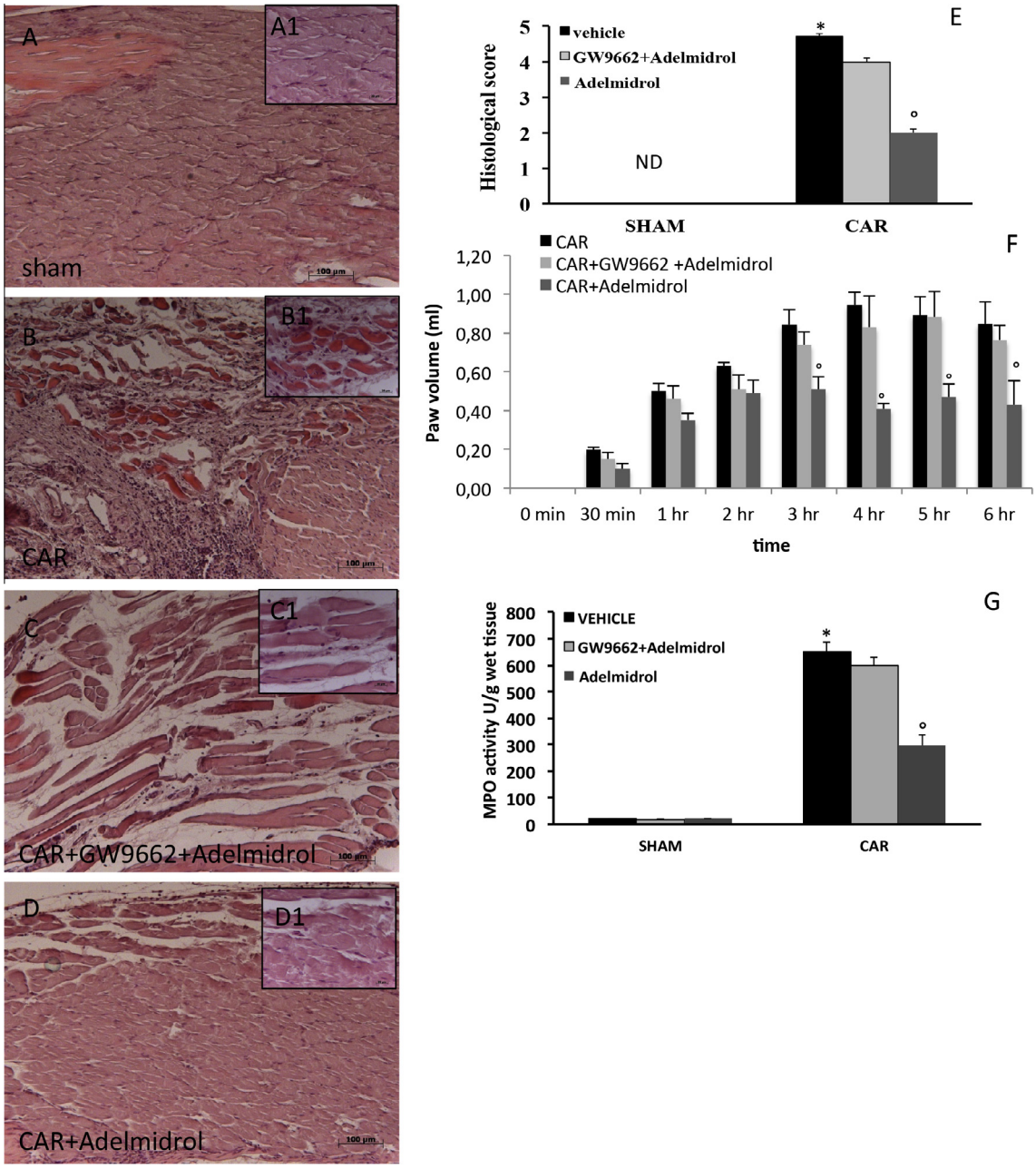

Fig. 1. Anti-inflammatory effects of adelmidrol on acute model of CAR induced paw damage. Histological evaluation was performed by H & E staining. Panels A-A1, sham group; panels B-B1, CAR group; Panels C-C1, CAR+GW9662+adelmidrol group; Panels D-D1, CAR+adelmidrol group. Panel E: histological score for the various groups. Adelmidrol administration caused significant improvements in paw volume and neutrophil infiltration in rats (F, G). Pretreatment with GW9662 antagonized the protective effects of adelmidrol on paw volume and MPO activity measurements (F, G). Values are means ± SD of 20 animals for each group. $^{*}p < 0.05$ vs sham-control and $^{\circ}p < 0.05$ vs CAR. $^{\circ\circ}p < 0.01$ vs CAR.

and p65 subunit translocation in rats subjected to CAR and treated with adelmidrol (Fig. 4B; see densitometry analysis B1).

3.4. Effect of adelmidrol on expression of COX-2 and iNOS

In order to characterize further the anti-inflammatory effects of adelmidrol, we also investigated COX-2 and iNOS expression that are under NF-κB control. COX-2 and iNOS expressions were also increased in inflamed paw tissues from CAR injected rats, while adelmidrol reduced COX-2 and iNOS levels (Fig. 4C and D). In contrast, pretreatment with GW9662 did not reduce these expressions in rats subjected to CAR and treated with adelmidrol (Fig. 4C and D).

3.5. Effect of adelmidrol treatment in the development of CIA

First clinical signs (periarticular erythema and edema) (Fig. 5B) of CIA in mice immunized with CII, were observed in hindpaws between 24 and 26 days after CIA induction, with a 100% of CIA incidence at day 28 (Fig. 5L). The maximum arthritis indices of approximately 12 were observed between days 32 and 35 after immunization (Fig. 5G) in CIA-control mice. The therapy with adelmidrol significantly reduced the development of joint

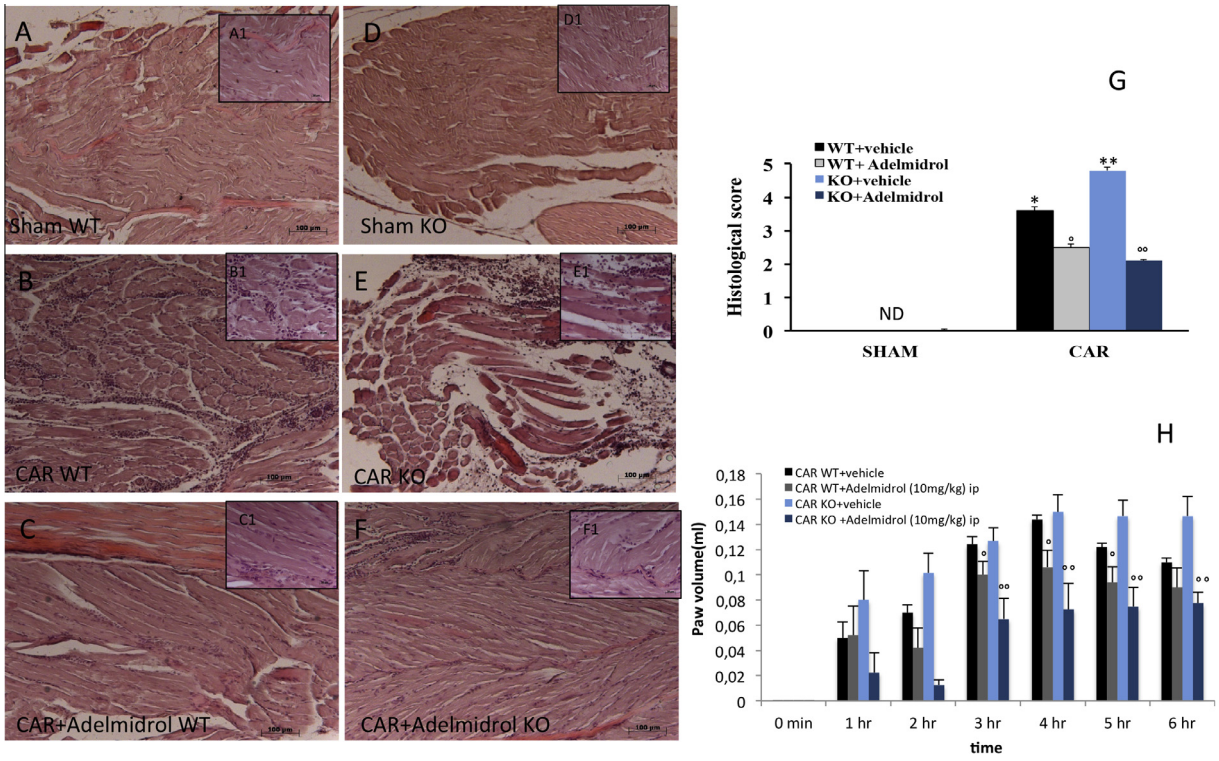

Fig. 2. The effects of adelmidrol on CAR induced inflammation in PPAR-α WT and KO mice. Histological evaluation was performed by H & E staining. Panels A-A1; D-D1, sham groups; panels B-B1; E, E1 CAR groups; Panels C-C1; F, F1 adelmidrol groups;. Panel G: histological score for the various groups. Paw inflammation was more evident in PPAR-α KO compared to PPAR-α WT mice. Adelmidrol administration caused significant improvements in the histological damage and in paw volume in PPAR-α WT as well as in PPAR-α KO (G, H). Thus, the deficiency of PPAR-α receptor did not inhibit the effects of adelmidrol in PPAR-α KO mice (H). Figures are representative of all the animals in each group. Values are means ± SD of 20 animals for each group. $^{*}p < 0.05$ vs sham-control $^{**}p < 0.01$ vs sham-control and $^{\circ}p < 0.05$ vs CAR. $^{\circ\circ}p < 0.01$ vs CAR.

inflammation (Fig. 5C). No clinical signs and histopathologic features of CIA were observed in the paws of sham controls (Fig. 5A). Adelmidrol reduced the arthritis index (Fig. 5G). The Fig. 5I demonstrated a time-dependent increase in paw volume (each value are the mean values of both hindpaws). Maximum paw volume was observed by day 35 in CIA mice. Treatment with adelmidrol exhibited a continuously significant reduction of hindpaw swelling from day 26–35 after induction (Fig. 5I). No increases in hindpaw volume were observed with normal control (data not shown).

The rate and the gain in body weight were similar in sham and in CIA mice in the first week (data not shown). From day 25, CII-injected mice gained less weight than the sham animals, until day 35 (Fig. 5M). Adelmidrol treatment determined a significant increase of body weight compared to CIA+vehicle mice (Fig. 5M). We also assessed whether daily treatment with adelmidrol affected the locomotor ability, by a non accelerating rotarod test, in CIA mice. Adelmidrol reduced the motor impairment in CIA mice (Fig. 6A).

3.6. Effect of adelmidrol on pain sensitivity and mechanical allodynia in CIA

At day 25 after CIA induction, mice showed thermal hyperalgesia, as indicated by a decrease in hindpaw withdrawal latency, with a maximum hypersensitive response between days 30 and 35 in CIA mice (Fig. 6C). Adelmidrol treatment was able to reduce thermal hyperalgesia (Fig. 6C). In addition, to better investigate the effects of adelmidrol in the mechanical hypersensitivity associated to CIA, Von Frey filament test was also performed. An increased mechanical allodynia was evident in CIA mice from 25 to 35 day that was less in CIA mice treated with adelmidrol (Fig. 6B).

3.7. Effect of adelmidrol on histopathology and radiographic analysis of CIA

At day 35, the histological evaluation of the paws from CIA+vehicle mice revealed signs of severe arthritis, with bone erosion and severe or moderate necrosis (Fig. 7A, A1, and see histologic score D). The bone erosion and the necrosis were decreased in the joint from adelmidrol-treated mice (Fig. 7B, B1, see for histologic score D). No joint damage was observed in sham animals (data not shown).

Radiographic analysis of knee joint and femoral growth plate in the femur from CIA+vehicle mice at 35 days after CII injection revealed bone erosion (Fig. 5E, see radiograph score H). Less bone resorption was found in the adelmidrol-treated mice (Fig. 5F, see radiograph score H). No evidence of bone resorption was found in sham mice (Fig. 5D, see radiograph score H).

3.8. Effect of adelmidrol on mast cells degranulation during CIA

To better examine the mast cell infiltration during CIA, toluidine blue staining was used. In particular, we observed a significant presence of mast cells in the joint tissues at day 35 after CIA induction (Fig. 7C), mainly localized in the articular space (see particles 7 C1). In contrast, less mast cell infiltration was found in the joint tissues from CIA mice treated with adelmidrol (Fig. 7D, D1). No resident mast cells were observed in the joint tissues from sham groups (data not shown).

In addition, we also analyzed the joint expression of chymase and tryptase by immunohistochemistry. An increased chymase and tryptase expression localized in mast cells, was observed in the joint tissues at 35 days after CIA induction (Fig. 7F, F1, H, H1,

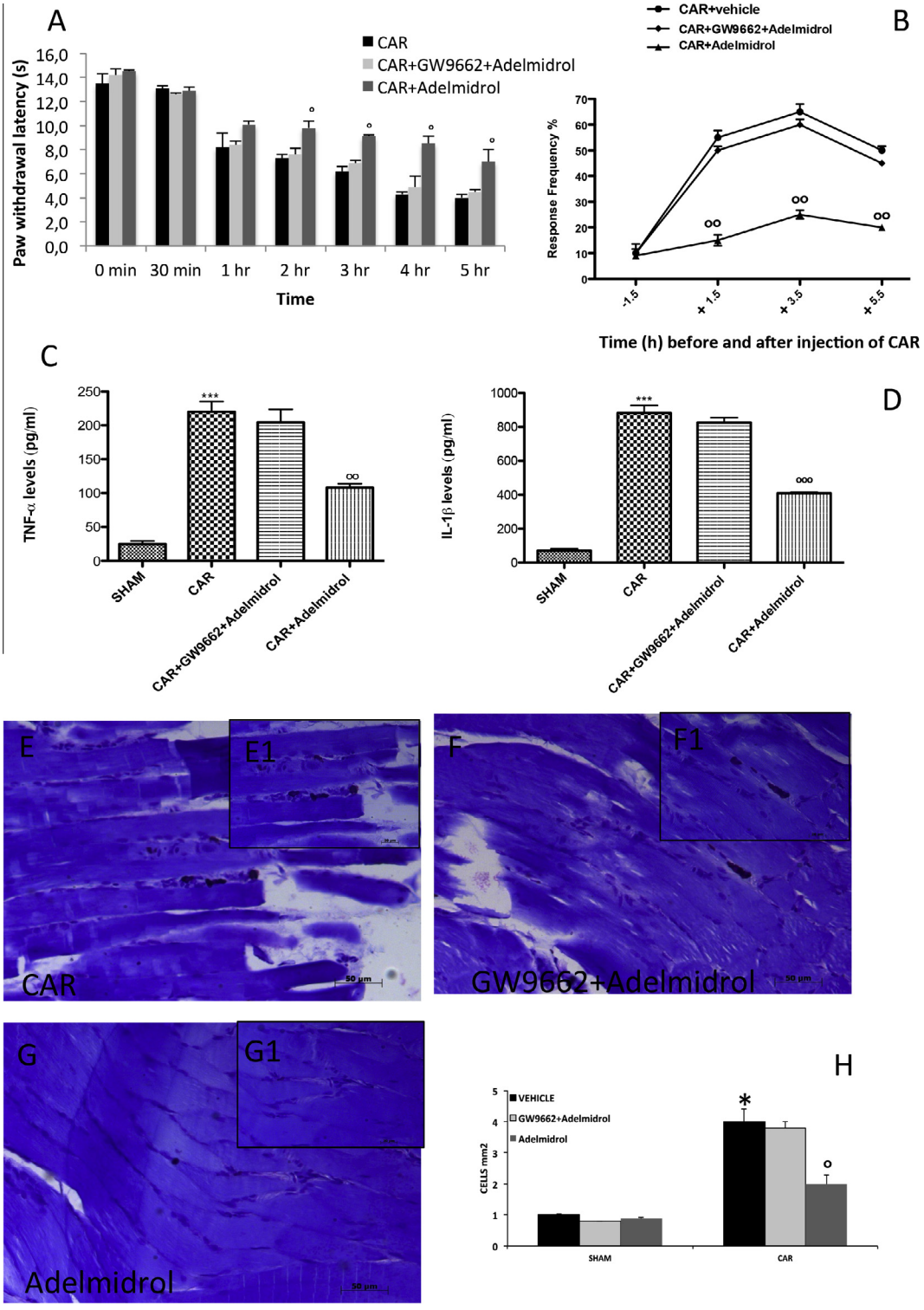

Fig. 3. The effects of adelmidrol on CAR-induced hypersensitivity, cytokines release and MCs infiltration. Heat hypersensitivity and mechanical allodynia were measured at different time points (A, B). CAR injection caused an increase in thermal and mechanical hypersensitivity (A, B). Adelmidrol administration determined important improvements (A, B) while pretreatment with GW9662 did not reduce thermal hyperalgesia and mechanical allodynia (A, B). CAR injection also caused an increase of pro-inflammatory cytokines production (C, D). Adelmidrol treatment was able to reduce TNF-α and IL-1β levels while pretreatment with GW9662 did not reduce cytokines levels (C, D). In addition, toluidine blue staining was also performed to measure mast cell infiltration. The mast cells were identified in the slides stained with acidified toluidine blue that have dark lilac blue granules, Panels E-E1, CAR group; Panels F-F1, CAR+adelmidrol group, Panels G-G1, GW9662+adelmidrol group. The number of mast cells per unit area of tissue (mast cell density) is shown (H). Values are means ± SD of 20 animals for each group. $^{*}p < 0.05$ vs sham-control $^{***}p < 0.001$ vs sham-control and $^{\circ}p < 0.05$ vs CAR. $^{\circ\circ}p < 0.01$ vs CAR. $^{\circ\circ\circ}p < 0.001$ vs CAR.

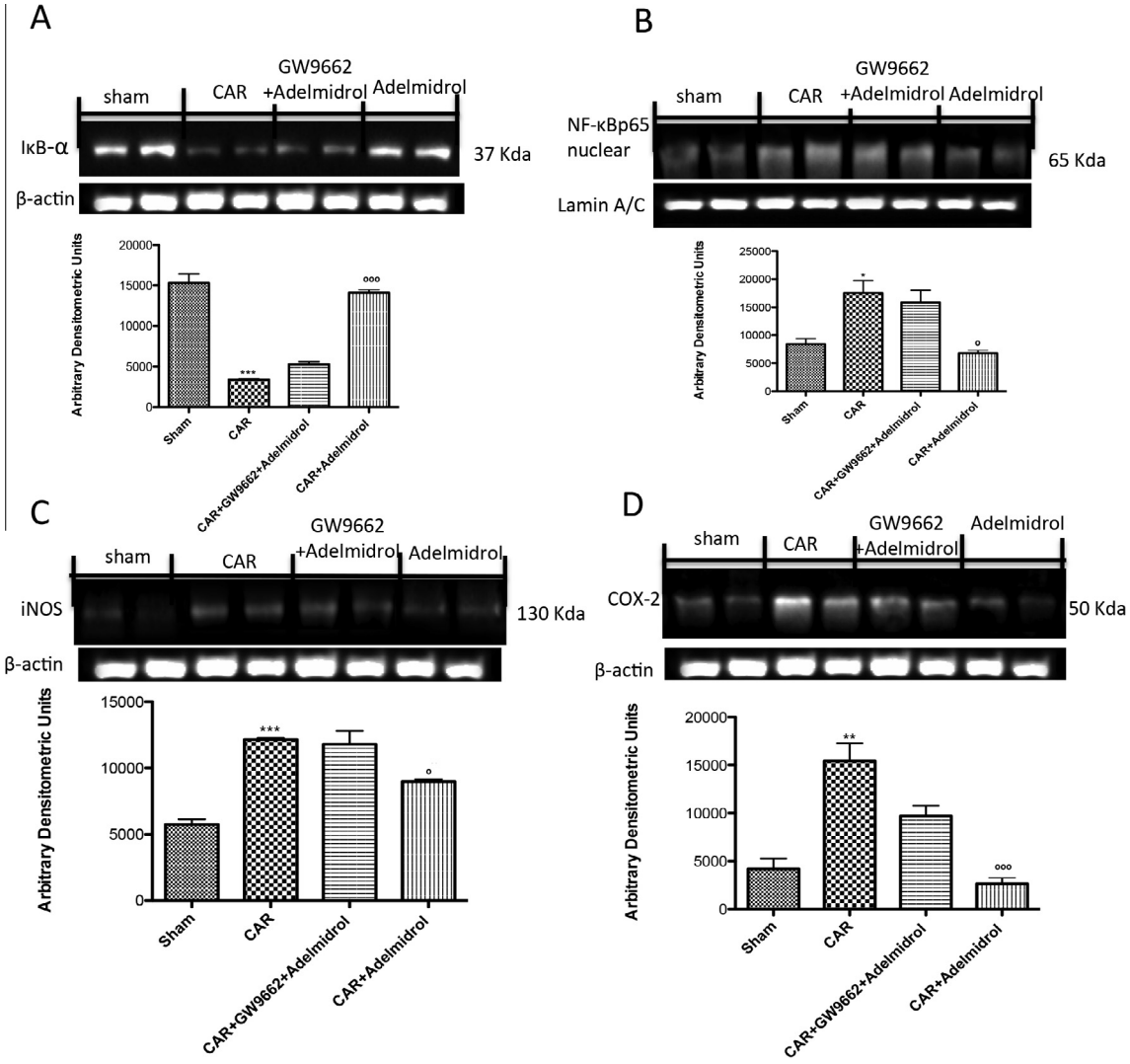

Fig. 4. The effects of adelmidrol on CAR-induced nuclear NF-κBp65 translocation and IKB-α degradation, iNOS and COX-2 expressions. Representative western blots showing the effects of adelmidrol on IκB-α degradation (A), NF-κB p65 translocation (B), iNOS expression (C) and COX-2 expression (D) after CAR injection. Adelmidrol treatment reduced IκB-α degradation (A), NF-κB p65 translocation (B), iNOS (C) and COX-2 (D) expressions. Pretreatment with GW9662 was not able to reduce NF-κB p65 translocation, IκB-α degradation, iNOS and COX-2 expressions (A, B, C, D). A representative blot of lysates obtained from 20 animals/group is shown, and densitometry analysis of all animals is reported. The results in A, B C and D are expressed as means ± SD of 20 animals for each group. $^{*}p < 0.05$ vs sham; $^{**}p < 0.01$ vs sham, $^{***}p < 0.01$ vs sham, $^{\circ}p < 0.05$ vs CAR; $^{\circ\circ}p < 0.01$ vs CAR, $^{\circ\circ\circ}p < 0.01$ vs CAR.

and see L). Joint expression of chymase and tryptase was significantly reduced in the joints from CIA mice treated with adelmidrol (Fig. 7G, G1, I, I1, and see L). No staining for chymase and tryptase was observed in sham mice (data not shown).

3.9. Effect of adelmidrol on cytokines, chemokines expression, and neutrophil infiltration after CIA induction

As shown in Fig. 8A and B, the expression of MIP-lα and MIP-2 was increased in the joint at 35 days after CII injection. MIP-1α and MIP-2 levels were reduced in CIA mice treated with adelmidrol compared to vehicle group (Fig. 8A and B). In addition, neutrophil infiltration was performed by MPO activity. MPO activity was increased in vehicle-treated CIA mice (Fig. 8F), whereas in the CIA+adelmidrol mice, MPO activity was reduced in a significant way (Fig. 8F).

In this study we also analyzed the levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the plasma. A substantial increase in TNF-α, IL-1β, and IL-6 (Fig. 8C–E) production was found in CIA mice 35 days after CII immunization. Levels of TNF-α, IL-1β, and IL-6 (Fig. 8C–E) were reduced in CIA mice treated with adelmidrol therapy.

3.10. Effect of adelmidrol on nitrotyrosine formation and lipid peroxidation after CIA induction

The release of ROS during chronic inflammation contributes to the tissue injury [26]. A positive staining for nitrotyrosine, a marker of nitrosative injury, was observed in joints of CIA+vehicle mice on day 35 (Fig. 8G, G1, and see I). Adelmidrol reduced the formation of nitrotyrosine in a significant way (Fig. 8H, H1, and see I).

In addition, MDA levels were also detected in the plasma as an indicator of lipid peroxidation, at 35 days after CIA induction, A significant increase of MDA levels (Fig. 8L) was observed in the plasma from mice subjected to CIA when compared to sham groups. MDA levels (Fig. 8L) were reduced in CIA mice treated with adelmidrol.

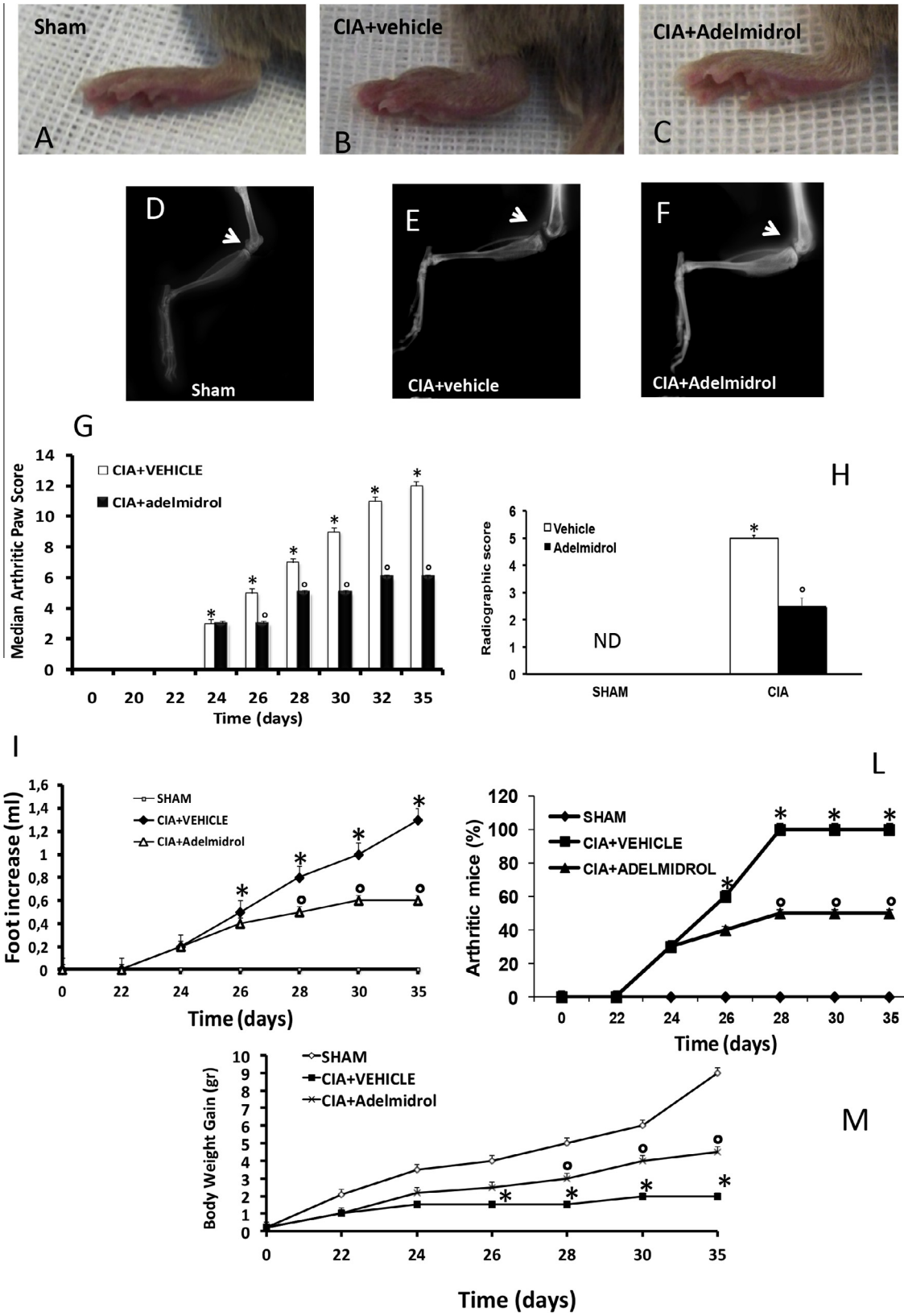

Fig. 5. The chronic effects of adelmidrol on the clinical and on radiographic analysis, paw edema and body weight during chronic inflammation. Clinical signs like periarticular erythema and edema were found in CIA+vehicle mice (B and G). Adelmidrol treatment showed a reduction of clinical signs of CIA (C and G). No clinical signs were found in sham animals (A). In addition, radiographic analysis was performed (H). No sign of pathology in the femoral growth plate and the tibiotarsal joints of normal mice was observed (D). Hindpaws from CIA+vehicle mice presented bone resorption in the femoral growth plate and in the tibiotarsal joints (E). Mice treated with adelmidrol have shown less bone erosion in the femoral growth plate, as well as in the tibiotarsal joints (F). In addition, the incidence of CIA was 100% at day 28 (L). CIA subjected mice gained significantly less weight, from day 25 to day 35 (M). CIA mice treated with adelmidrol presented a reduced weight loss (M), and reduced paw edema (I). Figures are representative of all the animals in each group. Values are expressed as mean ± SD of 20 animals for each group. $^{*}p < 0.01$ vs sham-control. $^{\circ}p < 0.01$ vs CIA.

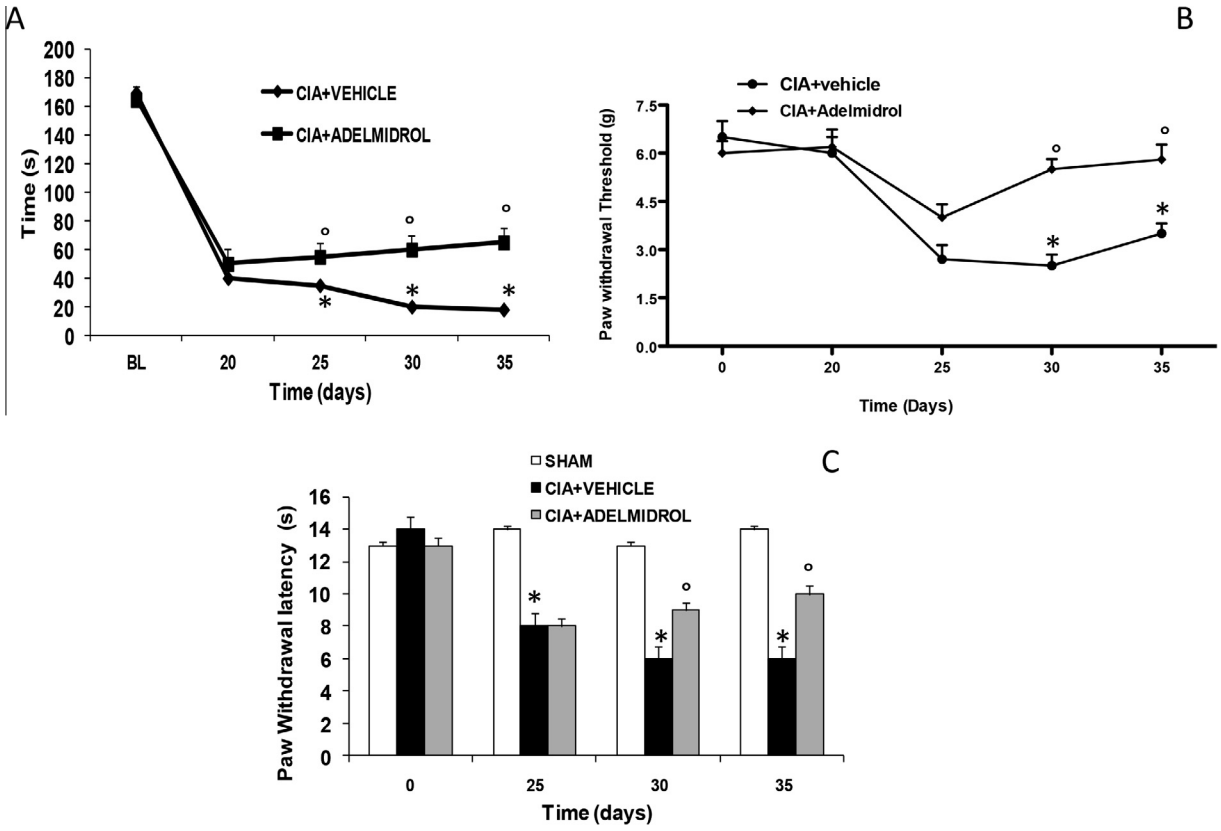

Fig. 6. The effects of adelmidrol on motility and pain during chronic inflammation. No alteration in motility was found in sham animals (A). Locomotor activities on the rotarod are ameliorated in CIA treated adelmidrol than in CIA+vehicle mice (A). In addition, CIA mice presented increased thermal hyperalgesia (C) and mechanical allodynia (B) compared to sham. Adelmidrol treatment decreased thermal hyperalgesia and mechanical allodynia in a significant way (B, C). Figures are representative of all the animals in each group. Values are given as mean ± SD of 20 animals for each group. $^{*}p < 0.01$ vs sham-control. $^{\circ}p < 0.01$ vs CIA.

3.11. Role of PPAR-γ and PPAR-α receptors on the anti-inflammatory effects of adelmidrol after CIA induction

In order to better investigate the mechanism of action of adelmidrol and the relation to PPAR-α or γ pathways activation during chronic inflammation, we also evaluated the pre-treatment effects of two potent antagonists of PPAR-γ (GW9662) and PPAR-α (GW6471) on adelmidrol action. As demonstrated during acute inflammation (CAR model), GW9662 pre-treatment was able to reduce the protective effect of adelmidrol in CIA mice, as evaluated by histological analysis (Fig. 9A, A1, B, B1, C, C1, D, D1 and E) MPO activity (Fig. 9G) and paw volume (Fig. 9F). On the contrary, GW6471 pre-treatment was not able to inhibit the anti-inflammatory effects of adelmidrol after CIA induction (Fig. 9).

4. Discussion

The present study demonstrated the acute and chronic effects of adelmidrol and the mechanism underlying such an action, in a model of CAR induced paw inflammation as well as in an experimental model of autoimmune disease CIA.

Adelmidrol belongs to the aliamide family [27] with similar anti-inflammatory and anti-nociceptive proprieties of PEA [28,29] and able to control MCs hyper-reactivity in several pathophysiological conditions [30,31]. Moreover, adelmidrol has been shown to negatively control canine skin MCs during pathological events [11]. Our previous work also demonstrated that PEA in combination with an antioxidant such as luteolin, was able to modulate MCs activation in paw tissues [7].

As PEA analogue, the pharmacological properties of adelmidrol can be related to the activation of a cell surface receptor cannabinoid CB2-like or the orphan GPR55 receptor, as well as a nuclear receptor of PPARs family [9,32]. Several studies have clearly demonstrated that PPAR-α and PPAR-γ agonists exert positive effects in experimental models of CNS injury [9]. A previous work of ours also showed that the beneficial effects of PEA were in part dependent on PPAR-α pathway in an experimental model of kidney I/R [33]. We also confirmed that PPAR-α is involved in protecting effects of PEA in a model of spinal cord trauma. Specifically, the anti-inflammatory effect obtained by PEA was antagonized by the antagonists administration for PPAR-γ (GW9662), and PPAR-δ (GSK0660) [9]. With this aim in mind, in this work, we also investigated whether the protective effects of adelmidrol were associated with PPARs signaling pathway such as PPAR-α or PPAR-γ.

ROS/RNS production in addition to cyclooxygenase (COX)-derived prostaglandins play a crucial role during inflammatory events associated with the development of altered pain sensitivity [34,35].

In that regard, here, we showed that adelmidrol treatment significantly reduced histological paw damage, thermal hyperalgesia and mechanical allodynia associated to CAR injection. Moreover, nitrooxidative stress could activate transcription factors such as nuclear factor-κB (NF-κB) which are known to induce COX-2 expression during inflammation [36–39]. IL-1β and TNF-α are initiators of the NF-κB activation cascade [40] and are under its transcriptional control, constituting a positive feedback loop. Several reports indicate that PEA can decrease the activation of the NF-κB system in different experimental models [41,42]. Furthermore, a previous work reported that adelmidrol reduced the levels of

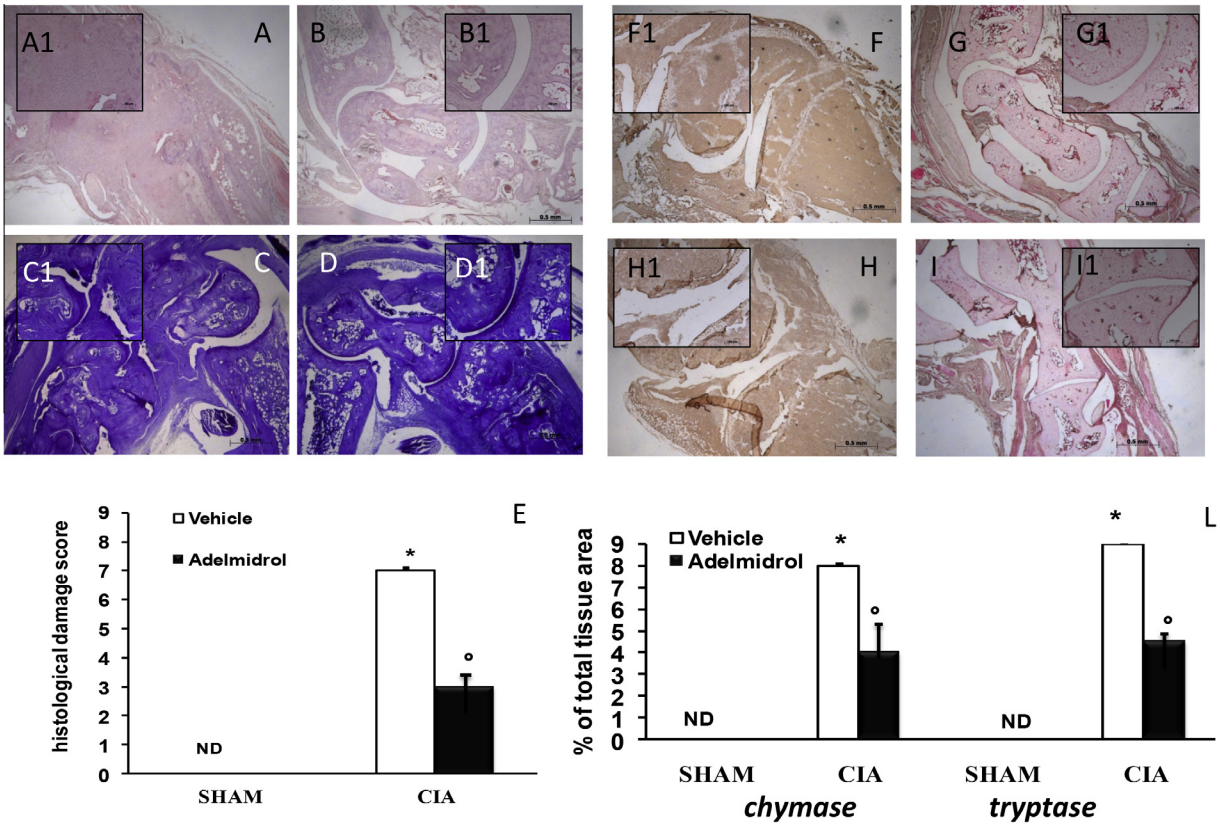

Fig. 7. The effects of adelmidrol on histological damage and chymase and tryptase expression on chronic CIA model. The histologic evaluation by hematoxylin/eosin-staining of joint sections showed inflammatory cell infiltration and bone erosion in CIA-control mice (A, A1 and E) compared to sham animals (data not shown). The histologic alterations were reduced in the tissues from CIA-adelmidrol treated mice (B, B1, and E). Toluidine blue staining was also performed to measure mast cell infiltration. An increased mast cell infiltration was found in joint tissues of CIA mice (C, C1) compared to sham (data not shown). Adelmidrol reduced mast cell infiltration (D, D1). A significant increase in chymase and tryptase expression was observed in the joint tissues after CIA induction (F, F1, H, H1 and L). Chymase and tryptase expression was significantly reduced in the joints from CIA-adelmidrol-treated mice (G, G1, I, I1 and L). Densitometry analysis of immunocytochemistry photographs ($n = 5$) for chymase and tryptase from paw sections was assessed (L). The assay was carried out by using computer-assisted color image analysis (Leica QWin V3, UK). Data are expressed as percentage of total tissue area. Figures are representative of all the animals in each group. Values are given as mean ± SD of 20 animals for each group. $^{*}p < 0.01$ vs sham-control. $^{\circ}p < 0.01$ vs CIA.

pro-inflammatory markers such as iNOS, TNF-α and nitric oxide [8]. Accordingly, the inhibition of NF-κB pathway with decreased inflammatory mediators levels under its control such as TNF-α, IL-1β, COX-2 and iNOS were found in CAR subjected rats treated with adelmidrol. In addition, the action of adelmidrol could be also due to the modulation of mast cells degranulation, as confirmed by toluidine blue staining (MCs infiltration) and by the inhibition of the release of several pro-inflammatory enzymes and mediators. These anti-inflammatory effects of adelmidrol could be dependent on PPAR-γ activation, as the *in vivo* antagonism of the receptor by GW9662 abolished the inhibitory action of adelmidrol in CAR induced paw edema. In the same time, through the use of PPAR-α KO mice we demonstrated that the absence of PPAR-α receptor did not modify the action of adelmidrol suggesting that its protective effect was independent to PPAR-α activation.

MC mediators released at early stage of the inflammatory process drive the inflammatory reaction to chronicity. In this study, we also demonstrated, for the first time, that adelmidrol exerts an anti-inflammatory effect in the studied model of chronic inflammation (CIA model) and that this effect may be due to the modulation of mast cell activation and mediator release. In particular, we observed a significant protection of cartilage/bone and inhibition of pro-inflammatory mediators and reduced MCs infiltration as well as the joint expression of chymase and tryptase compared to untreated collagen-immunized animals. The capacity of MCs to react to several stimuli facilitates the response to tissue injury, by stimulating a rapid release of pro-inflammatory mediators, mediators of hyperalgesia and itch mediators [43]. In our paper, we confirmed that the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) as well as the chemokines (MIP-Iα and MIP-2) are expressed in inflamed joints and contribute to the progress of chronic joint inflammation. Aldemidrol treatment demonstrated a more marked reduction of pro-inflammatory cytokines, chemokines and of leukocyte infiltration. The role of MCs in chronic inflammation-induced hyperalgesia has also been well documented. Previous data reported that PEA reduced granuloma-induced hyperalgesia by modulation of MCs activation [8] as well as a recent our paper showed that PEA comicronized with polydatin reduced thermal hyperalgesia and mechanical allodynia after CAR injection [15]. Pain hypersensitivity associated to CIA model seems to be mediated by both peripheral and central sensitization mechanisms [44]. Here, we demonstrated that the PEA analogue adelmidrol was also able to ameliorate peripheral sensitization (altered heat sensitivity) and central sensitization (mechanical hypersensitivity). Various studies have clearly demonstrated that the ROS/RNS production is also associated to MCs activation. In that regard, the role of ROS in degradation of cartilage and bone is also well documented [45,46]. Several evidences supported a link between ROS and cartilage oxidation/degradation [45]. Here, we demonstrated that adelmidrol treatment reduced oxidative and nitrosative stress in a model of CIA. Finally, as just showed in the acute CAR model, the correlation of adelmidrol action with

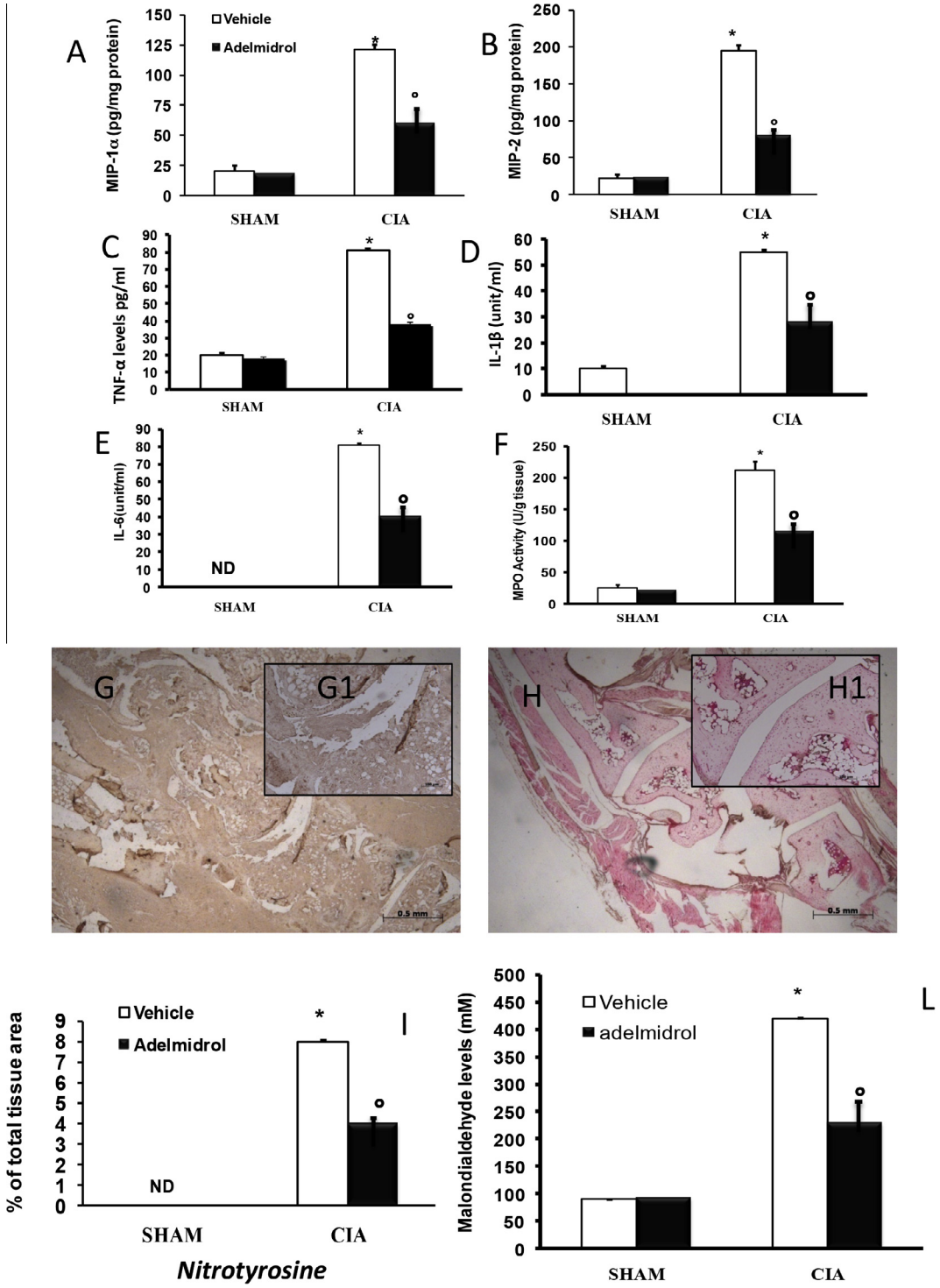

Fig. 8. The effects of adelmidrol on cytokines, chemokines production, neutrophil infiltration and on nitrotyrosine expression and MDA levels during CIA model. An increase of MIP-1α (A), MIP-2 (B), IL-1β (C), IL-6 (D), TNF-α (E) levels and MPO activity (F) were found in CIA+vehicle mice after CIA induction. CIA mice treated with adelmidrol exhibited a reduction of MIP-1α (A), MIP-2 (B), IL-1β (C), IL-6 (D), TNF-α (E) levels and MPO activity (F). In addition, an increase of nitrotyrosine (G, G1 and I), staining was detected in the paws after CIA induction. A substantial reduction of nitrotyrosine (H, H1 and I) staining was found in the paws of CIA mice treated with adelmidrol. Densitometry analysis of immunocytochemistry photographs (n = 5) for nitrotyrosine from paw sections (I) was carried out by using computer-assisted color image analysis (Leica QWin V3, UK). Data are expressed as percentage of total tissue area. $^{*}p < 0.01$ vs Sham-control. $^{\circ}p < 0.01$ vs CIA. In addition, MDA levels were evaluated. A substantial increase in MDA levels (D) was found in CIA-control mice 35 days after CIA induction. CIA-adelmidrol-treated mice showed a significant reduction in MDA levels (L). Values are shown as mean ± SD of 20 animals for each group. $^{*}p < 0.01$ vs sham-control. $^{\circ}p < 0.01$ vs CIA animals for each group.

PPAR-γ signaling pathway was also confirmed in the chronic CIA model. Our results are in accordance with a previous work in which it was shown that adelmidrol exerted an anti-inflammatory effect in a rat model of carrageenin-granuloma [8] and the administration of GW6471, a PPAR-α antagonist, didn't reverse the effect of adelmidrol.

In summary, in this study, we propose the possible mechanisms of action by which adelmidrol could act during acute and chronic

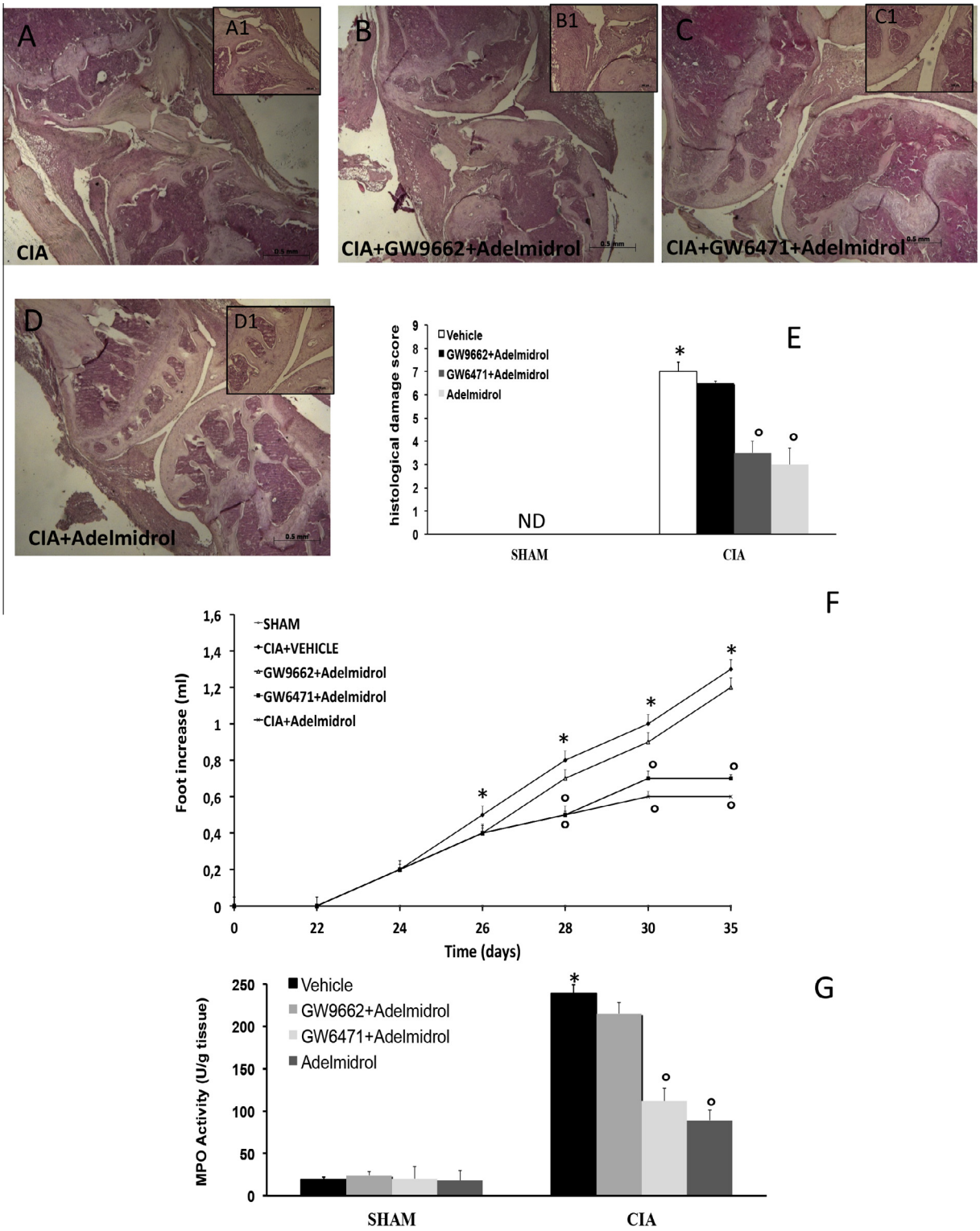

Fig. 9. Role of PPAR-α and PPAR-γ pathways on chronic effect of adelmidrol after CIA induction. Severe histological damage (A, A1 see histological score E), increased paw volume (F) and MPO activity (G) was found in CIA mice as well as in CIA+GW9662+adelmidrol mice groups (B, B1 see histological score E; F and G). CIA+adelmidrol and CIA +GW6471+adelmidrol groups showed a reduced histological damage, paw volume and MPO activity compared to vehicle groups (C, C1; D, D1 see histological score E; F and G). Figures are representative of all the animals in each group. Values are given as mean ± SD of 20 animals for each group. $^{*}p < 0.01$ vs sham-control. $^{\circ}p < 0.01$ vs CIA.

inflammation: PPAR-γ signaling pathway activation – inflammatory NF-κB pathway inhibition – nitrosative and oxidative stress reduction – MCs modulation – decrease of pro-inflammatory mediators levels – low pain sensitivity – reduced tissue injury.

In conclusion, we propose that adelmidrol may be useful in the therapy of conditions associated with local (CAR model) or systemic inflammation (CIA model). Although the present study showed that mast cell is a cellular target of adelmidrol, the exact

mechanism of action is not yet fully elucidated. Moreover, we have clearly demonstrated that the anti-inflammatory effects of adelmidrol are dependent on the PPAR-γ but not on the PPAR-α receptor in this experimental setting.

Conflict of interest

Salvatore Cuzzocrea is co-inventor on patent WO2013121449 A8 (Epitech Group Spa) which deals with compositions and methods for the modulation of amidases capable of hydrolysing N-acylethanolamines useable in the therapy of inflammatory diseases as well as is also a co-inventor on patent just submitted entitled ADEL-COLART "ADELMIDROL PER L'USO NELLE PATOLOGIE CARATTERIZZATE DA INSUFFICIENTE AGONISMO DEL RECETTORE PPAR-gamma" (Epitech Group Spa) which contains part of data related to the present study.

Moreover, Dr. Cuzzocrea is also a co-inventor with Epitech group on the following patent:

1. EP 2 821 083
2. MI2014 A001495
3. 102015000067344

In addition, no non-financial conflicts of interest exist for any of the authors.

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