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Nociceptive Effect of Subcutaneously Injected Interleukin-12 is Mediated by Endothelin Acting on ET_B Receptors in Rats

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Running title: IL-12 Hyperalgesia: Endothelin Action on ET_B Receptors

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Abbreviations: Adenosine 3'5' cyclic monophosphate (cAMP), cyclo[$_D$Trp-$_D$Asp-Pro-$_D$Val-Leu] (BQ123); N-*cys*-2,6 dimethylpiperidinocarbonyl-$_L$-γ-methylleucyl-$_D$-1-methoxycarboyl-$_D$-norleucine (BQ788), cytokine-induced neutrophil chemoattractant 1 (CINC-1), endothelin receptor type A (ET_A), endothelin receptor type B (ET_B), T helper 1 (Th1), tumor necrosis factor-alpha (TNF-α), interleukin (IL), 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid, Na (MK886).

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Abstract

Interleukin-12 (IL-12) is an inflammatory Th1 driving cytokine, which has been clinically used as immune therapy and vaccine adjuvant. Recently, it was reported that patients receiving IL-12 presented hyperalgesia. In the present study we investigated the mechanical hyperalgesic effect of IL-12 in rats using two tests: i. Paw constant pressure and ii. Electronic pressure-meter. In both tests, intraplantar administration of IL-12 (3-30 ng paw^{-1}) caused a dose- and time-dependent mechanical hyperalgesia, which peaked between 3-5 h, remaining significantly different from control levels until 7 h and resolved 24 h post injection. However, the same doses of IL-12 did not induce thermal hyperalgesia determined using the Hargreaves test. Pre-treatments with effective doses of indomethacin (2.5 mg kg^{-1}), atenolol (1 mg kg^{-1}), MK886 (5-lipoxygenase activating protein inhibitor, 1 mg kg^{-1}) or BQ123 (ET_A receptor antagonist, 30 nmol paw^{-1}) did not inhibit IL-12-evoked mechanical hyperalgesia (10 ng paw^{-1}). However, dexamethasone (2 mg kg^{-1}), morphine (3-12 μg paw^{-1}) and BQ788 (ET_B receptor antagonist, 3-30 nmol paw^{-1}) did inhibit IL-12 hyperalgesia. Furthermore, neither pre-treatment with effective doses of antiserum against rat-TNF-α (50 μl paw^{-1}) nor against IL-18 (10 μg paw^{-1}) inhibited the IL-12-induced hyperalgesia. Likewise, antiserum against IL-12 (10 ng paw^{-1}) did not alter IL-18-induced hyperalgesia. In conclusion, we demonstrated for the first time that IL-12 is a pro-hyperalgesic cytokine that induces mechanical hyperalgesia mediated by endothelin action on the ET_B receptor. Therefore, endothelin receptor antagonism could be beneficial in controlling IL-12 therapy induced pain or hyperalgesia.

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Introduction

IL-12 is the prototypic member of a heterodimeric family of cytokines that includes IL-23 and IL-27 (for review see Brombacher *et al.*, 2003). It is produced by a variety of cells including monocytes, neutrophils, B lymphocytes, macrophages and dendritic cells, stimulated by pathogenic organisms such as bacteria, parasites, viruses, and fungi. IL-12 regulates both innate and adaptive immunity, being a key cytokine that regulates Th1 differentiation (for review see Brombacher *et al.*, 2003; Watford *et al.*, 2004). Besides its importance in host-protective responses to most intracellular infectious microorganisms, the Th1 response is required for the development of autoimmune diseases including arthritis, myocarditis, encephalomyelitis, diabetes and lupus (Joosten *et al.*, 1997; for review see Brombacher *et al.*, 2003; Watford *et al.*, 2004).

Recent findings consistently demonstrated that IL-12 induces pain in humans. For instance, (a) patients that received intravenous rhIL-12 therapy for metastatic renal cancer or malignant melanoma presented arthralgias involving primarily the shoulders and fingers (Gollob *et al.*, 2000), (b) patients that received intraperitoneally injected rhIL-12 for Mullerian carcinoma, gastrointestinal primary malignancies, and mesothelioma treatment had headache and abdominal pain (Lenzi *et al.*, 2002), (c) pain and bladder spasms were adverse effects related to the intravesicular treatment with rhIL-12 for cell carcinoma of the bladder (Weiss *et al.*, 2003), and (d) mild to moderate pain at the site of injection has been reported in patients that received peritumoral injection of IL-12 transduced autologous fibroblasts (Kang *et al.*, 2001). However, the mechanisms underlying IL-12-induced pain have not yet been investigated.

At the present time, it is well accepted in the literature that a cascade of cytokines constitutes a link between inflammatory stimuli and release of the final mediators that directly sensitize the nociceptors, such as prostanoids and sympathetic amines (Cunha *et al.*, 1992). In rats, inflammatory stimuli induce resident cells to release TNF-α, which in turn activates two pathways: (i) TNF-α→ IL-6→ IL-1β→ prostaglandins (Cunha *et al.*, 1992) and (ii) TNF-α→ cytokine-induced neutrophil chemoattractant 1 (CINC-1; rat IL-8 related chemokine; Lorenzetti *et al.*, 2002)→ sympathetic amines (Nakamura and Ferreira, 1987; Cunha *et al.*, 1991). The sequential role of cytokines leading to the release of final mediators was further substantiated in rats by Safieh-Garabedian *et al.* (1997), and, more recently, in mice by Cunha *et al.* (2005). Furthermore, cytokines may also stimulate the release of other directly acting mediators, such as endothelin (Verri *et al.*, 2004).

Thus, in the present study we investigated whether the local injection of IL-12 induced mechanical hyperalgesia, as well as the involvement of other cytokines, prostanoid, sympathetic amines, leukotrienes and endothelin in this process. We found that IL-12 induces mechanical hyperalgesia in a dose- and time-dependent manner, mediated by endothelin acting via the ET_B receptor.

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Methods

Animals

Male Wistar rats (180-220 g) were housed in temperature-controlled rooms (22-25°C), with access to water and food *ad libitum*. All experiments were conducted in accordance with NIH guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirão Preto (University of São Paulo). The animals were used only in a single experimental group.

Mechanical hyperalgesic tests

Hyperalgesia was assessed using two different methods: the constant pressure rat paw and the electronic pressure-meter tests. A different investigator performed each test, as was the solution preparation and the subcutaneous injections in the hind paw of rats. Multiple paw treatments with saline did not alter basal reaction time, which was similar to that observed in non-injected paws.

The constant pressure rat paw test

Mechanical hyperalgesia was tested in rats as previously described (Ferreira *et al.*, 1978). In this method, a constant pressure of 20 mmHg (measured using a sphygmomanometer) is applied (via a syringe piston moved by compressed air) to a 15-mm^2 area on the dorsal surface of the hindpaw, and discontinued when the rat presents a typical “freezing reaction”. This reaction is comprised of brief apnea, concomitant with retraction of the head and forepaws and reduction in the escape movements that animals normally make to free themselves from the position imposed by the experimental

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situation. Usually, the apnea is associated with successive waves of muscular tremor. For each animal, the latency to the onset of the "freezing reaction" is measured before administration (zero time) and at different times after administration of the hyperalgesic agents. The intensity of mechanical hyperalgesia is quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first (Ferreira *et al.*, 1978). Reaction time was 31.9 ± 0.2 s (mean ± s.e.m.; n = 36) before injection of the hyperalgesic agents. A shortened reaction time is prevented by steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) treatment before an inflammatory stimuli injection (Cunha *et al.*, 1992, Lorenzetti *et al.*, 2002). This method has been used to demonstrate the peripheral effect of morphine (Ferreira *et al.*, 1978; Smith *et al.*, 1982), the contribution of eicosanoids, sympathetic amines, adenosine 3'5' cyclic monophosphate (cAMP) and of cytokines to the development of peripheral inflammatory hyperalgesia (Ferreira and Nakamura, 1979a; Cunha *et al.*, 1992; Ferreira *et al.*, 1993; Cunha *et al.*, 2000). These concepts and findings have been extensively confirmed with other methodologies such as formalin-induced flinching and others (Vinegar *et al.*, 1976; Vivancos *et al.*, 2004).

The Electronic pressure-meter test

The paw hyperalgesia was also measured with an electronic pressure-meter. The rats were placed in acrylic cages (12 x 20 x 17 cm high) with a wire grid floor, 15-30 min before beginning the tests. During this adaptation period, the paws were poked 2-3 times. Before paw stimulation, the animals should be quiet, without exploratory or toilet movements and not resting over the paws. In these experiments a pressure-meter, which consisted of a hand-held force transducer adapted with a 0.7 mm^2 polypropylene tip

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(electronic von Frey anesthesiometer, IITC Inc. Life Science Instruments, Woodland Hills, CA, USA) was used. The investigator was trained to apply the polypropylene tip perpendicularly in between the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hindpaw. The test consisted of poking the hindpaw to provoke a flexion reflex followed by a clear flinch response after the paw withdrawal. The electronic pressure-meter automatically recorded the intensity of stimulus when the paw was withdrawn. The stimulation of the paw was repeated until the animal presented three similar measurements (with the difference between the highest and the lowest measurement being no more than 10 g). If the results were inconsistent, the experimenter used another animal (~1:25 animals). The animals were tested before and after treatments and the results are expressed by the delta reaction force (g) that was calculated by subtracting the value of the measurements after treatment from that of the first measurement before treatment (Vivancos *et al.*, 2004). The reaction force was 43.6 ± 0.3 g (mean ± s.e.m.; n = 36) before injection of the hyperalgesic agents.

Thermal test

Hargreaves' plantar test

The Hargreaves' test was performed as previously described (Hargreaves *et al.*, 1988) using a standard apparatus (Ugo Basile). The test consists of placing the rat in a transparent acrylic box and applying a thermal radiant stimulus with a mobile infrared heat lamp positioned underneath the targeted hind paw. The latency of the paw withdrawal response was measured automatically with the help of a photoelectric-sensitive device. The latency of the withdrawal response of each hindpaw was

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determined before and at 1, 3 and 5 h after IL-12 (3, 10 and 30 ng in 50 μL) or saline (50 μL) i.pl. injection. The intensity of thermal hyperalgesia was expressed as the reduction in the reaction time, calculated by subtracting the value of the post-treatment measurement from the pre-treatment."

Protocols

The IL-12-induced mechanical or thermal hyperalgesia was assessed using the following protocols.

Dose- and time-dependent mechanical or thermal hyperalgesia induced by IL-12

In order to determine whether IL-12 induces mechanical and thermal hyperalgesia the cytokine (3-30 ng in 50 μL) was injected i.pl. and the nociceptive responses were measured 1, 3, 5, 7 and 24 h later.

Role of eicosanoids (prostanoids and leukotrienes), sympathetic mediators and of morphine treatment in IL-12-induced mechanical hyperalgesia

The participation of nociceptive mediators in IL-12 (10 ng in 50 μl)-induced mechanical hyperalgesia was determined 1, 3 and 5 h after i.pl. injection of IL-12. The rats were treated with dexamethasone (1 h before, 2.0 mg kg^{-1}, s.c., Verri *et al.*, 2004), indomethacin (30 min before, 2.5 mg kg^{-1}, s.c., diluted in Tris (2-amino-2-hydroxymethyl-propan-1,3-diol)/HCl, pH 8.0, Cunha *et al.*, 1992), atenolol (30 min before, 1.0 mg kg^{-1}, s.c., Nakamura and Ferreira, 1987) or 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid, Na (MK886; 24 h reinforcement dose 1 h before, 1.0 mg kg^{-1}, per oral, diluted in 0.1% methylcellulose in

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water, Tonussi and Ferreira, 1999). Additionally, it was determined the opioid modulation of IL-12-induced hyperalgesia. Because the peripheral effect of morphine lasts approximately 1 h (Ferreira and Nakamura, 1979b), morphine (3-12 μg in 50 μl, i.pl., Ferreira and Nakamura, 1979b) was injected 4 h after IL-12 administration (10 ng in 50 μl) and evaluated 1 h after its injection. Naloxone (1.0 mg Kg^{-1}, i.p., Ferreira and Nakamura, 1979b), was administrated 30 min before and evaluated 1 h after morphine (6 μg paw^{-1}). The selected doses of dexamethasone, indomethacin, atenolol, MK886 and morphine inhibit carrageenan- or LPS-induced mechanical hyperalgesia (Ferreira and Nakamura, 1979b; Nakamura and Ferreira, 1987; Cunha *et al.*, 1992; Tonussi and Ferreira, 1999; Lorenzetti *et al.*, 2002; Verri *et al.*, 2004), and did not affect the mechanical thresholds of normal animals (data not shown).

Role of TNF-α and IL-18 on IL-12-induced hyperalgesia, and of IL-12 on IL-18-induced hyperalgesia

Antiserum to rat TNF-α (15 min, 50 μl, i.pl., Ferreira *et al.*, 1993), anti-IL-18 antibody (15 min, 10 μg, 50 μl, i.pl.) or control serum (50 μl, i.pl.) was administered before IL-12 (10 ng, 50 μl) injection. Furthermore, anti-IL-12 antibody (15 min, 10 ng, 50 μl, i.pl.) or control serum (50 μl, i.pl.) was administered before IL-18 (40 ng, 50 μl) injection. The effects of the antiserum to rat TNF-α, anti-IL-18 antibody and anti-IL-12 antibody (doses described above) upon the TNF-α (2.5 pg in 50 μl), IL-18 (40 ng in 50 μl) or IL-12 (10 ng in 50 μl) induced-mechanical hyperalgesia were also determined, respectively. The hyperalgesic responses were measured 1, 3 and 5 h after stimulus i.pl. injection.

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Role of endothelin and its receptors in IL-12-induced mechanical hyperalgesia

BQ123 (30 min, 30 nmol in 50 μl, i.pl., an ET_A receptor antagonist) or BQ788 (30 min, 3-30 nmol in 50 μl, i.pl., an ET_B receptor antagonist) was injected before IL-12 (10 ng in 50 μl, i.pl.) or endothelin-1 (ET-1; 10 pmol in 50 μl, i.pl., Da Cunha *et al.*, 2004) administration. Animals may also be pre-treated with indomethacin or atenolol (doses described above) before ET-1 (10 pmol in 50 μl) injection. The hyperalgesic responses were measured 1, 3 and 5 h after IL-12 i.pl. injection.

Drugs, cytokines, antibodies and antisera

The following materials were obtained from the sources indicated: atenolol (Sigma, St. Louis, MO, USA); human IL-18 (referred to as IL-18; Peprotech Inc., Rocky Hill, NJ, USA), anti-human-IL-18 antibody (referred to as anti-IL-18 antibody; Peprotech Inc., Rocky Hill, NJ, USA, Lot 06993 J230); anti-human-IL-12 antibody (referred to as anti-IL-12 antibody; Peprotech Inc., Rocky Hill, NJ, USA); BQ123, sodium salt (cyclo[$_D$Trp-$_D$Asp-Pro-$_D$Val-Leu], Novabiochem, La Jolla, CA, USA, Lot A21510); BQ788, sodium salt (N-*cys*-2,6 dimethylpiperidinocarbonyl-$_L$-γ-methylleucyl-$_D$-1-methoxycarboyl-$_D$-norleucine, Calbiochem, La Jolla, CA, USA, Lot B32622); dexamethasone (Sigma, St. Louis, MO, USA); human endothelin-1 (referred to as ET-1, American Peptide Company, Sunnyvale, CA, USA); indomethacin (Prodome, Campinas, SP, Brazil); methylcellulose (Sigma, St. Louis, MO, USA); MK886 (3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid, Na, Calciochem, Darmstadt, Germany, Lot B39328); morphine sulfate (Cristália, Itapira, SP, Brazil); Naloxone, hydrochloride (Sigma, St. Louis, MO, USA), human IL-12 (referred to as IL-12, Lot 95/544), rat recombinant TNF-α, sheep antiserum to rat TNF-

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α and sheep pre-immune serum. The pre-immune serum was obtained from the sheep before the immunization procedure (NIBISC, National Institute of Biological Standards and Control, South Mimms, Hertfordshire, UK); tris (Merck, Darmstadt, Germany). The LPS content of the above materials, as measured in a Limulus Amoebocyte Lysate test, was of the order of 0.25 IU mg^{-1}, which is equivalent to a little over 10^{-15} g of LPS in a hypernociceptive dose of TNF-α (2.5 pg). The threshold hypernociceptive dose of LPS in the above model is 100 ng, i.e. 10^{-7} g (Ferreira *et al.*, 1993). Therefore, the doses of the hypernociceptive agents used contained amounts of LPS up to eight $\log_{10}$'s less than the threshold hypernociceptive dose of LPS.

Statistical analysis

Results are presented as means ± s.e.m. of measurements made on 4-5 animals in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Bonferroni's *t* test was performed for each time. On the other hand, when the hyperalgesic responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's *t* test. Statistical differences were considered to be significant at $P < 0.05$.

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Results

IL-12-induced dose- and time-dependent mechanical hyperalgesia

Injection of IL-12 into the hindpaw of rats induced significant dose- (3, 10 and 30 ng in 50 μl) and time- (1, 3, 5, 7 and 24 h) dependent mechanical hyperalgesia determined by either the constant pressure rat paw test (Figure 1, panel A) or the electronic pressure-meter test (Figure 1, panel B). The mechanical hyperalgesic time course of IL-12 determined by both methods peaked 3 h after the administration of the higher dose (30 ng), and 5 h after the injection of other doses (3 and 10 ng), and was maintained at a similar level until 7 h after injection, decreasing thereafter and returning to control levels 24 h later (Figure 1). All doses of IL-12 induced significant hyperalgesia after 1, 3, 5 and 7 h in both methods, except for the dose of 3 ng in the first hour. Therefore, for the other experiments the dose of 10 ng of IL-12 was used and the mechanical hyperalgesia was determined 1, 3 and 5 h after the cytokine injection, except in the morphine group, in which it was measured 5 h after IL-12 injection. In order to determine whether IL-12 induces thermal hyperalgesia the cytokine (same doses) was injected i.pl. and the thermal hyperalgesic response was measured 1, 3 and 5 h later. However, injection of IL-12 into the hindpaw of rats did not induce significant thermal hyperalgesia (data not shown).

Effects of dexamethasone, indomethacin, atenolol, MK886 and morphine on IL-12-induced mechanical hyperalgesia

The pre-treatment of the rats with a glucocorticosteroid (dexamethasone; 2.0 mg kg^{-1}) significantly inhibited IL-12- (10 ng) induced mechanical hyperalgesia determined by

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either the constant pressure rat paw test (Figure 2, panel A) or the electronic pressure-meter test (Figure 2, panel B). However, the treatment of the animals with a standard cyclooxygenase inhibitor (indomethacin; 2.5 mg kg^{-1}), β-adrenergic antagonist (atenolol; 1.0 mg kg^{-1}) or 5-lipoxygenase activating protein inhibitor (MK886; 1.0 mg kg^{-1}) was ineffective in inhibiting IL-12- (10 ng) induced mechanical hyperalgesia in both tests (Figure 2, panels A and B). These results suggest that prostanoids, sympathetic amines or leukotrienes are not involved in IL-12-induced mechanical hyperalgesia. The fact that dexamethasone inhibited the IL-12-induced hyperalgesia suggests that this cytokine is not directly sensitizing the nociceptor, but rather that it is acting via the release of glucocorticosteroid-sensitive secondary mediators. Moreover, the treatment with an opioid agonist (morphine, 3, 6 and 12 μg, i.pl.) also inhibited in a dose-dependent manner the IL-12- (10 ng) induced mechanical hyperalgesia, and an opioid antagonist (naloxone, 1.0 mg kg^{-1}, Figure 2, panels C and D) prevented the analgesic effect of morphine (6 μg, i.pl.).

Effects of antiserum against rat TNF-α or IL-18 antibody on IL-12-induced hyperalgesia, and of IL-12 antibody on IL-18-induced hyperalgesia.

The pre-treatment of rats with antiserum against rat TNF-α (50 μl) or anti-IL-18 antibody (10 μg) did not alter IL-12- (10 ng) induced mechanical hyperalgesia determined by both methods. Furthermore, anti-IL-12 antibody (10 ng) did not alter IL-18- (40 ng) induced mechanical hyperalgesia (Figure 3). As expected, the antiserum against rat TNF-α, IL-18 antibody and IL-12 antibody inhibited TNF-α- (2.5 pg in 50 μl), IL-18- (40 ng in 50 μl) and IL-12- (10 ng in 50 μl) induced mechanical hyperalgesia determined by the constant pressure rat paw or the electronic pressure-

meter tests (Table 1). These results suggest that IL-12 is not mediating IL-18-induced mechanical hyperalgesia, and neither TNF-α nor IL-18 is mediating the IL-12-induced mechanical hyperalgesia.

Effects of endothelin ET_A and ET_B receptor antagonists on IL-12-induced mechanical hyperalgesia

As shown in Figure 4, the ET_B receptor antagonist (BQ788, 3-30 nmol) inhibited the IL-12- (10 ng) induced mechanical hyperalgesia in both tests. The higher dose of BQ788 inhibited the IL-12 hyperalgesia at all selected times using the constant pressure test (Figure 4, panel A), and in the 3rd and 5th h using the electronic pressure meter (Figure 4, panel B). On the other hand, the ET_A receptor antagonist (BQ123) did not alter IL-12-induced hyperalgesia. In agreement with these results, in previous study using these same methods, we demonstrated that ET-1- (10 pmol) induced mechanical hyperalgesia is also inhibited by an ET_B receptor antagonist (BQ788, 10 nmol), while it is not affected by BQ123 (10 nmol). Moreover, neither indomethacin nor atenolol attenuate the ET-1-induced mechanical hyperalgesia in rats (Verri *et al.*, 2004; Da Cunha *et al.*, 2004). Therefore, these results suggest that ET-1 acting on ET_B receptors mediates the IL-12-induced mechanical hyperalgesia.

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Discussion

Interleukin-12 (IL-12) is a pro-inflammatory cytokine (for review see Watford *et al.*, 2004), and recently patients receiving IL-12 as an immune therapy for cancer treatment reported hyperalgesia (Gollob *et al.*, 2000; Kang *et al.*, 2001; Lenzi *et al.*, 2002; Weiss *et al.*, 2003). Therefore, in the present study we investigated the possible hyperalgesic effect of IL-12 and its pharmacological susceptibility. We report that IL-12 induced significant dose- and time-dependent mechanical hyperalgesia in rats determined by either constant pressure paw or electronic pressure-meter tests. The IL-12 effects were restricted to the ipsilateral paw (data not shown), and the hyperalgesic dose of IL-12 (10 ng/paw) was at least 50% lower than the local dose of IL-12 used in humans as a vaccine adjuvant (Portielje *et al.*, 2005). The IL-12-induced mechanical hyperalgesia was dose-dependently inhibited by the local administration of morphine, and the non-specific opioid receptor antagonist blocked this analgesic effect. These results are in line with the observation that opiates directly block hyperalgesia induced by various mediators such as endothelin (Menéndez *et al.*, 2003), prostaglandin E_2 and I_2 (Ferreira and Nakamura, 1979b). There is evidence that morphine, besides acting on the central nervous system has a peripheral effect (Ferreira and Nakamura, 1979b; Smith *et al.*, 1982). It is noteworthy that, differently of mechanical hyperalgesia, IL-12 i.pl. injection did not induce thermal hyperalgesia determined using the Hargreaves test. Similarly, doses of TNF-α and IL-1β that induced mechanical hyperalgesia did not induce thermal hyperalgesia (Cunha *et al.*, 1992; Ferreira *et al.*, 1993; Woolf *et al.*, 1997).

The IL-12-induced hyperalgesia was not affected by treatment of the rats with indomethacin, atenolol and MK886, suggesting that prostanoids, sympathetic amines and leukotrienes are not involved in the onset of the hyperalgesia induced by this cytokine. It has been demonstrated that hyperalgesia induced by IL-6/IL-1β and by the chemokines CINC-1 or IL-8 is dependent on prostaglandin synthesis and on the release of sympathetic amines, respectively (Cunha *et al.*, 1992; Ferreira *et al.*, 1993; Lorenzetti *et al.*, 2002; Cunha *et al.*, 2000). Furthermore, the release of these cytokines is stimulated by TNF-α, which is produced in response to inflammatory stimuli, such as carrageenan and LPS (Cunha *et al.*, 1992; Ferreira *et al.*, 1993; Cunha *et al.*, 2000; Lorenzetti *et al.*, 2002). It appears that TNF-α does not participate in the IL-12-induced hyperalgesia since it was observed that antiserum against TNF-α failed to alter the response. This is consistent with the negative results obtained with indomethacin and atenolol, because these compounds inhibit the hyperalgesia induced by TNF-α (Cunha *et al.*, 1992; Ferreira *et al.*, 1993).

Recently, we have shown that the mechanism by which IL-18-induces mechanical hyperalgesia in rats depends on endothelin acting on ET_B receptors (Verri *et al.*, 2004). IL-12 and IL-18 have synergic actions in several biological processes including IFN-γ production, T cells proliferation and enhancement of cell-mediated cytotoxicity (for review see Biet *et al.*, 2002). However, the IL-12 hyperalgesia is not dependent on IL-18 since an effective dose of anti-IL-18 antibody did not affect the IL-12-induced process. Further investigating the relation between IL-12 and IL-18, the IL-18-induced mechanical hyperalgesia was not affected by anti-IL-12 antibody, suggesting that the hyperalgesia induced by these cytokines are independent one of each other. The IL-12-induced hyperalgesia was inhibited by dexamethasone. It is important

to mention that the patients under IL-12 treatments which reported pain, described in above clinical study (Gollob *et al.*, 2000; Lenzi *et al.*, 2002; Weiss *et al.*, 2003) were not receiving glucocorticosteroid therapy. Glucocorticosteroids are known inhibitors of the synthesis of eicosanoids (prostaglandins and leukotrienes), pro-inflammatory cytokines such as TNF-α, IL-2, IL-1β, IL-6 and IL-18 (for review see Goulding, 1998; Kodama *et al.*, 2002) and endothelin (Dschietzig *et al.*, 2001). Thus, taking into account the above results indicating that prostanoids, leukotrienes, TNF-α and IL-18 were not involved in the IL-12-induced hyperalgesia, the possible involvement of endothelin and its receptor subtypes was addressed.

It appears that endothelin is involved in the IL-12-induced hyperalgesia and this effect is mediated via the ET_B receptor since BQ788 (ET_B receptor antagonist), but not BQ123 (ET_A receptor antagonist) inhibited the IL-12 hyperalgesia. In this context, ET-1 induces time and dose-dependent hyperalgesia, which was inhibited by BQ788, but not by BQ123 (Da Cunha *et al.*, 2004). Furthermore, neither indomethacin nor atenolol affects ET-1-induced hyperalgesia (Ferreira *et al.*, 1989, Da Cunha *et al.*, 2004; Verri *et al.*, 2004). In agreement with our results, ET_B receptors mediate phenylbenzoquinone-induced abdominal writhing (Griswold *et al.*, 1999), carrageenan-primed knee joints articular incapacitation (DeMelo *et al.*, 1998), and cytokine- (IL-18) or ET-1-induced mechanical hyperalgesia (Verri *et al.*, 2004; Da Cunha *et al.*, 2004). However, there is also evidence that ET_A mediates nociceptive behavior (flinching) induced by direct application of ET-1 on the sciatic nerve (Davar *et al.*, 1998), as well as ET-1-induced thermal hyperalgesia (Menéndez *et al.*, 2003) and ET-1 potentiation of capsaicin-induced nociception (Piovezan *et al.*, 1998). In fact, it has also been reported that the antinociception mediated by endothelin via the ET_B receptor is opioid-sensitive

(Khodorova *et al.*, 2003). However, other investigators have reported that both ET_A and ET_B mediate nociception in abdominal writhing (Raffa *et al.*, 1996) and carrageenan-induced mechanical hyperalgesia (Baamond *et al.*, 2004). These apparent discrepancies could be due to differences in experimental nociceptive models, the dose of endothelin, and also time intervals of the nociceptive responses, which might detect the hyperalgesia of different sets of primary sensory neurons.

In conclusion, we demonstrated here for the first time that IL-12 induces mechanical hyperalgesia mediated by endothelin action on ET_B receptors in an opioid-sensitive manner in rats. This finding not only highlights a possible adverse effect of IL-12 therapy, but also suggests that selective antagonism of the endothelin ET_B receptor could be of value to prevent IL-12 therapy-induced clinical pain and hyperalgesia in humans.

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Footnotes

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Legends for Figures

Figure 1: IL-12 induces dose- and time-dependent mechanical hyperalgesia. The intensity of hyperalgesia was measured 1, 3, 5, 7 and 24 h after IL-12 (3, 10 and 30 ng in 50 μl, i.pl.) administration by the constant pressure paw test (Panel A) or the electronic pressure-meter test (Panel B). Before the injection of the hyperalgesic agents, the reaction time and reaction force were 31.3 ± 0.3 s and 43.6 ± 0.7 g (means ± s.e.m.; n = 6 groups), respectively. Bars represent means ± s.e.m. of 5 rats per group. * $P < 0.05$ compared to saline control, and ** $P < 0.05$ compared to IL-12, 3 ng paw^{-1} (one-way ANOVA followed by Bonferroni's t-test).

Figure 2: Effects of dexamethasone, indomethacin, atenolol, MK886 and morphine on IL-12-induced mechanical hyperalgesia. Upper panels (A and B): The animals were pre-treated with dexamethasone (Dexa, 1 h, 2.0 mg kg^{-1}, s.c., glucocorticosteroid), indomethacin (Indo, 30 min, 2.5 mg kg^{-1}, s.c., cyclooxygenase inhibitor), atenolol (Atn, 30 min, 1.0 mg kg^{-1}, s.c., β-adrenergic antagonist) or MK886 (24 h plus 1 h, 1.0 mg kg^{-1}, per oral, 5-lipoxygenase activating protein inhibitor) before IL-12 (10 ng in 50 μl, i.pl.) administration. The intensity of hyperalgesia was measured 1, 3 and 5 h after IL-12 injection by the constant pressure paw (Panel A) or the electronic pressure-meter (Panel B) tests. Lower panels (C and D): After the administration of IL-12 (10 ng in 50 μl, i.pl.), the animals were treated with saline (Sal, 200 μL) or morphine (4 h after IL-12 injection, 3-12 μg in 50 μl, i.pl.). The animals also received an injection of naloxone (1.0 mg kg^{-1} in 500 μl, i.p.) 30 min before morphine (6 μg paw^{-1}) injection. The intensity of hyperalgesia was measured 5 h after IL-12 injection by the constant

pressure paw test (Panel C) or the electronic pressure-meter test (Panel D). Before the injection of the hyperalgesic agents, the reaction time and reaction force were 32.0 ± 0.2 s and 43.2 ± 0.7 g (means ± s.e.m.; n = 12 groups), respectively. Bars represent means ± s.e.m. of 4-5 rats per group, except for the vehicle bar in the upper panels (A and B) that represents means ± s.e.m of 4 groups (one for each drug treatment). * $P < 0.05$ compared to the respective control (one-way ANOVA followed by Bonferroni's t-test).

Figure 3: Effects of antiserum against rat TNF-α or IL-18 antibody on IL-12-induced hyperalgesia, and of IL-12 antibody on IL-18-induced hyperalgesia. The rats were pre-treated with antiserum against rat TNF-α (α-TNF-α, 15 min, 50 μl, i.pl.), with anti-IL-18 antibody (α-IL-18, 30 min, 10 μg in 50 μl, i.pl.) or control serum (α-control, 15 min, 50 μl, i.pl.) before IL-12 (10 ng in 50 μl, i.pl.) administration. Rats were also pretreated with anti-IL-12 antibody (α-IL-12, 15 min, 10 ng, 50 μl, i.pl.) or control serum (50 μl, i.pl.) before IL-18 (40 ng in 50 μl, i.pl.) injection. The intensity of hyperalgesia was measured 1, 3 and 5 h later by the constant pressure paw test (Panel A) or the electronic pressure-meter test (Panel B). Before the injection of the hyperalgesic agents, the reaction time and reaction force were 31.7 ± 0.2 s and 43.4 ± 0.6 g (means ± s.e.m.; n = 8 groups), respectively. Bars represent means ± s.e.m. of 4-5 rats per group. * $P < 0.05$ compared to the respective control (one-way ANOVA followed by Bonferroni's t-test).

Figure 4: Effects of endothelin ET_A and ET_B receptor antagonists on IL-12-induced mechanical hyperalgesia. The animals were pre-treated with BQ788 (3-30 nmol, in 50 μl, i.pl.) or BQ123 (30 nmol in 50 μl, i.pl.) 30 min before IL-12 (10 ng in 50 μl, i.pl.). The intensity of hyperalgesia was measured 1, 3 and 5 h after the IL-12 injection by the

constant pressure paw test (Panel A) or the electronic pressure-meter test (Panel B). Before the injection of the hyperalgesic agents, the reaction time and reaction force were 31.6 ± 0.2 s and 44.5 ± 0.4 g (means ± s.e.m.; n = 10 groups), respectively. Bars represent means ± s.e.m. of 4-5 rats per group. * $P < 0.05$ compared to the respective control (one-way ANOVA followed by Bonferroni's t-test).

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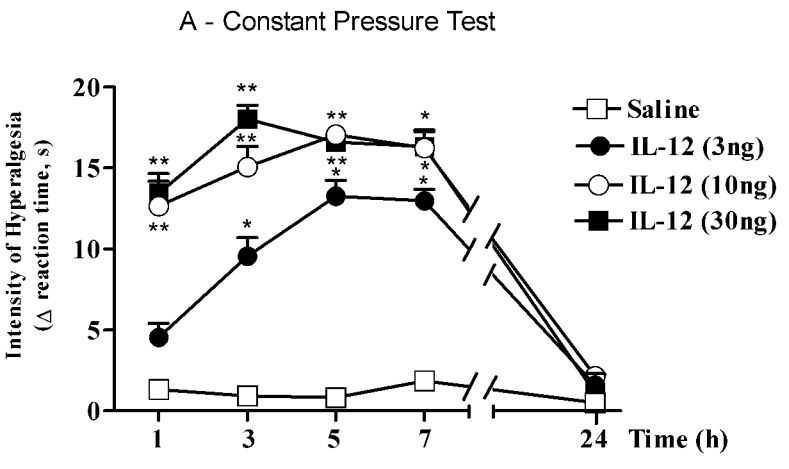
Table 1: Effects of antisera against TNF-α, IL-12 and IL-18 and their respective control sera on TNF-α-, IL-12- and IL-18-induced mechanical hyperalgesia, respectively.

	Constant Pressure Test (Δ Reaction time, s)			**Electronic Pressure-Meter Test (Δ Reaction force, g)**		
Group/Time	**1 h**	**3 h**	**5 h**	**1 h**	**3 h**	**5 h**
Saline + Saline	0.9±0.3	1.5±0.6	1.3±0.5	0.7±0.7	0.5±0.3	0.7±0.5
α-control + Saline	1.1±0.5	1,6±0.8	2.0±0,7	1,6±0,5	1,8±0,9	1,9±0,6
α-control + TNF-α	9.4±0.6*	16.2±0.4*	12.6±1.1*	12.9±0.8*	17.9±1.4*	14.5±1.6*
α-TNF-α + TNF-α	2.0±0.4†	3.9±1.1†	3.5±0.5†	8.1±1.9†	8.3±1.4†	8.1±0.5†
α-control + IL-12	15.5±0.6*	17.2±0.8*	18.6±0.5*	12.4±1.7*	18.4±1.6*	18.6±1.4*
α-IL-12 + IL-12	1.2±0.6†	1.5±0.7†	1.4±0.6†	6.8±0.7†	7.7±0.8†	8.7±1.4†
α-control + IL-18	8.3±1.4*	16.3±0.8*	14.7±0.5*	15.4±6.2*	27.6±3.8*	22.8±2.6*
α-IL-18 + IL-18	1.2±0.4†	4.6±1.1†	1.9±0.6†	1.0±0.6†	7.3±0.5†	10.0±0.9†

The rats were treated with antisera against rat TNF-α (α-TNF-α, 15 min, 50 μl, i.pl.), IL-12 antibody (α-IL-12, 30 min, 10 ng in 50 μl, i.pl.), IL-18 antibody (α-IL-18, 30 min, 10 μg in 50 μl, i.pl.) or control sera (α-control, 15 min, 50 μl, i.pl.) before TNF-α (2.5 pg in 50 μl, i.pl.), IL-12 (10 ng in 50 μl, i.pl.), IL-18 (40 ng in 50 μl, i.pl.) or saline (50 μl, i.pl.) administration, respectively. Each group represents means ± s.e.m. of 5 animals. * $P < 0.05$ compared to the α-control + saline group. † $P < 0.05$ compared to the α-control + respective cytokine group (one-way ANOVA followed by Bonferroni's t-test).

Figure 1



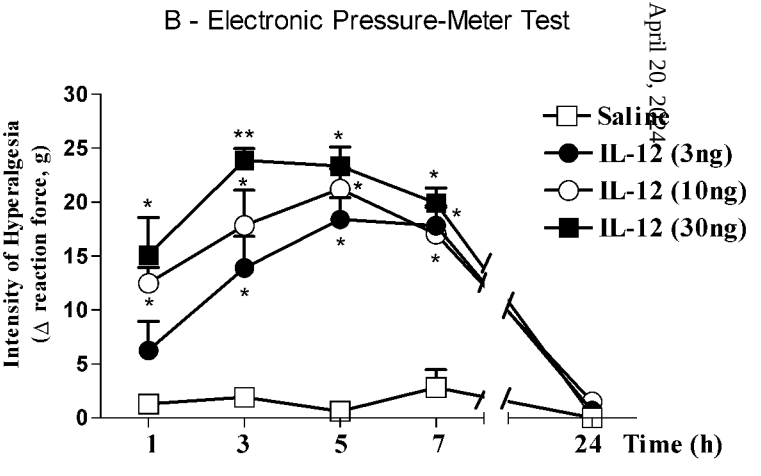


Figure 2

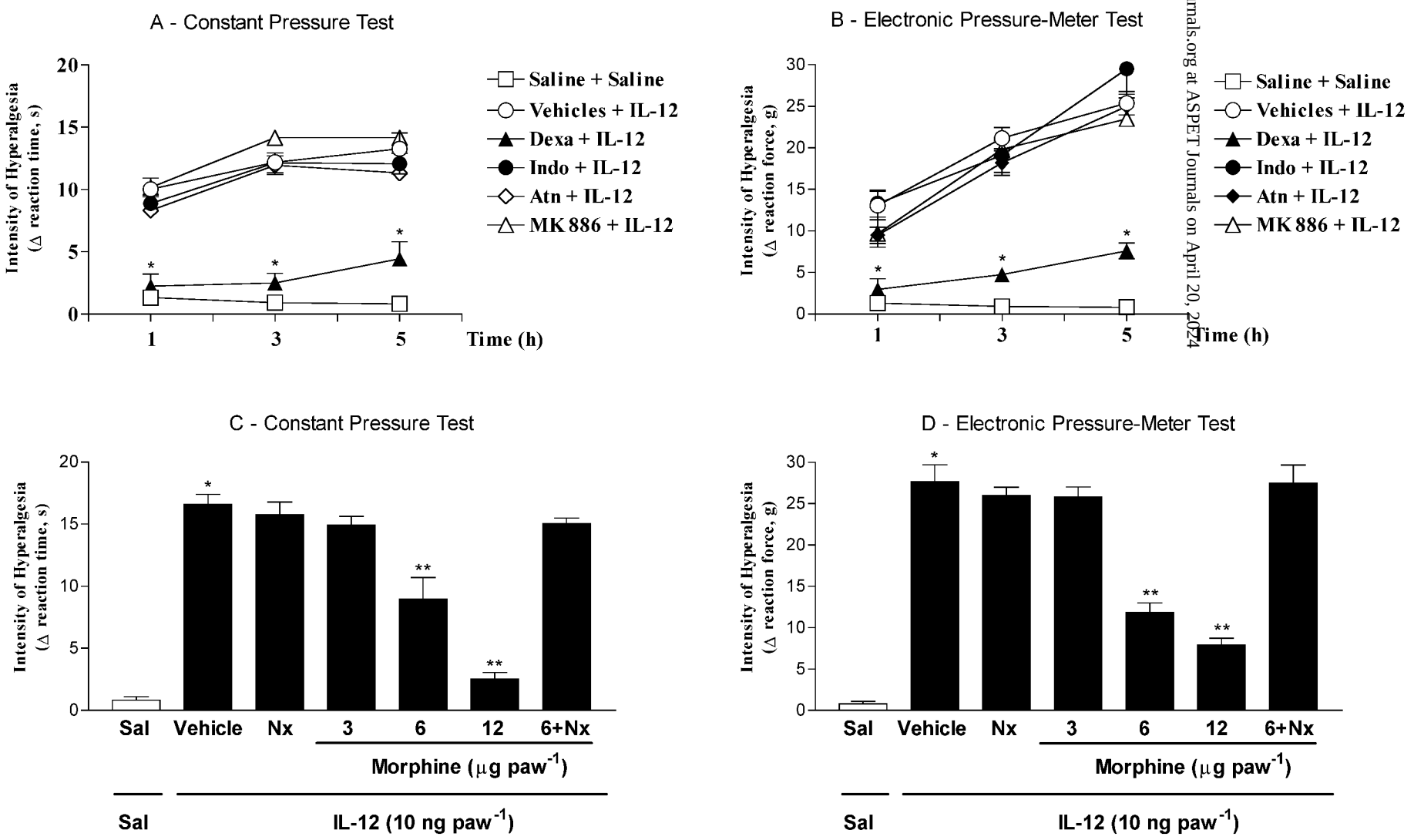


Figure 3

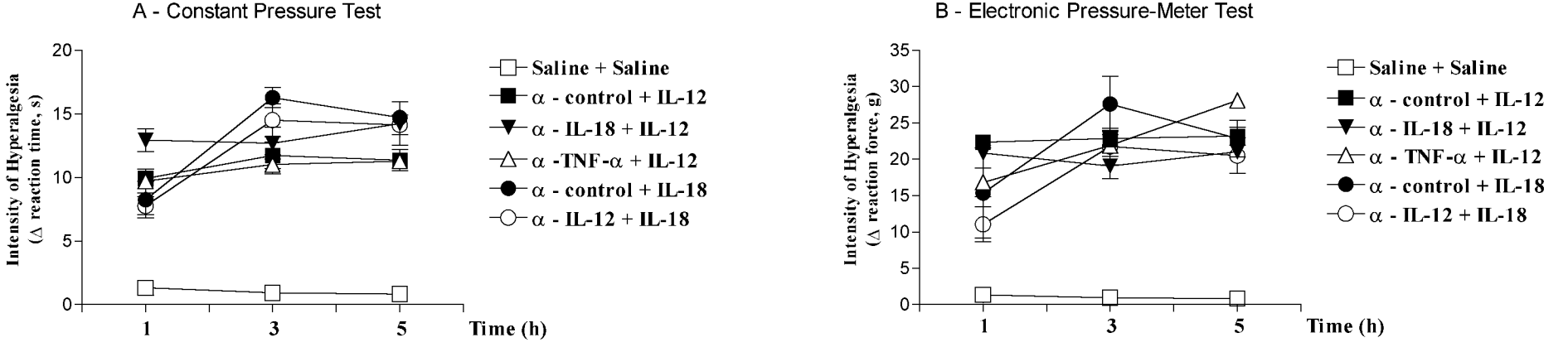
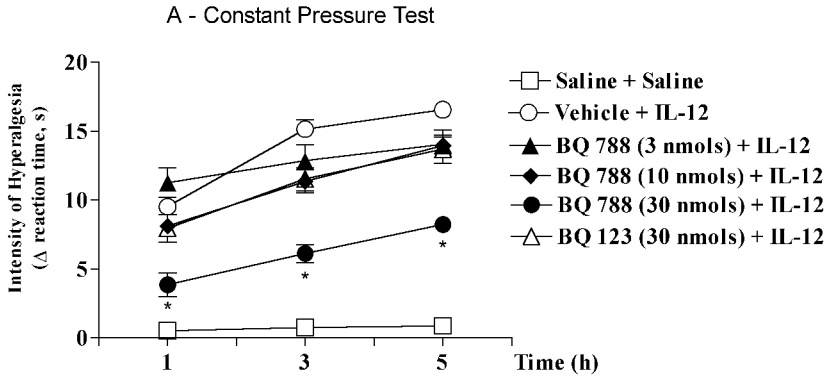
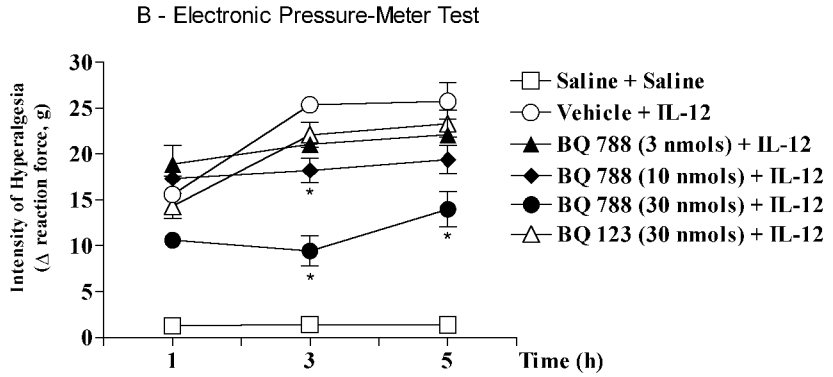

A - Constant Pressure Test
Intensity of Hyperalgesia (Δ reaction time, s)
20
15
10
5
0
1
3
5
Time (h)
Saline + Saline
α - control + IL-12
α - IL-18 + IL-12
α -TNF-α + IL-12
α - control + IL-18
α - IL-12 + IL-18
B - Electronic Pressure-Meter Test
Intensity of Hyperalgesia (Δ reaction force, g)
35
30
25
20
15
10
5
0
1
3
5
Time (h)
Saline + Saline
α - control + IL-12
α - IL-18 + IL-12
α - TNF-α + IL-12
α - control + IL-18
α - IL-12 + IL-18

Figure 4


A - Constant Pressure Test
Intensity of Hyperalgesia (Δ reaction time, s)
20
15
10
5
0
1
3
5
Time (h)
Saline + Saline
Vehicle + IL-12
BQ 788 (3 nmols) + IL-12
BQ 788 (10 nmols) + IL-12
BQ 788 (30 nmols) + IL-12
BQ 123 (30 nmols) + IL-12
*


B - Electronic Pressure-Meter Test
Intensity of Hyperalgesia (Δ reaction force, g)
30
25
20
15
10
5
0
1
3
5
Time (h)
Saline + Saline
Vehicle + IL-12
BQ 788 (3 nmols) + IL-12
BQ 788 (10 nmols) + IL-12
BQ 788 (30 nmols) + IL-12
BQ 123 (30 nmols) + IL-12
*