Nociceptive Effect of Subcutaneously Injected Interleukin-12 is Mediated by Endothelin Acting on ET_{B} Receptors in Rats


Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Avenida Bandeirantes, 3900, 14049-900- Ribeirão Preto, São Paulo, Brazil (W.A.V.J., R.O.M., I.R.S.S., T.M.C., C.A.P., S.H.F., F.Q.C.); and NIBISC, National Institute of Biological Standards and Control, South Mimms, Hertfordshire, UK (S.P.).
Running title: IL-12 Hyperalgesia: Endothelin Action on ET<sub>B</sub> Receptors

Author for correspondence: Prof. Dr. Fernando de Queiroz Cunha

Present address: Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, 14049-900- Ribeirão Preto, São Paulo, Brazil. Fax: + 55 16 633-0021, Tel: + 55 16 602-3205. E-mail address: fdqcunha@fmrp.usp.br

Number of text pages: 32
Number of figures: 4
Number of tables: 1
Number of references: 40
Number of words in the: Abstract: 242
Introduction: 448
Discussion: 957

Abbreviations: Adenosine 3'5' cyclic monophosphate (cAMP), cyclo[DTrp-DAsp-Pro-<br>Val-Leu] (BQ123); N-cys-2,6 dimethylpiperidinocarbonyl-L-γ-methyleucyl-D-1-<br>methoxycarboyl-D-norleucine (BQ788), cytokine-induced neutrophil chemoattractant 1 (CINC-1), endothelin receptor type A (ET<sub>A</sub>), endothelin receptor type B (ET<sub>B</sub>), T helper 1 (Th1), tumor necrosis factor-alpha (TNF-α), interleukin (IL), 3-[1-(p-chlorobenzyl)-5-<br>(isopropyl)-3-<br>-butylthioindol-2-yl]-2,2-dimethylpropanoic acid, Na (MK886).

Section: Inflammation & Immunopharmacology
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.
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 ETB receptor.
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-mm² 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
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 mm² polypropylene tip
(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
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-diyl)/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
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.
Role of endothelin and its receptors in IL-12-induced mechanical hyperalgesia

BQ123 (30 min, 30 nmol in 50 µl, i.pl., an ETA receptor antagonist) or BQ788 (30 min, 3-30 nmol in 50 µl, i.pl., an ETB 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[DTrp-DAsp-Pro-DVal-Leu], Novabiochem, La Jolla, CA, USA, Lot A21510); BQ788, sodium salt (N-cys-2,6 dimethylpiperidinocarbonyl-L-γ-methylleucyl-D,1-methoxycarbonyl-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-
\[ \alpha \] 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-\(\alpha\) (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 \(P < 0.05\).
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
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.
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 E₂ and I₂ (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 ETB 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$\alpha$ and ET$\beta$ 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$\beta$ 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$\beta$ receptor could be of value to prevent IL-12 therapy-induced clinical pain and hyperalgesia in humans.
Acknowledgments

We thank Sérgio Roberto Rosa for technical assistance.
References


Ferreira SH, and Nakamura MI (1979a) Prostaglandin hyperalgesia, a cAMP/ Ca\(^{2+}\) dependent process. *Prostaglandins.* **18**:179-190.


Footnotes

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa (CNPq), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Programa de Núcleos de Excelência (PRONEX), Brazil, and NIBISC, National Institute of Biological Standards and Control, South Mimms, Hertfordshire, UK.

W.A.V.J. and R.O.M. contributed equally.

Part of this work has been presented as an abstract at the XXXIV Brazilian Congress of Pharmacology and Experimental Therapeutics, Águas de Lindoia – SP - Brazil, October 28 – 31, 2002.

Address reprint requests to: Fernando de Queiroz Cunha

Present address: Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, 14049-900- Ribeirão Preto, São Paulo, Brazil. Fax: + 55 16 633-0021, Tel: + 55 16 602-3205.
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 pretreated 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 $31.6 \pm 0.2 \text{ s}$ and $44.5 \pm 0.4 \text{ g}$ (means $\pm$ s.e.m.; $n = 10$ groups), respectively. Bars represent means $\pm$ 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).
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.

<table>
<thead>
<tr>
<th>Group/Time</th>
<th>Constant Pressure Test (Δ Reaction time, s)</th>
<th>Electronic Pressure-Meter Test (Δ Reaction force, g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Saline + Saline</td>
<td>0.9±0.3</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>α-control + Saline</td>
<td>1.1±0.5</td>
<td>1.6±0.8</td>
</tr>
<tr>
<td>α-control + TNF-α</td>
<td>9.4±0.6*</td>
<td>16.2±0.4*</td>
</tr>
<tr>
<td>α-TNF-α + TNF-α</td>
<td>2.0±0.4†</td>
<td>3.9±1.1†</td>
</tr>
<tr>
<td>α-control + IL-12</td>
<td>15.5±0.6*</td>
<td>17.2±0.8*</td>
</tr>
<tr>
<td>α-IL-12 + IL-12</td>
<td>1.2±0.6†</td>
<td>1.5±0.7†</td>
</tr>
<tr>
<td>α-control + IL-18</td>
<td>8.3±1.4*</td>
<td>16.3±0.8*</td>
</tr>
<tr>
<td>α-IL-18 + IL-18</td>
<td>1.2±0.4†</td>
<td>4.6±1.1†</td>
</tr>
</tbody>
</table>

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

A - Constant Pressure Test

- Intensity of Hyperalgesia (Δ reaction time, s)

1. Saline
2. IL-12 (3ng)
3. IL-12 (10ng)
4. IL-12 (30ng)

Time (h): 1, 3, 5, 7, 24

B - Electronic Pressure-Meter Test

- Intensity of Hyperalgesia (Δ reaction force, g)

1. Saline
2. IL-12 (3ng)
3. IL-12 (10ng)
4. IL-12 (30ng)

Time (h): 1, 3, 5, 7, 24
**Figure 3**

**A - Constant Pressure Test**

- Open squares: Saline + Saline
- Solid squares: α - control + IL-12
- Inverted triangles: α - IL-18 + IL-12
- Triangles: α - TNF-α + IL-12
- Solid circles: α - control + IL-18
- Open circles: α - IL-12 + IL-18

Intensity of Hypoalgesia (Δ reaction time, s) vs. Time (h)

**B - Electronic Pressure-Meter Test**

- Open squares: Saline + Saline
- Solid squares: α - control + IL-12
- Inverted triangles: α - IL-18 + IL-12
- Triangles: α - TNF-α + IL-12
- Solid circles: α - control + IL-18
- Open circles: α - IL-12 + IL-18

Intensity of Hypoalgesia (Δ reaction force, g) vs. Time (h)