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Authors

Alvarez, Pedro
Bogen, Oliver
Green, Paul G
et al.

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Nociceptor interleukin 10 receptor 1 is critical for muscle analgesia induced by repeated bouts of eccentric exercise in the rat

Pedro Alvarez[1,2], **Oliver Bogen**[1,2], **Paul G. Green**[1,2], and **Jon D. Levine**[1,2,3,*]

[1]Department of Oral and Maxillofacial Surgery, University of California San Francisco, San Francisco, California, USA

[2]Division of Neuroscience, University of California San Francisco, San Francisco, California, USA

[3]Department of Medicine, University of California San Francisco, San Francisco, CA, USA

Keywords

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Introduction

Persistent skeletal muscle pain is an extremely common, disabling and costly condition, which still lacks safe and effective treatment. Although regular exercise can provide relief for chronic muscle pain conditions such as fibromyalgia [8,29,38] and myofascial pain [10], unaccustomed exercise may produce intense muscle pain [4,24] (for a review, see [37]). Indeed, muscle pain due to exertion is a major cause of limited compliance in exercise therapy, leading to a dropout rate of up to 44% in patients with fibromyalgia [8]. Thus, unraveling the mechanisms modulating muscle pain after exercise could not only clarify its physiological basis but also reveal new targets for the treatment of chronic muscle pain syndromes.

Exposure to strenuous eccentric exercise typically produces an intense and persistent mechanical hyperalgesia known as delayed onset muscle soreness (DOMS). Subsequent re-exposure to eccentric exercise, however, produces the so-called repeated bout effect (RBE), evidenced as a significant reduction in the intensity and duration of DOMS [23,30,37]. While the mechanism underlying the RBE has been elusive, recent studies indicate that exercise increases muscle and blood levels of interleukin 10 (IL-10) [9,18], an anti-inflammatory cytokine with well-established antinociceptive properties [41]. Local IL-10 acts on a number of different cell populations, including macrophages, neutrophils and skeletal muscle fibers, orchestrating muscle growth and regeneration after injury [16]. These IL-10 effects depend on its interaction with a heterodimer receptor complex, constituted by

*Corresponding author's contact information: Dr. Jon Levine, University of California San Francisco, 513 Parnassus Ave, Room S709, San Francisco, CA 94143-0440. Tel.: +1 415 476 5108; fax: +1 415 476 6305. jon.levine@ucsf.edu.

the high affinity subunit 1 (IL-10R1, also called subunit alpha) which is necessary for the binding of IL-10 [28,53], and the low-affinity IL-10 receptor 2 (IL-10R2, also called subunit beta and previously referred to as CRFB4), which is essential for IL-10 induced signal transduction events [25,53]. Interestingly, IL-10R1/subunit alpha, is expressed in dorsal root ganglia (DRG) [46] and intrathecal administration of recombinant IL-10 or adenoviral vectors containing IL-10 cDNA attenuates increased excitability of DRG neurons and abnormal nociceptive behavior in diverse neuropathic pain models [26,33,34,46,55]. Moreover, local and systemic administration of IL-10 attenuates muscle pain due to exercise [9] or to intramuscular injections of acidic saline [27] and inhibits increased muscle hyperalgesia due to exercise in IL-10 (−/−) mice [9]. It is however unknown as to whether IL-10 also contributes to blunted muscle hyperalgesia observed in RBE. Furthermore, whether IL-10 directly acts on muscle nociceptors to produce its antinociceptive effect remains to be established. Here we explored the hypothesis that blunted muscle pain to eccentric exercise observed in the RBE depends on a local effect of IL-10, acting on its receptor expressed by muscle nociceptors.

Materials and Methods

Animals

Adult male Sprague-Dawley (Crl:CD, Charles River, Hollister, CA) rats (n = 71) weighing 250–400 g were used in these experiments. They were housed in the Laboratory Animal Resource Center at UCSF, under environmentally controlled conditions (lights on 7 am to 7 pm; room temperature 21–23°C) with food and water available *ad libitum*. Animal care and use conformed to NIH guidelines; the University of California San Francisco Institutional Animal Care and Use Committee approved all experimental protocols.

Eccentric exercise

The method used to eccentrically exercise the rat hind limb was described in detail previously [4]. Briefly, rats were anesthetized with 2.5% isoflurane (Henry Schein Animal Health, Dublin, OH) and placed in supine position, on a heating pad (to maintain body temperature at 37°C), and the right hind paw affixed to the foot bracket of the exercise apparatus (Model RU-72, NEC Medical Systems, Tokyo, Japan) with surgical paper tape (Micropore® 3M), such that the angle of both the knee and ankle joints were ~90° (the paw 30° from vertical). After clipping and disinfecting the skin on the calf, the gastrocnemius muscle was stimulated via subcutaneous needle-type electrodes (25 G × 5/8″, Becton, Dickinson & Co., Franklin Lakes, NJ), attached to a Model DPS-07 stimulator (Dia Medical System Inc, Tokyo, Japan) that delivered trains of rectangular pulses (100 Hz, 700 ms, 3 V) every 3 seconds, to give a total of 300 contractions. During each of these stimulus-induced contractions of the gastrocnemius muscle, an electromotor system rotated the foot to produce extension of the gastrocnemius muscle. In order to study the RBE, rats were submitted to 2 different protocols. In the first protocol (RBE5) rats were exposed to eccentric exercise or a sham procedure, and 5 days later (i.e., after recovery to baseline) the same hind limb was again submitted to the same eccentric exercise procedure. In the second protocol (RBE12) rats were exposed to eccentric exercise or a sham procedure and 12 days later (one week after baseline recovery) the same hind limb was submitted to the eccentric

exercise protocol. The sham (control) procedure consisted in shaving and disinfecting the calf area, placing needle electrodes, and keeping the rats in the same anesthesia regimen used for eccentric exercise procedure, without electrically stimulating or lengthening the leg.

Measurement of hyperalgesia

Mechanical nociceptive threshold was quantified using a Chatillon® digital force transducer (model DFI2, Amtek Inc., Largo, FL). Rats were lightly restrained in a cylindrical acrylic restrainer that allows for easy access to the hind limb, and a 6 mm diameter probe attached to the force transducer applied to the gastrocnemius muscle, to deliver an increasing compression force. The nociceptive threshold was defined as the force, in miliNewtons (mN), at which the rat withdrew its hind leg. Baseline withdrawal threshold was defined as the mean of 2 readings taken at 5 min intervals. Rats were assigned randomly to each experimental group and behavioral readings were taken blind to the treatment.

Intrathecal oligodeoxynucleotide administration

To assess the role of IL-10 in eccentric exercise induced muscle hyperalgesia and RBE, the expression of its cognate receptor (IL-10R1/subunit alpha) on muscle nociceptors was attenuated by intrathecal (i.t.) injection of antisense (AS) oligodeoxynucleotides (ODN) directed against IL-10R1 mRNA. This approach has been shown to produce a reversible inhibition of the expression of several proteins involved in pain processing in DRG neurons (for a review see [49]) and to modulate a number of behavioral nociceptive responses, including mechanical hyperalgesia in skeletal muscle [2–4,13,17]. The AS ODN sequence, 5′-TTG TCC CGT AGA TGA AGC CGT TGC-3′, was directed against a unique sequence in rat IL-10R1 mRNA. The corresponding NCBI GenBank accession number and ODN position within the cDNA sequence are NM_057193 and 461–484. The mismatch (MM) ODN sequence, 5′-**A**TG TC**G** **T**GT A**AC** TGA AGC CGT **A**G**A**-3′, corresponds to the IL-10R1 AS sequence with 7 mismatched bases (denoted by bold letters). A nucleotide BLAST search was performed to confirm that the mRNA sequence targeted by the antisense ODN, or its MM control, were not homologous to any other sequences in the rat database. Rats received daily i.t. injections (40 μg/20 μl), with ODN either AS or MM to IL-10R1 mRNA, for 5 consecutive days. The AS and MM ODNs were synthesized by Invitrogen (Carlsbad, CA). To perform i.t. injections rats were anaesthetized with 2.5% isoflurane in 97.5% O_2. A 29-gauge hypodermic needle was then inserted into the subarachnoid space on the midline, between the L4 and L5 vertebrae. Proper i.t. injection was confirmed by observation of a tail-flick [32].

Tissue harvesting and Western blot analysis

Changes in IL-10R1 expression in lumbar DRGs were evaluated by Western blot analysis as previously described [13]. Briefly, rats were euthanized by exsanguination, while under deep isoflurane anesthesia, 24 h after the last i.t. injection of AS or MM ODN. The L4 and L5 DRGs were quickly dissected bilaterally and immediately transferred to cold homogenization buffer [150 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.4], supplemented with a 2x protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). DRGs were homogenized manually and

proteins solubilized by incubation in an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany) for 4 h at 25°C and 1400 rpm. Proteins were then extracted by centrifugation for 15 min at 4ºC and 14,000 rpm in an Eppendorf tabletop centrifuge, and the protein concentration of the samples determined using a micro BCA Protein Assay Kit (Thermo Fisher, Waltham, MA) with bovine serum albumin (BSA) as the standard. Mixtures of 40 μg of protein per sample were denatured by heating at 90°C for 10 min in sample buffer [3% SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.025% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) and electrophoresed on a 4% to 15% precast polyacrylamide gel (Biorad, Hercules, CA) in 25 mM Tris containing 192 mM glycine and 0.1% SDS. Proteins were then transferred to a nitrocellulose membrane using the semidry method (transfer time, 1 h at 10 V). The nitrocellulose membranes were saturated by shaking in antibody dilution buffer [5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST)] for 1 h at room temperature (RT), cut in half at ~50 kDa and incubated with either rabbit anti-IL-10R1 (sc-985, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-β-actin (ab8227, 1:1000, Abcam, Cambridge, MA) antibodies in antibody dilution buffer, at 4°C overnight. After washing with TBST (3 times at RT, 15 min each), the IL-10R1 blot was probed with a biotinylated goat anti-rabbit antibody (111-065-003, 1:2500, Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 2 h at RT, whereas the β-actin blot was probed with a horseradish peroxidase conjugated anti-rabbit antibody (NA934V, 1:2500, GE Healthcare, Piscataway, NJ) for 2 h at RT. Blots were washed with TBST (3 times at RT, 15 min each) and the IL-10R1 blot was then probed with a streptavidin-peroxidase polymer (S2438, 1:5000, Sigma-Aldrich, St. Louis, MO) for 1.5 h while shaking at RT. After washing with TBST (3 times at RT, 15 min each), immunoreactivity was visualized using the ultrasensitive West femto chemiluminescence detection system (Thermo Fisher). Results were analyzed using computer-assisted densitometry and levels of IL-10R1 immunoreactivity were normalized with respect to the β-actin control levels in each sample. The percentage decrease in IL-10R1 expression was calculated as: [normalized density for AS/normalized density for MM × 100]-100.

Measurement of IL-10 levels in the gastrocnemius muscle

Naïve (control) rats, or rats submitted to the RBE12 protocol, were deeply anaesthetized with 5% isoflurane in 95% O_2 and euthanized by exsanguination. The exercised (or control) gastrocnemius muscle was quickly dissected, placed in cold homogenization buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and homogenized with the aid of a hand-held tissue blender. The proteins of the homogenate were extracted by centrifugation at 14,000 g for 15 min at 4°C and the protein concentration was determined using the micro BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA as the standard. The IL-10 levels in muscle tissue extracts were measured by ELISA (rat IL-10 R1000, Quantikine® ELISA, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. All measurements were performed in duplicate and data are presented as pg of IL-10 per mg of protein.

Statistical analysis

Group data are expressed as mean ± SEM of n independent observations. Statistical comparisons were made using GraphPad Prism 6.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between treatments were made by means of Student's *t*-test or two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results

Attenuated hyperalgesia produced by repeated bouts of eccentric exercise

Comparison between experimental groups (RBE5 single [control] and double EE; RBE12 single [control] and double EE) by two-way repeated measures ANOVA showed a significant effect for treatment ($F_{3,20}$ =9.340, P = 0.0005), time ($F_{6,120}$ =362.2, P < 0.0001), and interaction ($F_{18,120}$ =80.78, P < 0.0001). Bonferroni's multiple comparisons post-hoc test showed that in RBE5 protocol, after first bout of eccentric exercise, significant differences in mechanical nociceptive threshold were observed between treatments, on days 1 (−38.2 ± 1.1% exercised group [1590.2 ± 24.1 mN] *vs* −5.8 ± 2.2% control group [2433.8 ± 62.8 mN]; P < 0.001) and 3 (−28.3 ± 3% exercised group [1845.5 ± 70.6 mN] *vs* −0.8 ± 1.1% control group [2562.7 ± 28.5 mN]; P < 0.001) after eccentric exercise (n=6/group, Fig. 1A). No significant differences were observed at day 5 after eccentric exercise (−2.1 ± 1.7% exercised group [2518.7 ± 29.8 mN] *vs* 3.1 ± 0.9%, control group [2664.3 ± 21.1 mN]; P > 0.05, Fig. 1A). Similar responses were observed in rats submitted to RBE12 protocol after first bout of eccentric exercise, with significant differences on days 1 (−39.5 ± 0.9% exercised group [1539 ± 20.5 mN] *vs* −0.5 ± 0.5% control group [2541.5 ± 11 mN]; P < 0.001) and 3 (−26.2 ± 2% exercised group [1878.5 ± 45.4 mN] *vs* −0.2 ± 0.4% control group [2550.3 ± 15.9 mN], P < 0.001), but not at day 5 (1.1 ± 0.8% exercised group [2571.2 ± 9.7 mN] *vs* 0.8 ± 0.4% control [2575.7 ± 8.5 mN]; P > 0.05) after eccentric exercise (n=6/group; Fig. 1B).

Bonferroni's post-hoc analysis also showed that exposure to a second bout of eccentric exercise, 5 days after first treatment (RBE5 protocol), produced significant differences in nociceptive threshold between rats previously submitted to the sham (control) procedure or eccentric exercise. These differences were observed on days 1 (−38.5 ± 1.6% single eccentric exercise group [1589.3 ± 42.6 mN] *vs* −23.5 ± 2.7% double eccentric exercise group [1968.7 ± 71.7 mN], P < 0.001) and 3 (−33.2 ± 1%, single eccentric exercise [1727.7 ± 27.4 mN] *vs* −21.1 ± 3.6% double eccentric exercise group [2032.7 ± 98.2 mN], P < 0.01), but not on day 5 (−4.2 ± 0.7% single eccentric exercise [2475.5 ± 12.1 mN] *vs* −1.9 ± 0.9% double eccentric exercise [2525.3 ± 22.8 mN], P > 0.05) after last bout of eccentric exercise (Fig. 1A). Similarly, experimental groups submitted to eccentric exercise 12 days after initial treatment (RBE12 protocol) produced significant differences in nociceptive threshold between rats previously submitted to the sham (control) procedure or eccentric exercise on days 1 (−40.2 ± 1.0% single eccentric exercise group [1526.8 ± 24.2 mN] *vs* −16.1 ± 1.3% double eccentric exercise [2135.8 ± 37.6 mN], P < 0.001), 3 (−31.8 ± 1.3% single eccentric exercise group [1741.3 ± 32.4 mN] *vs* −16.7 ± 2.2% double eccentric exercise group [2119 ± 52.3 mN], P < 0.001), and 5 (−7.7 ± 0.8% single eccentric exercise group [2357.3 ± 20.6

mN] *vs* −0.9 ± 0.8% double eccentric exercise group [2521.7 ± 20.4 mN], $P < 0.05$) after last bout of eccentric exercise (Fig. 1B). Furthermore, comparison of nociceptive thresholds between rats submitted to double eccentric exercise in RBE5 and RBE12 protocols showed a significant difference at day 1 ($P < 0.05$), but not at days 3 or 5 ($P > 0.05$), after last bout of eccentric exercise. Therefore, given the greater antinociceptive effect of the RBE12 protocol, it was used to explore the involvement of IL-10 and its canonical receptor in the RBE phenomenon (see below).

Antisense knockdown of IL-10R1 expression in nociceptors

In order to determine whether IL-10 acting on nociceptors is involved in the repeated bout effect, we designed an AS ODN targeting IL-10R1 mRNA. Western blot analysis of L4–L5 DRG extracts from rats submitted to 5 consecutive days of i.t. AS ODN treatment demonstrated a significant decrease in IL-10R1 expression (−22.9 ± 5.3%, Student's *t*-test, n = 5 rats/group, $P < 0.05$, Fig. 2A) when compared to the MM ODN treated group. Comparison of IL-10R1 protein expression, normalized to the reference protein β-actin, also showed a significant difference between rats treated with AS ODN (13.2 ± 1.2 arbitrary units, a.u.) or MM ODN (17.6 ± 1.7 a.u., $P < 0.05$, Fig. 2B). These results indicate that i.t. AS ODN treatment produces significant knockdown of IL-10R1 in L4–L5 DRGs, which contain the somas of the sensory neurons that innervate the gastrocnemius muscle.

Knockdown of nociceptor IL-10R1 does not affect baseline muscle nociception

We next determined whether IL-10R1 plays a role in the maintenance of baseline mechanical nociceptive threshold. Baseline nociceptive threshold values were similar in rats to be subsequently treated with AS (2586 ± 17.6 mN, n=6) or MM (2576.3 ± 15.3 mN, n=6, $P > 0.05$, Fig. 3A). The daily i.t. ODN treatment for 5 days, did not significantly affect nociceptive threshold in rats injected with AS (2582.5 ± 20.1 mN, $P > 0.05$, Fig. 3A) or MM (2581.8 ± 20.3 mN, $P > 0.05$, Fig. 3A).

Knockdown of IL-10R1 in muscle nociceptors disrupts RBE analgesia

To evaluate the relative contribution of IL-10 in the attenuated hyperalgesia induced by repeated exposure to eccentric exercise, the expression of its canonical receptor was knocked down by AS ODN treatment from day 8 to day 12 (corresponding to day 0 after second bout of eccentric exercise) after the first bout of eccentric exercise, for a total of 5 days (Fig. 3B,C).

Comparison between experimental groups (AS ODN group *vs* MM ODN [control] group) by two-way repeated measures ANOVA showed a significant effect for treatment ($F_{1,60} = 347.4$, $P = 0.0001$), time ($F_{5,60} = 103.3$, $P < 0.0001$), and interaction ($F_{5,60} = 24.05$, $P < 0.0001$). Bonferroni's multiple comparisons post-hoc test revealed no significant differences in nociceptive threshold to a first bout of eccentric exercise (Fig 3B) or immediately before starting ODN treatment (AS group [2498 ± 24.9 mN] *vs* MM group [2535.8 ± 12.6 mN]; n=6/group, $P > 0.05$, Fig 3C). However, on day 12 after exposure to the first bout of eccentric exercise, a significant change in nociceptive threshold was observed in rats receiving AS ODN (−24.7 ± 1.6% [1880.2 ± 52.7 mN]) *vs* MM ODN-treated rats (0.8 ± 1.1% [2555 ± 29.5 mN], $P < 0.001$, Fig 3C). Significant differences were also observed on

days 1 (−44.5 ± 1.7% AS group [1386 ± 43.3 mN] *vs* −15.7 ± 2.5% MM group [2136.7 ± 55.1 mN], $P < 0.001$), 3 (−40.4 ± 2.1% AS group [1491 ± 61.2 mN] *vs* −15.9 ± 3.1% MM group [2131.8 ± 69.8 mN], $P < 0.001$), and 5 (−32.3 ± 1% AS group [1692.5 ± 33.9 mN] *vs* −6.8 ± 2.8% MM group [2362.3 ± 63.4 mN], $P < 0.001$) after the second bout of eccentric exercise (Fig. 3C). No differences were observed on day 8 after the second bout of eccentric exercise (−3.6 ± 0.8% AS group [2407.3 ± 26.9 mN] *vs* 0.2 ± 1.1% MM group [2541.7 ± 24.1 mN], $P > 0.05$)

Increased IL-10 muscle levels after repeated bouts of eccentric exercise

To assess whether repeated exposure to eccentric exercise modify IL-10 levels in the exercised muscle we measured this cytokine in naïve (control) rats (n=6) and in rats submitted to the RBE12 protocol (n=6) (Fig. 4). Twenty-four hours after last exposure to eccentric exercise, Student's *t* test revealed significant difference in IL-10 muscle levels between naïve rats (0.04 ± 0.01 pg/mg protein) and rats submitted to double eccentric exercise (0.1 ± 0.03 pg/mg protein; $P = 0.0259$, Fig. 4).

Discussion

Re-exposure to eccentric exercise produces a marked attenuation in DOMS [23,30,37], one of the most common types of muscle pain. This protective adaptation, together with a reduction in the force loss, attenuated histological damage, less muscle swelling and release of muscle fiber-specific proteins (e.g., creatine kinase, lactate dehydrogenase, myoglobin) into the blood, is known as the RBE [23,30,37]. Although RBE is a well-established phenomenon, its underlying mechanism remains poorly understood. The present results suggest that blunted muscle pain to eccentric exercise observed in RBE depends on IL-10 acting on its high affinity receptor IL-10R1 expressed by muscle nociceptors.

1. Eccentric exercise-induced muscle hyperalgesia and RBE

As previously reported [4,36,50], rats exposed to a protocol of strenuous eccentric exercise exhibited persistent mechanical hyperalgesia reminiscent of DOMS. A number of well-established proalgesic mediators are locally released in muscle after eccentric exercise and thus likely involved in DOMS, including pro-inflammatory cytokines [4], trophic factors [2,35,36] and prostanoids [12]. Importantly, after recovery from this hyperalgesia, rats exhibited attenuated muscle pain upon exposure to a second bout of eccentric exercise. This effect was anti-hyperalgesic rather than analgesic, since it did not increase nociceptive threshold above the initial baseline. These observations are in line with a recent report demonstrating complete inhibition of muscle mechanical hyperalgesia in rats exposed to a second bout of eccentric exercise [52].

One day after the last eccentric exercise, we observed that rats with 12 days between eccentric exercise bouts displayed significantly less hyperalgesia than rats with a delay of 5 days. Time course studies have shown that significant tissue injury is still present even if DOMS is no longer present [37]. Moreover, since a single bout of eccentric exercise may induce microscopic changes indicative of muscle injury noticeable for up to 9 days in rats [5], it is likely that full expression of the antihyperalgesic effect of RBE would occur after

full recovery from the first bout of eccentric exercise. Growing evidence shows that several types of muscle injury, including eccentric exercise, trigger a time-dependent change in the phenotype of local macrophages [1,45,54], from classically activated (pro-inflammatory) macrophages (M1), to alternatively activated macrophages (M2), which display anti-inflammatory features and are necessary for myogenesis and complete tissue repair/regeneration at resolution stages [1,45,54]. Importantly, M2 macrophages present in skeletal muscle produce and release IL-10 [16,51], which can in turn act on a number of different cell types involved in local responses to muscle injury [51]. This is consistent with our finding of increased IL-10 levels in the gastrocnemius muscle submitted to the RBE protocol. In good agreement with these observations, the protective effect of RBE on muscle pain (i.e., attenuated DOMS) is absent when exposure to a second bout of eccentric exercise occurs 2 days after an initial injury [39], and daily re-exposure to eccentric exercise for 3 days after initial bout does not produce a protective effect on DOMS but rather aggravates symptoms, in humans [14]. A similar lack of RBE is observed in rats upon daily re-exposure to eccentric exercise for up to 2 weeks [19]. Thus, the enhanced anti-hyperalgesic effect of RBE observed after a longer bout-to-bout interval (i.e., RBE12) is likely due to a more advanced stage of recovery of tissue injury induced by the first bout, with a higher number of local macrophages displaying the M2 phenotype at the moment of the second bout of eccentric exercise. This is consistent with our observation that, even at late stages of recovery from an initial bout of eccentric exercise, there is latent hyperalgesia that is masked by local IL-10 acting on muscle nociceptors (see below).

2. Role of IL-10 in nociceptive baseline, recovery from eccentric exercise and RBE

We observed that AS treatment was devoid of effect in control (not exposed to eccentric exercise) rats. This is consistent with the lack of changes on baseline mechanical nociceptive threshold in skin [47] or muscle [15] in IL-10 knockout mice, or after administration of IL-10R blocking antibodies to control mice [27]. Conversely, administration of systemic recombinant IL-10 [27] or intrathecal viral vectors encoding IL-10 [33,34] do not change baseline nociceptive mechanical threshold in control rodents, nor the mechanosensitivity of C-fibers from mice recorded *in vitro* [22]. Hence, the latent mechanical hyperalgesia unveiled by knockdown of IL-10R1 was unexpected since it occurred at a time point after eccentric exercise when nociceptive threshold is recovered [4,36,50]. Therefore, the physiological recovery of nociceptive threshold after eccentric exercise appears not to be merely due to a decrease in local proalgesic mediators from muscle injury but to an ongoing antinocicepive effect of local IL-10 acting on muscle nociceptors. Of note, local production of IL-10 by M2 macrophages during the resolution phase of muscle injury is critical to regulate the termination of the inflammatory response and to achieve complete tissue regeneration [16,45,51].

While the mechanism of the RBE is poorly understood, our results point to a critical contribution of IL-10. Indeed, we observed increased IL-10 levels in the gastrocnemius muscle after repeated eccentric exercise. Consistent with this finding, repeated muscular exercise, in different schedules of regularity and intensity, reduces DOMS [23,37,48] and increases local and circulating IL-10 levels [21,48], This functional relationship between exercise and IL-10 levels appears to also underlie the antinociceptive effect of exercise on

muscle pain induced by injections of acidic saline [27] or pain due to nerve injury [18]. Conversely, increased muscle pain after strenuous swimming has been observed in IL-10 knockout mice compared to wild type littermates [9]. Furthermore, systemic or contralateral intramuscular injections of recombinant IL-10 inhibit acidic saline-induced muscle hyperalgesia in mice [27]. This fits well with the fact that RBE is also observed when first and second bouts of eccentric exercise are applied to different limbs [11,23,37], suggesting a role of a diffusible antinociceptive mediator, such as IL-10.

3. RBE analgesia depends on IL-10 acting at nociceptor IL-10R1

Since only sensory neurons innervating skeletal muscle were exposed to both eccentric exercise-induced muscle injury and knockdown of IL-10R1, the effect of AS ODN is likely due to a disruption of the interaction of endogenous IL-10 with its receptor on the nociceptor. This is an important finding given that IL-10 acts on a number of different cell populations present at the site of muscle injury [6,16,40,51], making them potential players in the expression of RBE. Indeed, although previous studies have shown that endogenous IL-10 attenuates muscle hyperalgesia after exercise [9] or insults such as intramuscular acidic saline [27], they do not clarify whether such an action is due to an effect on immune resident or infiltrating cells, satellite cells, muscle fibers or a combined effect. Similarly, systemic neutralizing antibodies directed against IL-10 [27] may prevent its inhibitory effect on muscle pain by acting on many target cells.

We specifically targeted the IL-10R1/subunit alpha for knockdown because of its main role in the binding of IL-10 and the assembling of the IL-10R receptor complex [6,25,53]. Besides its contribution to the IL-10R complex, IL-10R2 (beta subunit) also associates with IL-20 R alpha, IL-22 R alpha or IL-28 R alpha to form the receptor complexes for IL-22, IL-26, IL-28, and IL-29 [53], making it unsuitable as specific target for our experimental knockdown approach. Importantly, IL-10R1 is widely expressed in sensory neurons from L4–L5 DRGs [46], which innervate the gastrocnemius muscle [7], where it is co-expressed with the marker of neuronal identity NeuN, but not with the satellite cell marker glial fibrillary acidic protein, indicating that in DRGs it is strictly expressed by sensory neurons [46]. *In vitro* studies have shown that recombinant IL-10 reduces the increased tetrodotoxin sensitive (TTX-S) and tetrodotoxin resistant (TTX-S) sodium currents induced by exposure of DRG neurons to tumor necrosis alpha (TNFα) or after peripheral nerve injury [46]. A potential mechanism of the antihyperalgesic effect of IL-10 is the downregulation of mRNA and protein expression of alpha subunits of voltage-dependent sodium channels contributing to TTX-S ($Na_v1.6$) and TTX-R ($Na_v1.8$) currents [46] expressed in sensory neurons innervating the gastrocnemius muscle [42–44]. These observations are in good agreement with the inhibitory effect of IL-10 on the mechanical hyperalgesia induced by algogens such as bradykinin, TNFα, and interleukin 6 [41], which also produce muscle mechanical hyperalgesia and/or sensitize muscle nociceptors upon local administration [17,20,31,36] and likely contribute to eccentric-exercise induced mechanical hyperalgesia [4,36].

Conclusions

In summary, our results provide evidence for the involvement of IL-10 in the attenuation of muscle hyperalgesia typically observed in the RBE. Although RBE depends on the length of

the interval between exercise bouts, it is not merely due to reduced levels of nociceptive mediators produced after the second bout of eccentric exercise. Finally, these data indicate that IL-10 produces its antihyperalgesic effect by acting locally on muscle nociceptors. These findings underline the importance that IL-10 plays in the control of pain due to exercise and could help to tailor exercise-based therapies for the clinical management of chronic muscle pain.

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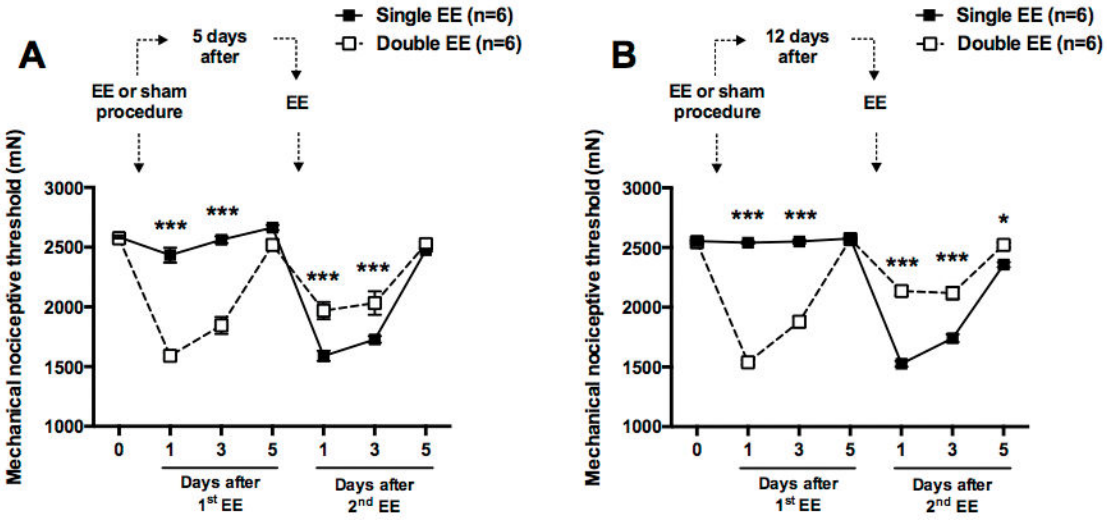

Figure 1. Effect of single and repeated exposure to eccentric exercise

The RBE was studied by evaluating the effect of repeated exposure to bouts of eccentric exercise and 2 different length intervals between these bouts (5 or 12 days) on muscle mechanical nociceptive threshold. **A**. After assessment of baseline nociceptive threshold (0), rats were submitted to eccentric exercise (double EE, open symbols, n=6) or sham procedure (single EE, solid symbols, n=6). Five days later, both groups were exposed to a bout of eccentric exercise (EE). **B.** A similar approach was used to study RBE after an interval of 12 days between bouts of eccentric exercise. $*P < 0.05$; $***P < 0.001$.

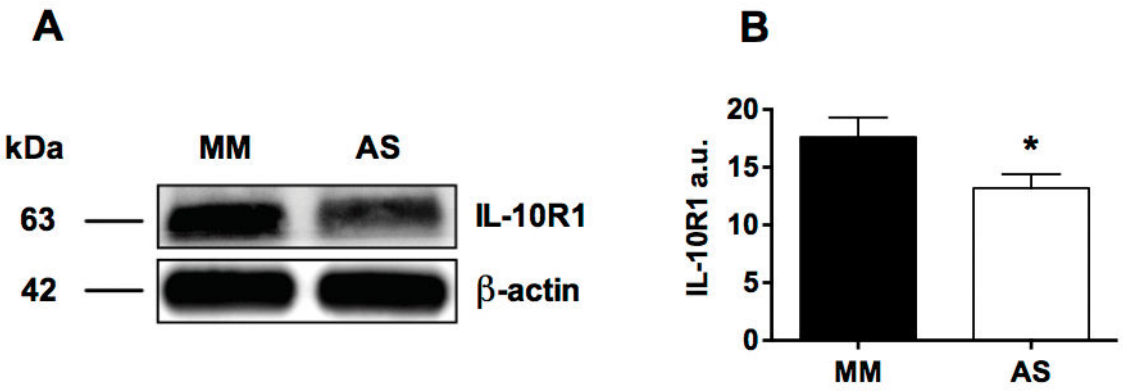

Figure 2. Effect of AS/MM ODN treatment directed against IL-10R1 mRNA on protein expression of IL-10R1 in L4 and L5 DRG neurons

A. Western blot analysis of DRG extracts from rats injected i.t. daily with 40 μg of AS or MM ODNs for 5 consecutive days revealed significant down-regulation of IL-10R1 (see *results* section for details). The calculated molecular weight of IL-10R1 is 63 kDA (according to UniProtKB database entry G3V830). β-actin was used as a housekeeping gene product in this analysis, which has a calculated molecular weight of ~42 kDa (according to UniProtKB database entry P60771). **B.** Comparison of the protein expression by Western blotting demonstrated a significant decrease in IL-10R1 immunoreactivity (arbitrary units, a.u.) of DRG extracts from rats treated with AS ODN compared to MM ODN treated group. $*P < 0.05$

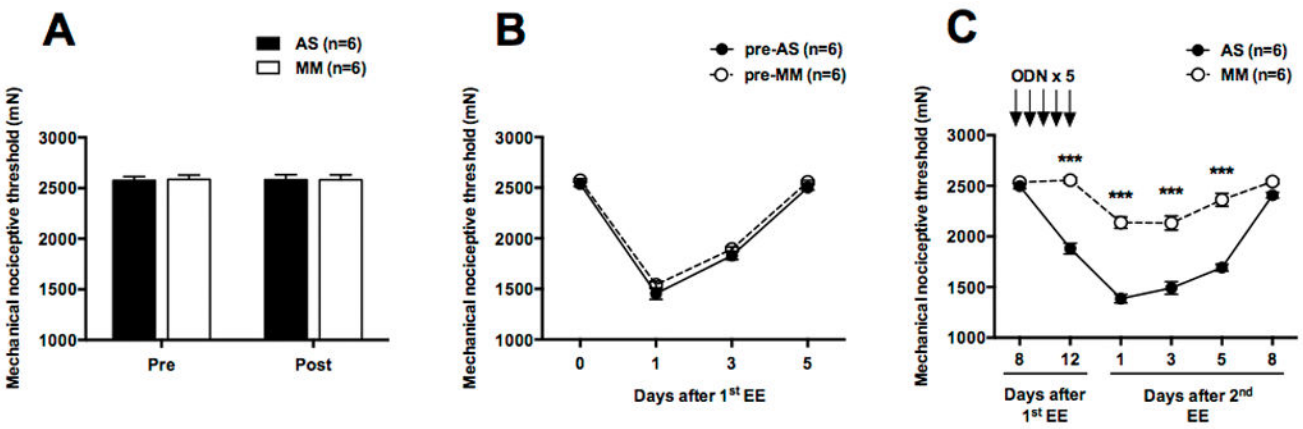

Figure 3. Effect of intrathecal treatment with AS/MM ODN directed against IL-10R1 mRNA on muscle nociceptive threshold

(**A**) Effect of AS/MM ODN (40 μg/20 μl, i.t.) injections for 5 days on muscle mechanical nociceptive threshold in naïve rats. Bars were plotted from readings taken before (Pre) and after (Post) ODN treatment. **B.** Effect of a first bout of eccentric exercise (EE) on muscle mechanical nociceptive threshold in rats to be submitted to AS or MM ODN treatment. **C.** Effect of daily AS/MM ODN (40 μg/20 μl) i.t. injections (starting on day 8 after first bout of EE) on muscle mechanical nociceptive threshold in rats submitted to a first bout of EE 12 days prior. The effect of this treatment on RBE12 was also evaluated on days 1 to 8 after second bout of EE. *** $P < 0.001$.

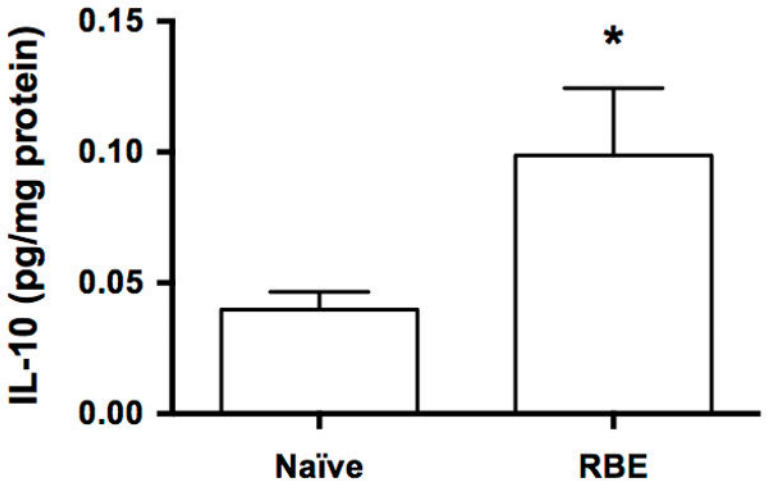

Figure 4. Effect of repeated exposure to eccentric exercise on IL-10 levels in the gastrocnemius muscle

The right gastrocnemius muscle was sampled in control rats (n=6, Naïve) and rats submitted to double bout of eccentric exercise (n=6, RBE). The IL-10 levels were assessed by ELISA from muscle samples (in duplicate) obtained 24 h after last exposure to EE. $*P < 0.05$