ORIGINAL ARTICLES

Molecular and physiological events in respiratory muscles and blood of rats exposed to inspiratory threshold loading

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High-intensity exercise induces oxidative stress and inflammatory events in muscles. Tumor necrosis factor (TNF)-α may alter muscle protein metabolism or promote muscle regeneration. We hypothesized that a program of noninvasive chronic inspiratory loading of different intensities induces a differential pattern of physiological, molecular, and cellular events within rat diaphragms. Antioxidants and TNF-α blockade may influence those events. In the diaphragm, gastrocnemius, and blood of rats exposed to high-intensity inspiratory threshold loads (2 hour every 24 hours for 14 days), with and without treatment with N-acetyl cysteine or infliximab (anti-TNF-α antibody), inflammatory cells and cytokines, superoxide anion production, myogenesis markers, and muscle structure were explored. In all animals, maximum inspiratory pressure (MIP) and body weight were determined. High-intensity inspiratory loading for 2 weeks caused a decline in MIP and body weight, and in the diaphragm induced a reduction in fast-twitch fiber proportions and sizes, whereas inflammatory cells and cytokine levels, including TNF-α immunohistochemical expression, superoxide anion, internal nuclei counts, and markers of myogenesis were increased. Blockade of TNF-α improved respiratory muscle function and structure, and animal weight, and, in the diaphragm, reduced inflammatory cell numbers and superoxide anion production drastically while inducing larger increases in protein and messenger RNA levels and immunohistochemical expression of TNF-α, internal nuclei, and markers of muscle regeneration. Blunting of TNF-α also induced a reduction in blood inflammatory cytokines and superoxide anion production. We conclude that TNF-α synthesized by inflammatory cells or myofibers could have differential effects on muscle structure and function in response to chronic, noninvasive, high-intensity inspiratory threshold loading. (Translational Research 2014;163:478–493)

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Abbreviations: COPD = chronic obstructive pulmonary disease; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; m-cadherin = muscle calcium-dependent cell adhesion; MIP = maximal inspiratory pressure; mRNA = messenger RNA; myf-6 = myogenic factor 6; MyHC = myosin heavy chain; NAC = N-acetylcysteine; TNF = Tumor necrosis factor

AT A GLANCE COMMENTARY

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Background

Chronic noninvasive inspiratory loading of different intensities induces a differential pattern of physiological, molecular, and cellular events within rat diaphragms, eventually influenced by antioxidants and tumor necrosis factor (TNF)-α blockade.

Translational Significance

Chronic, noninvasive, high-intensity inspiratory threshold loading reduced respiratory muscle function and body weight, altered muscle structure, and increased levels of inflammatory cells, cytokines, superoxide anions, internal nuclei, and myogenesis markers. TNF-α blockade improved respiratory muscle function and structure, and animal weight, and reduced inflammatory cell numbers and superoxide anions, while increasing TNF-α levels and muscle regeneration in diaphragms. These findings have therapeutic implications in exercise training of chronic respiratory patients with muscle dysfunction.

Skeletal muscle dysfunction, which is a major systemic manifestation in highly prevalent conditions such as chronic obstructive pulmonary disease (COPD), has relevant implications in patients' exercise capacity and quality of life.[1,2] General exercise and inspiratory muscle training have been shown to exert beneficial effects on clinical outcomes such as respiratory muscle performance and underlying structure among patients with severe COPD.[3-9] Nonetheless, other studies have also shown an increase in oxidative stress markers[10] and proinflammatory cytokines[11] in response to the administration of high-intensity inspiratory loads in animal models. In fact, strong muscle contractions leads to enhanced oxidant production,[12,13] which may result in the development of oxidative stress.[10] These are relevant findings, because patients with COPD already exhibit a greater production of oxidants in their diaphragms at rest.[14-16]

Inflammatory makers such as the pleiotropic cytokine tumor necrosis factor (TNF)-α, has long been considered to exert catabolic actions in muscles and to induce contractile dysfunction in chronic inflammatory conditions including cancer and COPD.[17] Furthermore, TNF-α was also shown to block protein synthesis, to enhance protein breakdown,[18] and to inhibit myogenesis through several mechanisms in *in vitro* studies.[19,20] Nevertheless, TNF-α is also involved in other processes such as muscle repair and regeneration,[21] growth,[22] and differentiation,[23] as well as in the delay of muscle proteolysis in dystrophic mice.[24] Other proinflammatory cytokines such as interleukin (IL)-6 and IL-1 seem to participate in muscle metabolism in contracting muscles. As such, muscle messenger RNA (mRNA) levels of IL-6 were shown to increase as early as 30 minutes after exercise and to reach their peak at the end of the exercise bout.[25] In another relevant investigation, high inspiratory loads were also shown to induce an increase in the synthesis of the cytokines TNF-α, IL-6, and IL-1 in the rat diaphragm.[11] More important, in humans, exercise of moderate to high intensity also enhanced the production of IL-6 and its release into the bloodstream.[26,27]

On the other hand, the contractile dysfunction induced by oxidants in the diaphragms of dogs exposed to several degrees of inspiratory resistive breathing[10] and in septic rats[28] was attenuated in response to antioxidant treatment with N-acetyl cysteine (NAC). Nevertheless, whether inspiratory threshold loading of high intensity may induce deleterious molecular events that could counteract the potential beneficial effects of chronic exercise on muscle structure and function should still be explored further. In addition, identification of whether cytokine release from the contracting muscles may be associated with muscle regeneration also needs to be elucidated. Moreover, the potential beneficial effects of blocking the actions of oxidants or proinflammatory cytokines (eg, TNF-α) within the contracting muscles also remain unidentified.

On this basis, we hypothesized that a program of chronic, noninvasive inspiratory threshold loading of different intensities would induce a differential pattern of physiological, molecular, and cellular events within the diaphragm myofibers of rats at the end of the study period. Furthermore, we also attempted to assess whether concomitant treatment of the animals with the antioxidant NAC or the anti-TNF-α antibody infliximab may interfere differentially with such responses. Accordingly, the study objectives were to determine (1) whole body weight and respiratory muscle function (force), (2) diaphragm levels of inflammatory and regeneration markers (cell proliferation and differentiation),

and (3) diaphragm muscle structural abnormalities and fiber type features in an *in vivo* model characterized by the noninvasive administration of several degrees of inspiratory threshold loading (mild, moderate, and high intensity) daily (2 hours) to adult rats for 2 consecutive weeks. In addition, the effects of the antioxidant NAC and those of the anti-TNF-α antibody infliximab were also assessed in the diaphragm and plasma of the animals exposed to the highest inspiratory loads, in which molecular and cellular alterations were, indeed, observed.

METHODS

See the online supplementary material for additional information on all methodologies.

Ethical approval. All animal experiments were conducted in the animal facilities of the Biomedical Research Unit at Hospital de Cruces (Barakaldo, Bizkaia, Spain). This controlled study was designed in accordance with both the ethical standards on animal experimentation (EU 609/86 CEE, Real Decreto 1201/05 BOE 252, Spain) at Hospital de Cruces and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). The study conforms to the current international ethical guidelines for animal research. Ethical approval was obtained by the Animal Research Committee at Hospital de Cruces.

Animal experiments and study design.

Animals. Pathogen-free male Wistar rats (Harlan, Horst, Netherlands) of identical age (8 weeks, 250–300 g) at the start of the study were used. Food and water was supplied *ad libitum* during the study period. In all rats, maximal inspiratory pressure (MIP) and total body weight were determined twice: at baseline and at the end of the 2-week study period by the same investigators.

Pharmacologic agents. NAC powder (kindly provided by Dr A. Esteras, Zambon S.A., Barcelona, Spain) was dissolved in distilled water and prepared to obtain a final dose of 3 mmol/kg. In the treated rats, NAC was administered daily using a 14-mm-gauge needle (oral gavage). The NAC dose was chosen on the basis of previous studies.[10,28] The anti-TNF-α antibody infliximab was administered intraperitoneally (0.01 mg/g/0.3 mL dose) on days 1 and 7 of the study protocol, also following previous studies.[24] Infliximab is a chimeric immunoglobulin containing 25% mouse-derived amino acids in the variable region, which is in turn, linked by disulfide bonds to a human constant region. Infliximab is produced by cell culture using Chinese hamster ovary cells.[29] After subcutaneous injection, infliximab is absorbed slowly, probably via lymphatic drainage, reaching maximum concentrations 8–10 days after administration. This anti-TNF-α antibody has been used previously in a wide range of investigations in which different types of experimental models (mainly mice and rats) were studied.[30-35] Furthermore, pharmacologic blockade of TNF-α with infliximab was shown specifically to delay and decrease the catabolism of dystrophic muscles in mouse mutant deficient in distrophin mice.[24]

MIP measurements. The animals were first placed, fully awake, in a polyurethane plethysmographic chamber, the anterior part of which contained a 2-way threshold valve (model 2200; Hans Rudolph, Kansas City, Mo, USA). Following similar procedures reported previously by our group,[28,36] MIP was determined by total occlusion of the inspiratory circuit of the Hans Rudolph valve for 30 seconds, and this maneuver was performed at least 3 times, with an interval of less than 60 seconds between them. MIP was measured at 2 different time points: at baseline on day 1 at the start of the protocol and on day 14, immediately after the loading period in each study group.

Experimental protocol. During the study period, the experimental setting was similar to that used for the estimation of MIP in all animal groups, including the use of the plethysmographic chamber and the 2-way valve. Briefly, a 1-way valve (Hans Rudolph, model 1230) was attached to the inspiratory branch of the 2-way valve. The inspiratory loads were administered by means of an elastic metal spring that was inserted into the 1-way valve. Inspiratory loads were modified by compressing or expanding the metal spring. Resting baseline MIP values were taken into consideration for the application of the desired inspiratory loads. For the purpose of the investigation, several degrees of inspiratory threshold loads were established—mild, moderate, and high intensity—defined as 33%, 50%, and 70% of those resting baseline MIP values. All study animals remained fully awake and alive during the entire duration of the protocol.

Study groups and protocol. A total of 48 rats were studied in the investigation. All rats were assigned randomly to the different experimental groups (n = 8 per group). The following study groups were established: (1) the control group, in which animals were exposed to identical experimental conditions without application of any loads to control for stress-related factors; (2) the 33% MIP group; (3) the 50% MIP group; and (4) the 70% MIP group. Furthermore, during the molecular analyses, because differences were observed mainly among animals exposed to high-intensity loads (70% MIP), 2 additional groups were included in the study: (5) the 70% MIP-NAC group, in which animals received treatment concomitantly with 3 mmol/kg NAC/24 h (oral gavage) for 14 consecutive days; and (6) the 70% MIP-anti-TNF-α group, in which animals received treatment concomitantly with a single dose of anti-TNF-α antibody every 7 days for 14 consecutive

Table I. Probes used for quantitative real-time polymerase chain reaction of the target genes

Genes	Assay identification	Nucleotide sequences (5′-3′)	GenBank accession no.
M-cadherin	Rn01432568_m1	CTTCATCAGTGACGGCTTGGAGGCT	NM_207613.1
myf6	Rn00565920_m1	GCAAGAAATTCTTGAGGGTGCGGAT	NM_013172.1
TNF-α	Rn99999017_m1	CCCTCACACTCAGATCATCTTCTCA	NM_012675.2
TNF-RI	Rn01492348_m1	TGCAGCCACTGCAAGAAAAATCAGG	NM_013091.1
TNF-RII	Rn00709830_m1	ACCATGGTGCCTCATCTGCCTGATG	NM_130426.4
ACTB	Rn00667869_m1	GCCTTCCTTCCTGGGTATGGAATCC	NM_031144.2

Abbreviations: ACTB, actin-β; M-cadherin, muscle calcium-dependent cell adhesion; myf-6, myogenic factor 6; R, receptor; TNF, tumor necrosis factor.

Table II. Body weight in control and loaded rats with and without pharmacologic treatment

Groups	Initial body weight, g	Final body weight, g	*P* value	Percentage of body weight gain
Control	263 (10)	283 (11)	<0.05	+8 (7)
33% MIP	267 (20)	285 (17)	<0.05	+7 (15), ns
50% MIP	260 (5)	289 (13)	<0.05	+11 (5), ns
70% MIP	264 (11)	260 (11)	ns	−1.6% (3)*
70% MIP + NAC	260 (5)	271 (9)	<0.05	+4% (3)†
70% MIP + anti-TNF-α	266 (6)	268 (9)	ns	+0.7% (3)†

Abbreviations: MIP, maximal inspiratory pressure; NAC, N-acetyl cysteine; ns, not significant; TNF, tumor necrosis factor.
Values are expressed as means (standard deviation). Paired analyses between postloading and baseline time points, $P < 0.05$ and ns.
*$P < 0.05$, between any loaded group and unloaded control animals.
†$P < 0.05$ between any of the pharmacologically treated groups exposed to 70% MIP and 70% MIP-only rats.

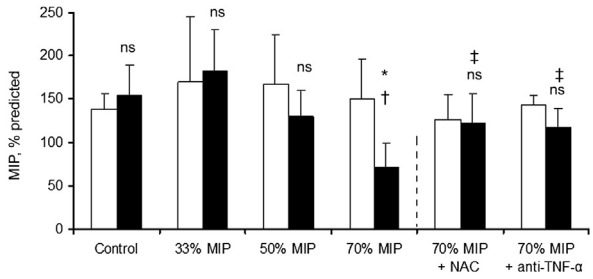

Fig 1. Mean value and standard deviation (n = 8 per group, all groups) of maximum inspiratory pressure (MIP) expressed as the percentage of predicted values obtained previously in rats. After the study protocol (black bars), MIP values decreased significantly only in rats exposed to 70% MIP compared with baseline (white bars). MIP values were significantly greater in animals exposed to high inspiratory loads treated with either N-acetyl cysteine (NAC) or anti-tumor necrosis factor (anti-TNF)-α antibody compared with 70% MIP-only rats. *$P < 0.01$, comparisons between 70% MIP and the unloaded control rats. †$P < 0.05$, MIP values after the study period compared with baseline. ‡$P < 0.05$, comparisons between 70% MIP receiving either NAC or anti-TNF-α antibody and the nontreated animals exposed to 70% MIP only. ns, not significant.

days (2 intraperitoneal injections in total). All animals were exposed to the corresponding inspiratory threshold loading level for 2 h/d for 14 consecutive days (including weekends). None of the animals died during the study period. Moreover, 2 additional groups of control rats were included for which treatment included either 3 mmol/kg NAC/24 h or anti-TNF-α antibody administration every 7 days for 14 consecutive days without exposure to any inspiratory loads. On day 14 of the study protocol, animals from the different experimental groups were sacrificed. Animals were exsanguinated and blood was collected from the cardiac cavities and stored at −80°C until further use. The diaphragm and gastrocnemius were then excised quickly. All muscle samples were either frozen immediately in liquid nitrogen and stored subsequently at −80°C until further use (molecular analyses) or immersed in an alcohol-formol bath for 2 hour to be embedded later in paraffin (tissue analyses).

Muscle biology analyses. ***Detection of superoxide anion radicals in muscle compartments.*** Frozen muscle specimens from all muscles were fractionated into cytosolic, membrane, and mitochondria compartments following previous reports by some of us.[16,37]

Detection of superoxide anion radicals in blood. The reagents used in these methodologies were all purchased from Sigma (Sigma, St. Louis, Mo, USA). To quantify superoxide anion production, lucigenin-derived chemiluminescence signals were determined in all blood (serum) samples using a luminometer (Lumat LB 9507; Berthold Technologies GmbH, Bad Wildbad, Germany) as described previously.[16,37]

Cytokine enzyme-linked immunosorbent assay. The protein expression of the cytokines TNF-α, IL-1β, IL-6, and interferon-γ was quantified in the diaphragm, gastrocnemius, and plasma of controls and all groups of loaded rats using specific-species sandwich enzyme-linked immunosorbent assay (ELISA) kits (Bender Medsystems GmbH, Austria) following similar previously published methodologies.[37-41] In addition, protein levels of the TNF-α receptors I and II were also determined in the diaphragm and blood

(plasma) of control, 70% MIP, 70% MIP-NAC, and 70% MIP-anti-TNF-α rodents using species-specific kits (SunRed Biological Technology Company, Shanghai, China).

RNA isolation, reverse transcription, and real-time polymerase chain reaction. Total RNA was extracted from skeletal muscle using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, Calif, USA) and previous studies[40,41] (Table I). The relative copy number was calculated according to the comparative threshold cycle method as published previously.[42]

Muscle fiber counts, and morphometry and immunohistochemical expression of markers. On 3-μm-muscle paraffin-embedded sections from both diaphragms and gastrocnemius muscles from all animal groups, myosin heavy chain (MyHC)-I and -II isoforms, TNF-α, muscle calcium-dependent cell adhesion (m-cadherin), and myogenic factor 6 (myf-6) expression were identified using anti-MyHC-I (clone NOQ7.5.4D; Sigma), anti-MyHC-II (clone MY-32; Sigma), anti-TNF-α (Santa Cruz), anti-m-cadherin (Santa Cruz), and anti-myf-6 antibodies (Santa Cruz), respectively (Santa Cruz Biotechnology Inc, Calif, USA), as published elsewhere.[14,16,37,39]

Muscle structure abnormalities. The area fraction of normal and abnormal muscle (abnormal cellular events taking place in the muscle fibers) was evaluated on 3-μm paraffin-embedded sections of diaphragm and gastrocnemius of all rats following similar methodologies published elsewhere.[37,43]

Statistical analyses. Data are presented as mean (standard deviation). Physiological variables (MIP and body weight) were analyzed as follows: (1) comparisons were made between the baseline and postloading periods in each experimental group of rats, (2) comparisons were made among each experimental group of rats (33% MIP, 50% MIP, and 70% MIP) and nonloaded control animals right at the end of the study protocol, and (3) comparisons were made between rats exposed to 70% MIP treated with either NAC or anti-TNF-α antibody and rodents exposed to 70% MIP only, which was the control group in this type of analysis.

All biologic variables (from muscles and blood) were analyzed at the end of the study period and comparisons were made as follows: (1) among each experimental group (33% MIP, 50% MIP, and 70% MIP) and nonloaded control animals and (2) between rats exposed to 70% MIP treated with either NAC or anti-TNF-α antibody and animals exposed to 70% MIP only, which was the control group in this type of analysis.

Comparisons of physiological variables between postloading and baseline time points were explored using a paired parametric t test. At the end of the study period, differences in both physiological and biologic variables among groups were analyzed using 1-way analysis of variance. Tukey's post hoc analysis was used to adjust for multiple comparisons. Pearson's correlation coefficient was used to assess relationships among different variables within specific groups of rats. The level of significance was established at $P \leq 0.05$.

RESULTS

Physiological characteristics. As shown in Table II, animals from all groups, except for 70% MIP and 70% MIP-TNF-α, gained a significant amount of body weight after the 2-week exercise period compared with baseline. Rats in the unloaded control groups treated with either NAC or anti-TNF-α antibody also gained weight, although showed no differences in MIP values after the study period compared with baseline (data not shown). Moreover, when comparisons were made among the different study groups, in 70% MIP rats, the gain in total body weight was significantly less than in control animals (Table II), despite the fact that no differences in the amount of food intake were observed between the 2 groups. In 70% MIP-NAC and 70% MIP-TNF-alpha rats, body weight gain improved significantly compared with 70% MIP rodents (Table II). Animals in the 70% MIP group exhibited a significant decline in MIP values, expressed as a percentage of predicted, after the 2-week study period compared with baseline ($^*P < 0.05$) and with unloaded control rats ($P < 0.01$; Fig 1). The decrease in MIP among rodents exposed to 70% MIP was attenuated significantly by the concomitant administration of either NAC or anti-TNF-α antibody treatments compared with 70% MIP-only animals ($P < 0.05$).

Systemic and muscle inflammatory markers. Protein levels of TNF-α were significantly greater in the diaphragm of 70% MIP rats than in the controls (Fig 2, *A*, top panel). Interestingly, treatment of 70% MIP rats with anti-TNF-α antibody elicited a further increase in protein levels of the cytokine TNF-α in their diaphragms compared with 70% MIP-only rodents (Fig 2, *A*, top panel). In the gastrocnemius, TNF-α protein levels did not differ among any of the study groups (data not shown). In plasma, TNF-α protein levels showed a significant increase in 70% MIP compared with controls (Fig 2, *A*, bottom panel). Concomitant treatment of these animals with either NAC or anti-TNF-α antibody elicited a significant decline in plasma TNF-α levels compared with 70% MIP-only rats (Fig 2, *A*, bottom panel). More important, the immunohistochemical expression of TNF-α was more intense in the diaphragms of both 70% MIP-only rodents and 70% MIP rats treated with anti-TNF-α antibody (Fig 2, *B*, middle and right top panels, respectively) compared with the controls (Fig 2, *B*, left

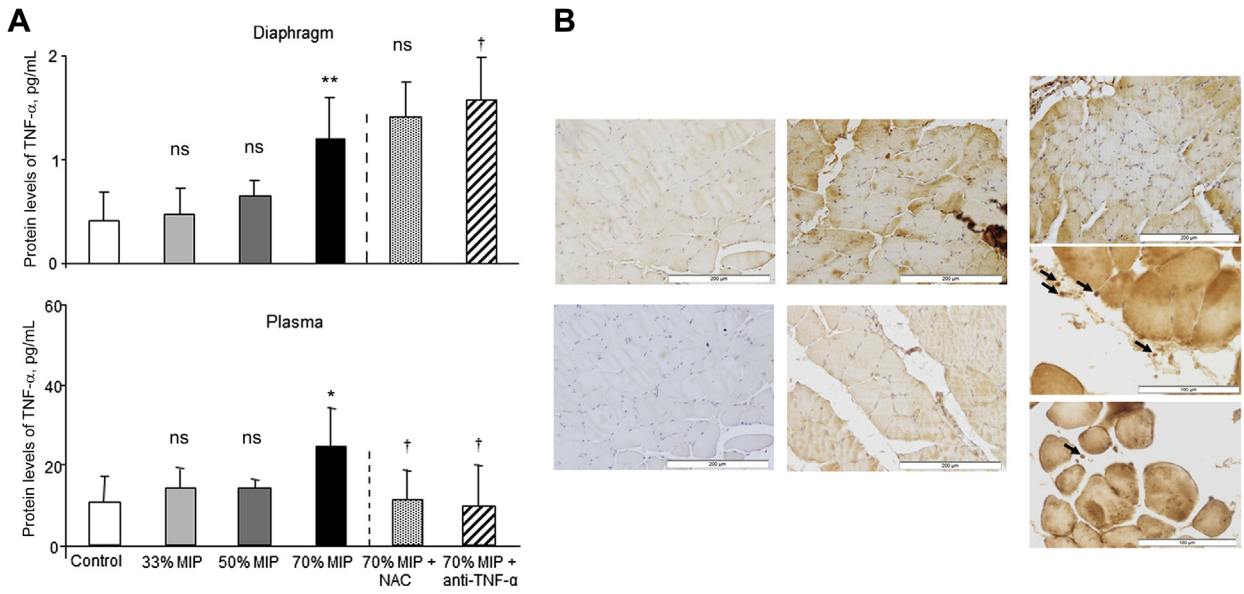

Fig 2. (**A**) Mean values and standard deviations of tumor necrosis factor (TNF)-α protein levels measured in the diaphragm (**top**) and plasma (**bottom**) compartments (n = 8 per group, all groups). In the diaphragm, protein levels of TNF-α were increased significantly in rats only exposed to 70% maximum inspiratory pressure (MIP) loads compared with the controls ($^{**}P < 0.01$). In addition, the respiratory muscles of rats treated with anti-TNF-α antibody exhibited a larger increase ($\dagger P < 0.05$) in TNF-α protein levels compared with rats exposed to 70% MIP only. In plasma, TNF-α was significantly greater ($^{*}P < 0.05$) in rats exposed to 70% MIP than in the controls, whereas a significant decrease was observed in animals treated with anti-TNF-α antibody ($\dagger P < 0.05$). (**B**) Immunohistochemical expression of the cytokine TNF-α within the muscle fibers of the 70% MIP-only rats and 70% MIP treated with anti-TNF-α antibody (middle and right top panels, respectively) was more intense than in control animals (left top panel) and gastrocnemius (middle bottom panel) ($\times$400, light microscopy). The immunohistochemical expression of TNF-α within infiltrating inflammatory cells was also strong in the diaphragms of 70% MIP, and 70% MIP and anti-TNF-α rats (arrows in right middle and bottom panels, respectively) ($\times$400, light microscopy). A negative control in which anti-TNF-α primary antibody was omitted in the immunohistochemical preparations is also shown (left bottom panel) from a 70% MIP-only rat ($\times$400, light microscopy). Scale bars are shown in each histological image. (**C**) Mean values and standard deviations of protein levels of the TNF-α receptors I and II (top and bottom panels, respectively) measured in the diaphragm (n = 8 per group, all groups). No significant differences were observed among any of the study groups in TNF-α receptor I (RI) or receptor II (RII) in the rat diaphragms. (**D**) Mean values and standard deviations of protein levels of the TNF-α RI and RII (top and bottom panels, respectively) measured in the plasma (n = 8 per group, all groups). No significant differences were observed among any of the study groups in plasma levels of TNF-α RI or RII. (**E**) Mean values and standard deviations of interleukin (IL)-1β protein levels measured in the diaphragm and plasma compartments (top and bottom panels, respectively; n = 8 per group, all groups). Protein levels of IL-1β were increased significantly ($^{*}P < 0.05$) in the diaphragm of 70% MIP rats compared with the controls. Moreover, protein levels of that cytokine were even greater ($\dagger P < 0.05$) in the diaphragms of rats treated with either N-acetyl cysteine (NAC) or anti-TNF-α antibody than in 70% MIP-only rats. No significant differences were observed among any of the study groups in IL-1β protein levels in plasma. (**F**) Mean values and standard deviations of IL-6 protein levels measured in the diaphragm and plasma compartments (top and bottom panels, respectively; n = 8 per group, all groups). In the respiratory muscle, IL-6 levels were significantly greater ($^{*}P < 0.05$) in the 70% MIP rats compared with the controls. In addition, an even greater increase ($\dagger P < 0.05$) in IL-6 protein levels was observed in the diaphragms of rats treated with either NAC or anti-TNF-α antibody compared with rats exposed to 70% MIP only. In plasma, IL-6 protein levels were greater in 70% MIP rats compared with the controls ($^{*}P < 0.05$). Plasma levels of IL-6 were lower in animals treated with either NAC antibody ($\dagger P < 0.05$) or anti-TNF-α antibody ($\dagger P < 0.05$) compared with 70% MIP only rats. (**G**) Mean values and standard deviations of interferon-γ protein levels measured in the diaphragm and plasma compartments (top and bottom panels, respectively; n = 8 per group, all groups). In the respiratory muscle, interferon-γ levels were significantly greater in the 70% MIP rats compared with the controls ($^{*}P < 0.05$). Concomitant treatment with either NAC or anti-TNF-α did not induce any difference compared with 70% MIP-only rats. In plasma, protein levels of interferon-γ were significantly greater in the 70% MIP rats than in the controls Moreover, interferon-γ plasma levels were significantly decreased in rats treated with either NAC or anti-TNF-α antibody ($\dagger P < 0.05$). ns, not significant.

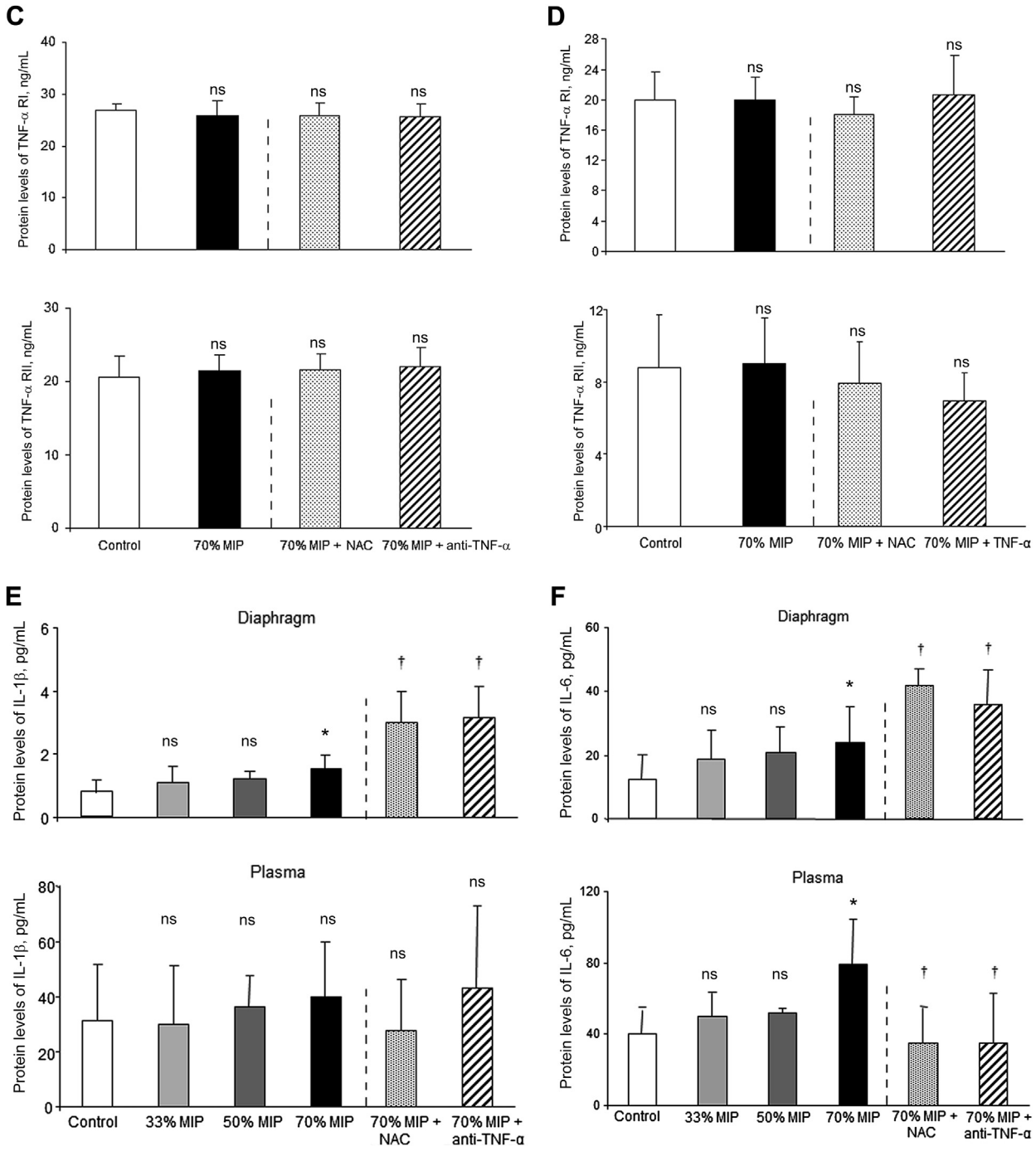

Fig 2. (*continued*).

top panel) and gastrocnemius muscle (Fig 2, *B*, middle bottom panel). The immunohistochemical expression of TNF-α within infiltrating inflammatory cells was also strong in the diaphragms of both 70% MIP and 70% MIP-anti-TNF-α antibody rats (Fig 2, *B*, right middle and bottom panels, respectively). Moreover, protein levels of both receptors I and II did not differ significantly among the experimental study groups in either diaphragm or plasma (Fig 2, *C* and *D*, respectively).

Protein levels of IL-1β were significantly greater in the diaphragms of 70% MIP rats than in the controls (Fig 2, *E*, top panel). Interestingly, protein levels of such a proinflammatory cytokine were increased further in the respiratory muscle of both 70% MIP-NAC and 70% MIP-anti-TNF-α rodents compared with 70%

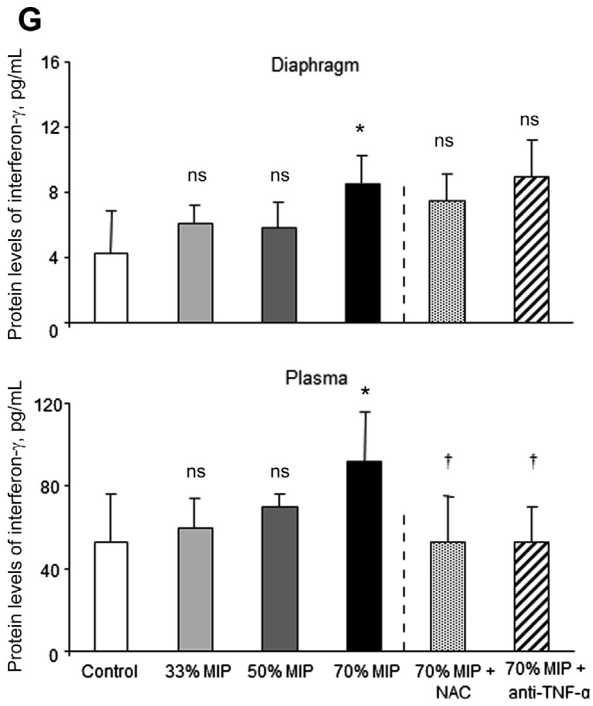

Fig 2. (*continued*).

MIP-only rats (Fig 2, *E*, top panel). Protein levels of IL-1β did not differ significantly among any of the study groups in either gastrocnemius (data not shown) or plasma (Fig 2, *E*, bottom panel).

Protein levels of IL-6 were increased significantly in the diaphragms of 70% MIP rats compared with the controls (Fig 2, *F*, top panel). More important, IL-6 protein levels were even greater in the respiratory muscle of both 70% MIP-NAC and 70% MIP-anti-TNF-α animals than in muscles of 70% MIP-only rodents (Fig 2, *F*, top panel). In the limb muscle, no significant differences were observed in IL-6 protein levels among the study groups (data not shown). In plasma, protein levels of IL-6 showed a significant increase in 70% MIP rats compared with the controls (Fig 2, *F*, bottom panel). IL-6 plasma levels exhibited a significant decline in both 70% MIP-NAC and 70% MIP-anti-TNF-α rodents compared with 70% MIP-only animals (Fig 2, *F*, bottom panel).

Protein levels of interferon-γ were increased in the diaphragms of the 70% MIP rats compared with the controls (Fig 2, *G*, top panel). Treatment with either NAC or anti-TNF-α antibody did not elicit any significant modification in interferon-γ protein levels in the diaphragms of 70% MIP rats (Fig 2, *G*, top panel). In the gastrocnemius, no significant differences were observed in interferon-γ levels among the study groups (data not shown). A significant increase in plasma interferon-γ levels was detected in the 70% MIP rodents compared with control animals (Fig 2, *G*, bottom panel). Interestingly, 70% MIP-NAC and 70% MIP-anti-TNF-α rodents exhibited a significant decline in plasma interferon-γ levels compared with 70% MIP-only rats (Fig 2, *G*, bottom panel).

Interestingly, TNF-α mRNA levels were increased significantly within the diaphragms of 70% MIP-anti-TNF-α rats compared with the controls, whereas no differences were observed in this marker between 70% MIP and control rats (Fig 3, *A*). Similarly, mRNA levels of the 2 TNF-α receptors I and II were also significantly greater in the diaphragms of 70% MIP-anti-TNF-alpha rodents, but not in those of 70% MIP-only rats, than in control animals (Fig 3, *B* and *C*, respectively).

Systemic and muscle oxidant production. Generation of superoxide anion was greater within the mitochondrial and membrane compartments in the diaphragm of 70% MIP rats than in the controls (Fig 4, top and medium panels, respectively). Interestingly, diaphragms of both 70% MIP-NAC and 70% MIP-anti-TNF-α rats exhibited a significant reduction in superoxide anion synthesis within the mitochondrial and membrane compartments compared with 70% MIP-only rodents (Fig 4, top and medium panels, respectively). In the diaphragm cytosolic fraction, superoxide anion levels were very low and did not differ significantly among the study groups (Fig 4, bottom panel). In the gastrocnemius and plasma, levels of superoxide anion were much lower within the mitochondrial, membrane, and cytosolic compartments compared with those observed within the

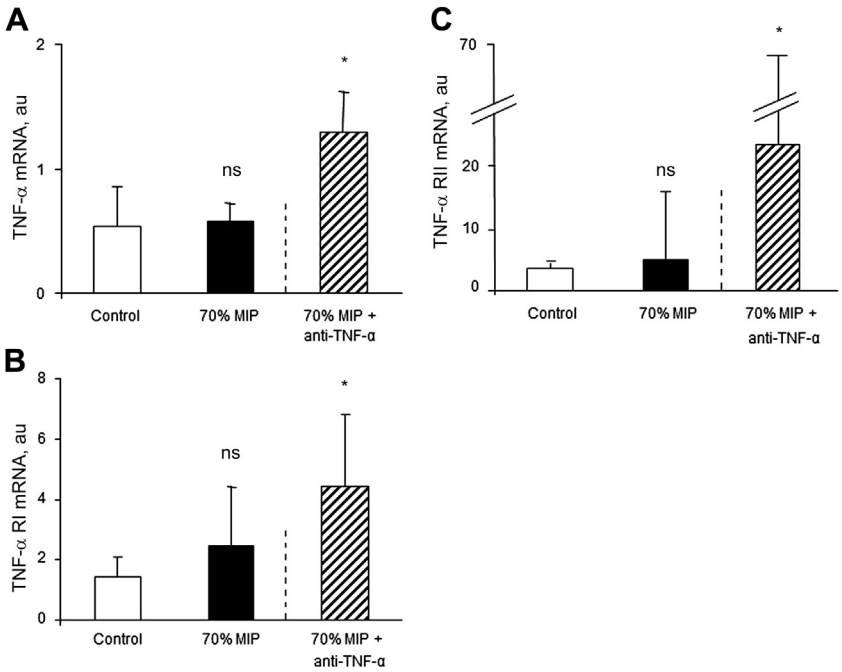

Fig 3. (**A**) In the diaphragm, messenger RNA (mRNA) levels of tumor necrosis factor (TNF)-α did not differ significantly between 70% maximum inspiratory pressure (MIP) and control rats (n = 8 per group, all groups). However, mRNA TNF-α levels were significantly greater in the diaphragms of rats treated with the anti-TNF-α antibody compared with 70% MIP rats only (*$P < 0.05$). (**B, C**) Similarly, mRNA TNF-α receptor I (RI) (**B**) and receptor II (RII) (**C**) levels were significantly greater in rats treated with the anti-TNF-α antibody compared with animals exposed to 70% MIP only (*$P < 0.05$). No differences were observed in mRNA RI or RII levels between 70% MIP and control animals. au, arbitrary units.

diaphragms, and no differences were detected among the study groups (data not shown).

Muscle regeneration markers. M-cadherin and myf-6 mRNA levels were significantly greater in the diaphragms of both 70% MIP and 70% MIP-anti-TNF-α rats, especially in the latter, than in the control rodents (Fig 5, *A* and *B*). Levels of m-cadherin and myf-6, however, did not differ in the gastrocnemius muscle in any of the study groups (data not shown). In addition, the immunohistochemical expression of both m-cadherin and myf-6 was more intense in the diaphragms of both 70% MIP-only rodents and 70% MIP rats treated with anti-TNF-α antibody (Fig 5, *C*, middle and right top and bottom panels, respectively) compared with the control rodents (Fig 5, *C*, left top and bottom panels, respectively).

Muscle structure. Proportions of total structural abnormalities were increased within the diaphragm of 70% MIP rats compared with the controls (Table III). Interestingly, respiratory muscles of 70% MIP-NAC rats exhibited a significant decline in total muscle abnormalities compared with control rats, whereas 70% MIP-anti-TNF-α rodents showed a significant increase (Table III). In the same diaphragms, counts of internal nuclei were greater in 70% MIP than in control animals. More important, in respiratory muscles of 70% MIP-anti-TNF-α rats, internal nuclei levels were even greater than in 70% MIP-only animals (Table III). NAC treatment did not elicit any modification in the numbers of diaphragm internal nuclei in the 70% MIP rodents compared with 70% MIP-only rats (Table III). Inflammatory cell counts were increased significantly in the respiratory muscles of the 70% MIP rats compared with the controls (Table III). Interestingly, diaphragm levels of inflammatory cells were decreased significantly in both 70% MIP-NAC and 70% MIP-anti-TNF-α rodents compared with 70% MIP-only rats (Table III). Levels of other structural abnormalities such as signs of necrosis within the diaphragm muscle fibers were also significantly greater in 70% MIP rodents than in the controls, whereas those levels were much lower (and similar to those in the unloaded controls) in the diaphragm of both 70% MIP-NAC and 70% MIP-anti-TNF-α compared with 70% MIP-only rodents (Table III). In the gastrocnemius, no differences were observed in any of these parameters among the study groups (data not shown).

Compared with control animals, proportions of fast-twitch fibers were reduced, whereas those of type I fibers were increased in the diaphragms of 70% MIP rats (Table IV). The size of type II fibers, but not that of type I, was diminished significantly in the respiratory muscles of the 70% MIP rodents compared with the controls (Table IV). In diaphragms of both groups of 70% MIP-NAC and 70% MIP-anti-TNF-α rats, proportions of fast-twitch fibers were increased (control levels), whereas those of slow-twitch were decreased compared with 70% MIP-only rodents (Table IV). Moreover, the size of type II fibers was greater in the respiratory muscle of 70% MIP-NAC and 70% MIP-anti-TNF-α animals than in 70% MIP-only rats (Table IV). Eventually, no differences among groups were observed in either muscle type proportions or sizes within the limb muscles (Table IV).

DISCUSSION

From a molecular standpoint, protein levels, but not mRNA, of the cytokine TNF-α, were increased in the respiratory muscle and blood of rodents exposed to high inspiratory loads compared with controls. These findings are consistent with previous reports in which an increase in TNF-α was observed in the diaphragm of rats exposed to inspiratory-resistive loading,[11] and in the external intercostals of COPD patients.[41] Moreover, as also demonstrated in former investigations,[11,41] the respiratory muscle exhibited an increase in protein levels of other proinflammatory cytokines in response to high inspiratory threshold loads, whereas the peripheral muscle did not exhibit any differences in the analyzed molecular events in any of the studies.[11,41] From the reported findings, including the current results, it is possible to conclude that strong muscle contractions induce the release of proinflammatory cytokines to the bloodstream whereas resting muscles do not.[25–27,44,45]

Animals exposed to the highest inspiratory loads exhibited a reduction in body weight gain compared with control rats, and treatment with either NAC or infliximab reversed that effect significantly. In line with a previously report,[46] it could be hypothesized that exposure to strenuous exercise (70% MIP) for 2 consecutive weeks may have altered the anabolic/catabolic balance toward a rather catabolic profile, accounting for the reduced body weight gain observed in those rodents. Although weights of viscera such as liver or lungs were not obtained in the study, it is likely that enhanced catabolism may have induced a decrease in the magnitude of those organs to a similar degree of that observed in the diaphragm fiber sizes. Moreover, despite the fact that a control group of rats exposed to identical experimental conditions (except for the inspiratory loads) was used in the investigation, the influence of general stress-related factors on body weight should not be neglected.

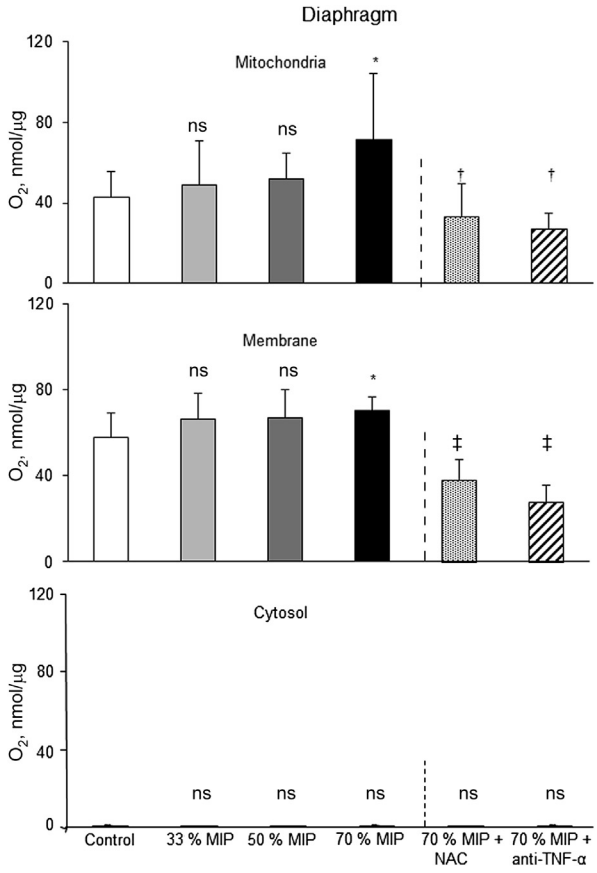

Fig 4. In both mitochondrial and membrane diaphragm muscle compartments, levels of superoxide anion were significantly increased in the 70% MIP group of rats compared to the controls ($*P < 0.05$), while concomitant treatment with either NAC or anti-TNF-alpha antibody induced a significant decline in superoxide anion levels in both muscle fractions ($^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$). No significant differences were observed in superoxide anion levels produced within the cytosol compartment among any of the study groups (n = 8 per group, all groups). ns, non-significant.

Another relevant outcome in the investigation is the differential fiber type distribution exhibited by the respiratory muscle in response to the highest inspiratory loads, characterized by a significant decrease (7%) in the proportions of fast-twitch fibers. Moreover, the size of type II fibers was also reduced significantly in the diaphragms of the 70% MIP rodents compared with the controls. These findings may also account for the lower respiratory muscle force generated by the animals after the high-intensity loading period compared with control animals (Fig 6, *A*). Indeed, among several factors, muscle force generation is highly dependent on the proportions of fast-twitch fibers.[47] Interestingly,

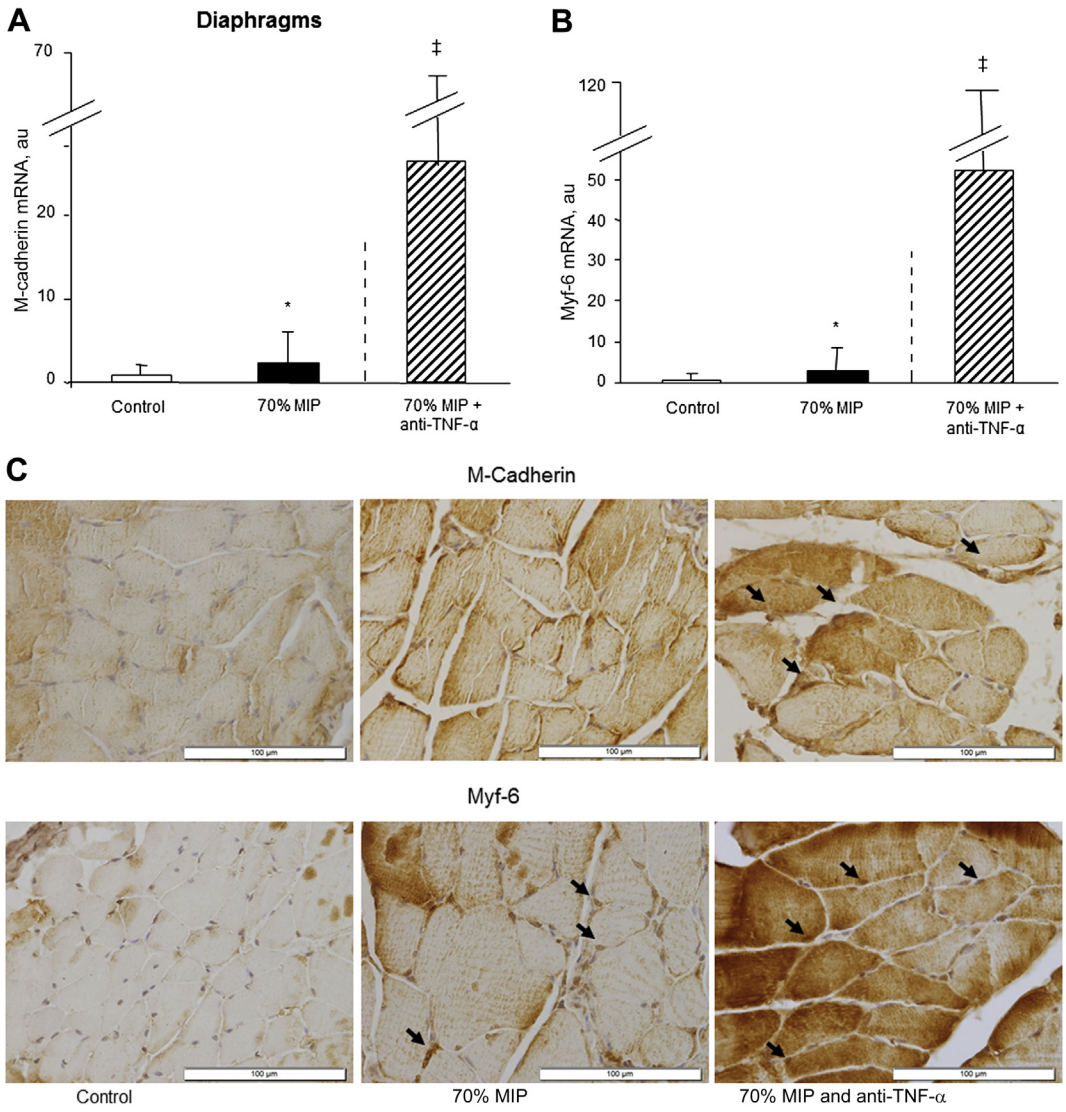

Fig 5. (**A, B**) Messenger RNA (mRNA) levels of the markers of muscle repair and growth—muscle calcium-dependent cell adhesion (m-cadherin) and myogenic factor 6 (myf-6)—were increased significantly in the diaphragms of the 70% maximum inspiratory pressure (MIP) rats compared with the controls (*$P < 0.05$). Furthermore, diaphragms of the 70% MIP rats treated concomitantly with the antitumor necrosis factor (anti-TNF)-α antibody exhibited an even greater increase in mRNA m-cadherin and myf-6 levels than animals exposed to 70% MIP only (†$P < 0.01$ and ‡$P < 0.001$). (**C**) Immunohistochemical expression of the adhesion molecule m-cadherin and that of the transcription factor myf-6 was more intense in the diaphragms of the 70% MIP, and 70% MIP and anti-TNF-α rats (middle and right top and bottom panels, respectively) was more intense than in the diaphragms of the control animals (left top and bottom panels, respectively, ×400, light microscopy). Scale bars are shown in each histologic figure. au, arbitrary units.

concomitant treatment with either NAC or anti-TNF-α antibody elicited an improvement in type II muscle fiber size and proportions within the diaphragms of the rodents exposed to high inspiratory loads. These findings may also account for the improvement in respiratory muscle force observed in the pharmacologically treated rats (Fig 6, *A*). Although diaphragm muscle mass was not determined in the study, it could possibly be argued that enhanced proteolysis of structural muscle proteins (eg, myosin), triggered by oxidants and inflammation, may have occurred in the rat diaphragm in response to chronic strenuous exercise, leading to fiber atrophy and lower body weight gain. Indeed, similar results were shown to occur in the vastus lateralis of healthy humans.[48]

More important, compared with control rodents, protein levels of proinflammatory cytokines, including TNF-α, were increased in the diaphragms and plasma

Table III. Structural abnormalities in the diaphragm of control and loaded rats with and without pharmacologic treatment

Groups	Total abnormalities, %	Internal nuclei	Inflammatory cells	Lipofuscin	Abnormal viable	Inflamed/necrotic
Control	3.98%	21.83 (11)	54.17 (13)	0	4.83 (8)	2.17 (1)
33% MIP	4.66%	32.3 (3), ns	48.5 (5), ns	0, ns	0, ns	0, ns
50% MIP	4.97%	31.83 (15), ns	50.50 (21), ns	0, ns	0.83 (1), ns	3.50 (2), ns
70% MIP	8.08%*	55.83 (24)†	80 (26)‡	1.0 (2), ns	5.16 (4), ns	30.16 (10)*
70% MIP + NAC	4.66%§	59.75 (31), ns	17.37 (14)‖	0, ns	0§	0.25 (0.4)‖
70% MIP + anti-TNF-α	11%§	120 (42)¶	34 (18)¶	8, ns	0.25§	2.5 (3)‖

Abbreviations: MIP, maximal inspiratory pressure; NAC, N-acetyl cysteine; ns, not significant; TNF, tumor necrosis factor.
Values are expressed as mean (standard deviation). Values in the different categories are expressed as absolute values of the total amount of each item counted in the fields (1890) analyzed in the muscles. However, total abnormalities are expressed as the percentage of items considered to be abnormal (see Methods) within the total number of fields (1890, normal and abnormal) examined in all the muscles.
*$P < 0.001$, between any loaded group and control animals.
†$P < 0.01$, between any loaded group and control animals.
‡$P < 0.05$, between any loaded group and control animals.
§$P < 0.05$, between any of the treated groups exposed to 70% MIP and 70% MIP-only rats.
‖$P < 0.001$, between any of the treated groups exposed to 70% MIP and 70% MIP-only rats.
¶$P < 0.01$, between any of the treated groups exposed to 70% MIP and 70% MIP-only rats.

Table IV. Fiber type composition in the muscles of control and loaded rats with and without pharmacologic treatment

Morphometric analyses	Control	33% MIP	50% MIP	70% MIP	70% MIP + NAC	70% MIP + anti-TNF-α
Diaphragm						
Type I fibers, %	32 (4)	30 (4), ns	33 (5), ns	37 (6)*	31 (5)†	33 (2)†
Type II fibers, %	68 (4)	70 (4), ns	67 (4), ns	63 (5)*	69 (6)†	67 (2)†
Cross-sectional area, type I fibers; μm^2	624 (100)	630 (110), ns	605 (48), ns	600 (80), ns	611 (153), ns	617 (90), ns
Cross-sectional area, type II fibers; μm^2	803 (90)	817 (128), ns	785 (161), ns	740 (51)*	831 (195)†	810 (113)†
Gastrocnemius						
Type I fibers, %	26.6 (7)	27.9 (1), ns	27.4 (5), ns	26 (8), ns	21.3 (8), ns	25.9 (4), ns
Type II fibers, %	73.4 (7)	72.1 (1), ns	72.5 (5), ns	74 (8), ns	78.6 (7), ns	74.1 (4), ns
Cross-sectional area, type I fibers; μm^2	907 (103)	1075 (275), ns	1079 (263), ns	981 (206), ns	997 (112), ns	1104 (86), ns
Cross-sectional area, type II fibers; μm^2	1171 (347)	1353 (191), ns	1352 (247), ns	1135 (426), ns	1072 (294), ns	1155 (161), ns

Abbreviations: MIP, maximal inspiratory pressure; NAC, N-acetylcysteine; ns, not significant; TNF, tumor necrosis factor.
Values are expressed as mean (standard deviation).
*$P < 0.05$, between any loaded group and control animals.
†$P < 0.05$, between any of the treated groups exposed to 70% MIP and 70% MIP-only rats.

of rats exposed to the highest inspiratory loads, whereas levels of the TNF-α receptors I and II did not differ. In addition, the number of inflammatory cell counts was also increased significantly in the respiratory muscles of those rats, suggesting they could contribute greatly to the synthesis of TNF-α. On the other hand, the redundant actions of other proinflammatory cytokines such as IL-6 and IL-1β may account for the additional increase detected in their levels after TNF-α blockade in the rodents exposed to the highest loads that were treated concomitantly with infliximab. In addition, TNF-α blockade also induced an additional increase in TNF-α protein levels (ELISA and immunohistochemistry) in the diaphragms of the 70% MIP rodents, whereas protein levels of this cytokine and those of IL-6, IL-1β, or interferon-γ decreased significantly or remained unmodified in the plasma of the same animals. Concomitantly, mRNA levels of TNF-α and its 2 receptors (I and II) were increased in the diaphragms of the 70% MIP rats treated with infliximab but not in the muscles of the rodents exposed to identical levels of loading without the treatment.

In the current study, infliximab induced differential effects on muscles and blood compartments: a decline in protein levels of proinflammatory cytokines including TNF-α in the plasma, whereas an increase in TNF-α protein levels was detected in the diaphragms of the same animals. On this basis, it may be speculated that 2 different sources of TNF-α may be at stake in the current experimental model. On the one hand, "systemic" TNF-α produced primarily by inflammatory cells (afflux of these cells to the exercising diaphragm) would be responsible for the damaging effects observed in the

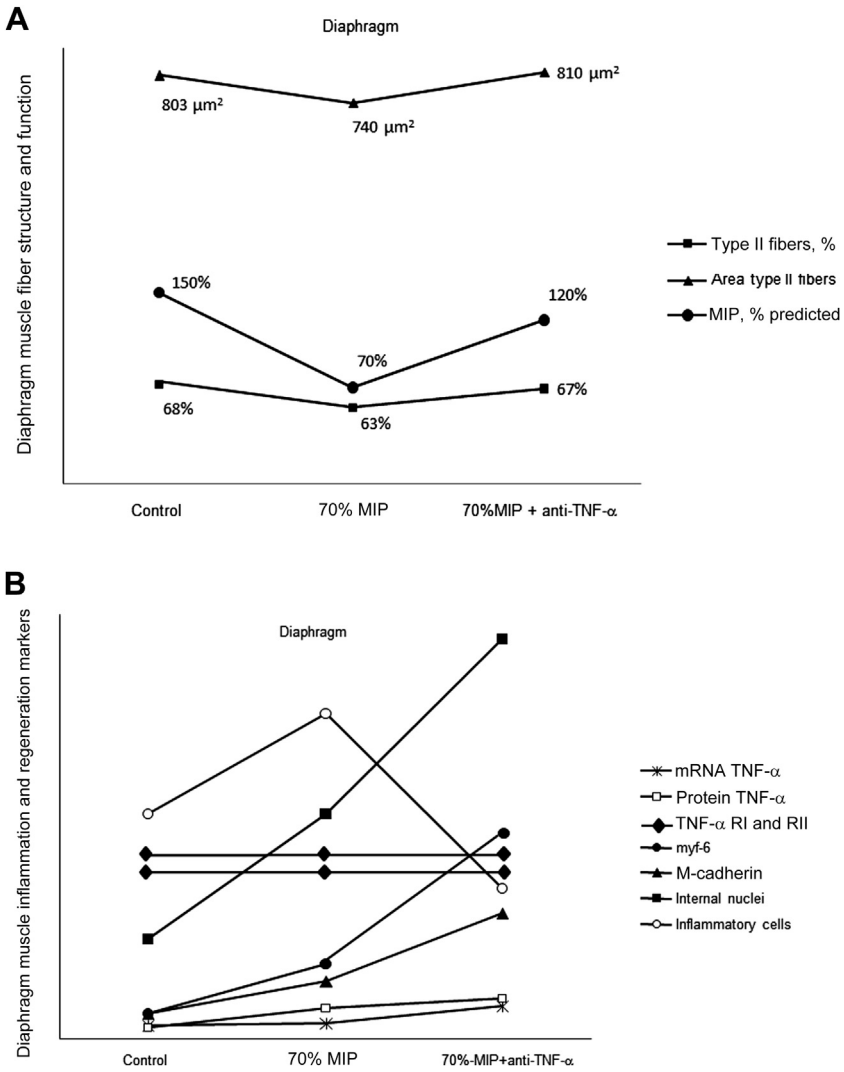

Fig 6. (**A**) Schematic representation of the levels of maximal inspiratory pressure (MIP) and variables of muscle fiber structure (proportions and cross-sectional area of type II fibers) in the diaphragms of unloaded control rats and animals exposed to 70% MIP with and without treatment with antitumor necrosis factor (anti-TNF)-α antibody (n = 8 per group, all groups). (**B**) Schematic representation of the levels of the markers TNF-α (both messenger RNA [mRNA] and protein levels), TNF-α receptor I (RI) and receptor II (RII), myogenic factor 6 (myf-6), muscle calcium-dependent cell adhesion (m-cadherin), internal nuclei, and inflammatory cell counts in the respiratory muscles of unloaded control rats and animals exposed to 70% MIP with and without treatment with anti-TNF-α antibody (n = 8 per group, all groups).

muscles of rodents exposed to the highest inspiratory loads: a decline in respiratory force production, reduced proportions and sizes of type II fibers, increased inflammatory cell counts, and increased protein levels, but not mRNA, of several proinflammatory cytokines (including TNF-α) in both diaphragm and plasma. All these effects were reversed significantly by treatment with infliximab.

On the other hand, TNF-α possibly synthesized by the myofibers (diaphragms), probably in much lower concentrations than those of "systemic" TNF-α, increased significantly (mRNA and protein levels) in rodents exposed to the highest loads treated with infliximab, probably as a trigger component of the regeneration process taking place in response to high-intensity loading. Additional data supporting this postulate are the reduced counts of inflammatory cells, the increase (mRNA and protein levels) in myogenic factors (m-cadherin and myf-6, involved in terminal muscle differentiation[49] and regulation of muscle phenotype,[50] respectively), and the greater number of internal nuclei detected in the diaphragms of the animals exposed to high-intensity loads receiving infliximab concomitantly, but not NAC. Hence, it would be possible to

conclude that in response to high-inspiratory loading, infliximab may have exerted its effects mainly on the production of "systemic/inflammatory" TNF-α by inflammatory cell counts, which may in turn account for the deleterious effects observed in MIP and diaphragm fiber structure, while having very little effect, if any, on TNF-α levels synthesized by the actual diaphragm fibers. Moreover, the decrease in systemic/inflammatory TNF-α synthesis induced by infliximab, as confirmed by the reduced inflammatory cell counts seen in the diaphragms, would have allowed the process of repair and regeneration to take place in these muscles, probably mediated by muscle TNF-α. In line with this, the ability of the myofibers to synthesize TNF-α has already been demonstrated in rodents.[22] Moreover, the stronger immunohistochemical expression of TNF-α cytokine within the muscle fibers and infiltrating inflammatory cells of the 70% MIP rats receiving infliximab reinforces this concept. In addition, programs of muscle regeneration have been shown to occur after chronic strenuous exercise in muscles of patients with COPD[40,41,51] and in rats[52] (Fig 6, *B*).

More important, an increase in both mRNA and protein levels of TNF-α was observed in the diaphragms of the animals treated with infliximab, but not in rodents exposed to the high-intensity loads without the treatment, in which only protein levels were, indeed, increased together with inflammatory cell counts. Again, we believe that whether TNF-α was synthesized by inflammatory cells or the actual myofibers, this may account for these findings. In this regard, the afflux of inflammatory cells to the exercising diaphragm would be responsible for the increase in proinflammatory cytokines (protein levels) in muscle and blood compartments (plasma), as also shown previously.[25-27,44,45] The concomitant process of muscle repair and regeneration occurring in the diaphragm after the blockade of TNF-α actions may account for the increase in mRNA and protein levels of this cytokine in those muscles. The decline in plasma levels of TNF-α, IL-6, and interferon-γ would reinforce this conclusion further. Moreover, the differential effects induced by the antioxidant NAC on cytokine protein levels, including TNF-α, in the respiratory muscle (greater increase) or plasma (significant decline) would also support this concept.

As expected, levels of superoxide anion were significantly greater within the mitochondria and membrane compartments, but not the cytosol, in the diaphragms of animals exposed to high inspiratory loads compared with controls and animals exposed to moderate and low levels of loading. These findings are consistent with findings reported by some of us, in which superoxide anion levels were shown to be increased in the same myofibrillar compartments of respiratory[16] and limb muscles of patients with COPD.[37] Indeed, in resting muscles, oxidants are generated at low levels, and they promote physiological functions, including regulation of the contractile process. During strong contractions or under pathophysiological conditions,[12] oxidants are synthesized at higher rates. Another interesting finding in our study was the decrease in superoxide anion production identified within the diaphragm mitochondria and membrane compartments of rats exposed to high loads treated concomitantly with either the antioxidant NAC or anti-TNF-α antibody. The significant reduction in inflammatory cell counts observed in the respiratory muscles of these groups of rodents may account in part for these results.

Study limitations. A first limitation of the study has to do with the potential formation of immune complexes in the muscles and blood of animals treated with infliximab as well as the identification of its bioactivity. Nonetheless, we do not believe that immune complexes may have influenced the levels of TNF-α detected using either ELISA or immunohistochemistry, because proper control groups, in which animals were exposed to exactly the same inspiratory loads without receiving concomitant treatment with infliximab, were also used in the study.

A second limitation refers to the lack of longitudinal experiments in the study, in which rodents were sacrificed at different time points after the loading period. However, assessment of the cellular and molecular events occurring in the muscle remodeling process was clearly beyond the scope of the current investigation and will be the focus of future research.

A third limitation is related to the lack of information on the weights of the muscles analyzed in the study. Nevertheless, because a control group of rodents was used in the investigation, the reported findings were all referred to the control rodents.

Last, it should also be underscored that the approach taken in the investigation, in which inspiratory loads were administered noninvasively to rats that remained fully awake and alive during the study protocol (14 consecutive days), reinforces its translational components. study limitations: See the online supplement for this section.

Speculations. Chronic, noninvasive, high-intensity inspiratory threshold loading causes a decline in respiratory muscle function and body weight. Furthermore, in the diaphragm, a reduction in fast-twitch fiber proportions and sizes was observed, whereas levels of inflammatory cells and cytokines (protein levels) and superoxide anion were increased, as well as internal nuclei counts and markers of myogenesis. Blockade of TNF-α with a specific antibody improved respiratory

muscle function, structure, animal weight, and in the diaphragm reduced drastically inflammatory cell numbers and superoxide anion production while inducing greater increases in protein and mRNA levels and immunohistochemical expression of TNF-α, internal nuclei counts, and markers of muscle regeneration. We conclude that TNF-α synthesized by inflammatory cells or myofibers could have differential effects on muscle structure and function in response to chronic, noninvasive, high-intensity inspiratory loading. These findings could have potential therapeutic implications in the administration of exercise training programs to patients with chronic respiratory conditions and muscle dysfunction, at least those of a rather short duration. Only muscle regeneration that may eventually take place at later stages could offset the deleterious physiological and biologic events that occur after 2 weeks of training.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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M. Dominguez-Alvarez conducted the animal experiments, obtained the biologic samples, performed part of the *in vivo* physiological and molecular experiments, and contributed to manuscript writing. M. Sabaté-Brescó conducted a great part of the molecular laboratory experiments and measurements, and expanded the database sheet. M. Vilà-Ubach performed part of the molecular biology experiments. J.B. Gáldiz participated in the animal experiments and physiological *in vivo* measurements. F.J. Alvarez participated in the animal experiments and physiological *in vivo* measurements. C. Casadevall conducted part of the molecular biology experiments and data interpretation. J. Gea contributed to the study design, data analyses, organization of the study results, and manuscript writing. E. Barreiro contributed to the study design; supervision of the molecular biology experiments, data analyses, and interpretation; and wrote the manuscript. All authors approved the final version of the manuscript.

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Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.trsl.2013.12.004.

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