

Theodoros Vassilakopoulos and Sabah N. A. Hussain

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HIGHLIGHTED TOPIC | *Free Radical Biology in Skeletal Muscle*

Ventilatory muscle activation and inflammation: cytokines, reactive oxygen species, and nitric oxide

Theodoros Vassilakopoulos¹ and Sabah N. A. Hussain²

¹Department of Critical Care and Pulmonary Services, University of Athens Medical School, Evangelismos Hospital, Athens, Greece; and ²Critical Care and Respiratory Divisions and Meakins-Christie Laboratories, Department of Medicine, McGill University Health Centre, Montreal, Quebec, Canada

Vassilakopoulos T, Hussain SN. Ventilatory muscle activation and inflammation: cytokines, reactive oxygen species, and nitric oxide. *J Appl Physiol* 102: 1687–1695, 2007. First published December 21, 2006; doi:10.1152/jappphysiol.01273.2006.—Strenuous diaphragmatic contractions that are induced by inspiratory resistive breathing initiate an inflammatory response that involves the elevation of pro- and anti-inflammatory cytokines within the diaphragm, which may then spill into the circulation. The production of reactive oxygen species within working respiratory muscles increases in response to these strenuous diaphragmatic contractions. At the same time, diaphragmatic nitric oxide (NO) production declines significantly, despite a time-dependent increase in NO synthase isoform protein expression. The increase in adhesion molecule expression and infiltration of granulocytes and macrophages that follows may contribute to the contraction-induced diaphragm injury. Enhanced generation of reactive oxygen species, oxidative stress augmentation, reduced NO production, and glycogen depletion are potential stimuli for the cytokine induction that is secondary to strenuous diaphragmatic contractions. This production of cytokines within the diaphragm may contribute to the diaphragmatic muscle fiber injury that occurs with strenuous contractions or to the expected repair process. TNF- α is a cytokine that compromises diaphragmatic contractility and may contribute to muscle wasting. IL-6 is a cytokine that may have beneficial systemic effects by mobilizing glucose from the liver and free fatty acids from the adipose tissue and providing them to the strenuously working respiratory muscles. Thus cytokine upregulation within the working diaphragm may be adaptive and maladaptive.

control of breathing; immune response; respiratory diseases; immune challenge

RESISTIVE BREATHING is encountered in many disease states, such as asthma, chronic obstructive pulmonary disease, and upper airway obstruction. When strenuous enough, inspiratory resistive breathing produces diaphragmatic fatigue (62) and diaphragmatic structural injury (50, 60). Strenuous diaphragmatic contractions associated with inspiratory resistive breathing also induce an inflammatory response that results in the elevation of pro- and anti-inflammatory cytokines within the diaphragm. These cytokines do not originate from monocytes but are, instead, produced within the diaphragm, secondary to the increased muscle activation. This review addresses the idea that resistive breathing per se is an “immune challenge” that initiates an inflammatory response within the respiratory muscles. This review is organized into two major parts. 1) The relations between ventilatory muscle activation and the production of cytokines and reactive oxygen and nitrogen species are reviewed. Then the stimuli responsible for muscle activation-induced cytokine production are discussed. 2) The func-

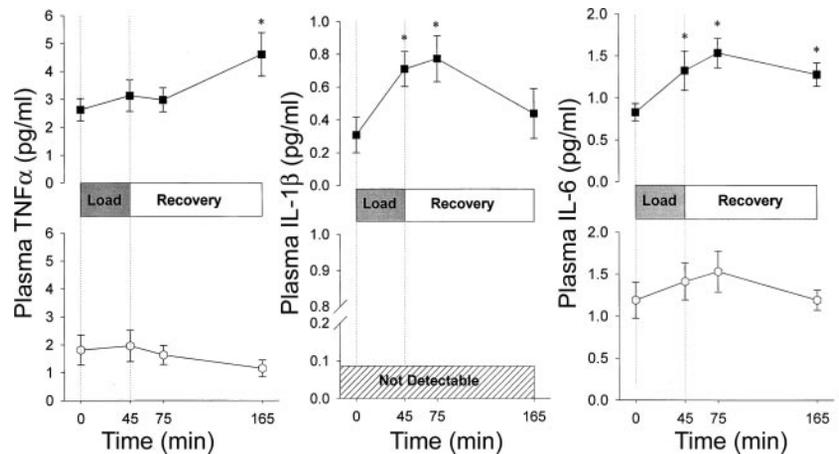
tional significance of cytokines in the ventilatory muscles is reviewed. The possible implications of the immune response in ventilatory muscle function and biology are addressed. The roles of cytokines in muscle regeneration, muscle contractility, metabolism, and endurance are highlighted. By speculating on how ventilatory muscle activation and inflammation intersect, this review is intended to stimulate debate and encourage further research in this area.

VENTILATORY MUSCLE ACTIVATION AND CYTOKINE PRODUCTION

Levels of circulating cytokines, including TNF- α , TNF receptors, IL-1 β , IL-1 receptor antagonist, IL-10, IL-8, and macrophage inflammatory protein-1, are increased by strenuous whole body physical exercise (51, 78). In the respiratory system, inspiratory resistive breathing represents a form of “exercise” for the ventilatory muscles. Accordingly, when healthy normal volunteers are subjected to strenuous resistive breathing (45 min at 75% of their maximum inspiratory pressure through a linear inspiratory resistance with unloaded expiration), plasma levels of TNF- α , IL-1 β , and IL-6 are

Address for reprint requests and other correspondence: S. N. A. Hussain, Dept. of Medicine, McGill Univ., Montreal, PQ, Canada H3A 1A1 (e-mail: sabah.hussain@mhcc.mcgill.ca).

Fig. 1. Mean plasma TNF- α , IL-1 β , and IL-6 in normal humans before resistive breathing (0 min), at the end of resistive breathing (load, 45 min), and 30 and 120 min after the end of resistive breathing (recovery, 75 and 165 min after the beginning of the resistive breathing session). *Top*: before antioxidant supplementation. *Bottom*: after antioxidant administration. * $P < 0.05$ compared with baseline values (0 time). [Adapted from Vassilakopoulos et al. (79).]



significantly elevated (Fig. 1) (79, 80). The origin, however, of these resistive breathing-induced cytokines has not been definitively established.

Resting skeletal muscles express low levels of various cytokines, including TNF- α (63), IL-1 β (3), and IL-6 (29). However, intramuscular cytokine production increases significantly in response to strenuous contraction. This is clearly the case in the diaphragm of spontaneously breathing rats that underwent

inspiratory resistive loading corresponding to 45–50% of maximum inspiratory pressure. In these animals, mRNA expression of IL-6 and, to a lesser extent, IL-1 β , TNF- α , IL-4, IL-10, and IFN- γ significantly increased inside the diaphragm in a time-dependent manner (Fig. 2, left) (76). Elevation of cytokine mRNA levels inside the diaphragm is also accompanied by commensurate increases in levels of cytokine protein, at least IL-6 and IL-1 β (76). Furthermore, immunohistochemistry re-

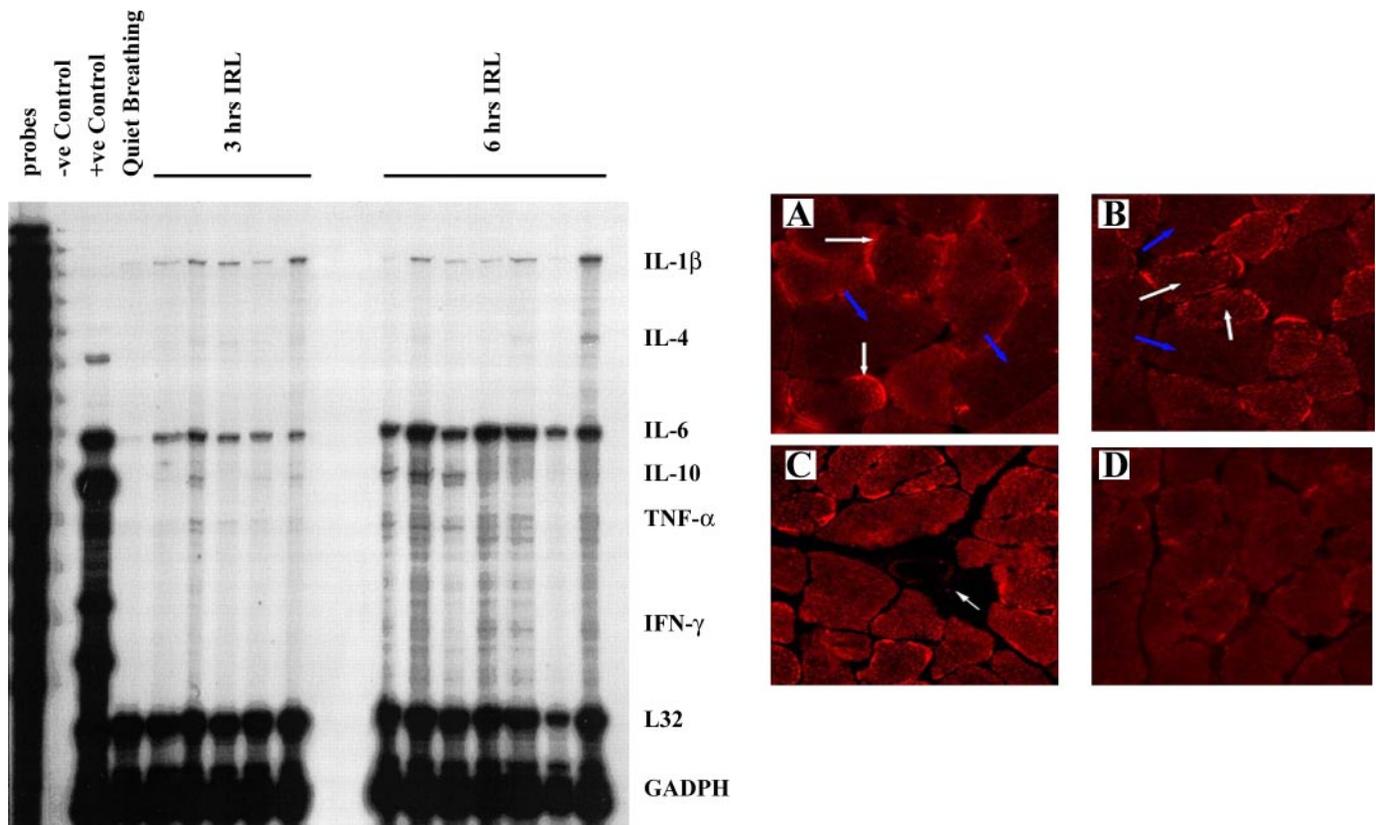


Fig. 2. Regulation of cytokine production inside the diaphragm in response to inspiratory resistive loading (IRL) in rats. *Left*: representative autoradiograph of RNase protection assay of diaphragm muscle samples obtained after 3 h (lanes 5–9) and 6 h (lanes 10–16) of inspiratory resistive loading. Lanes 1–3, probe [negative (–ve) and positive (+ve) control]; lane 4, diaphragm of a quietly breathing rat. A total of 10 μ g RNA was used in each lane. *Right*: localization of IL-6 protein expression in rat diaphragms. Goat anti-rat IL-6 (A) and rabbit anti-rat IL-6 (B) antibodies detected positive IL-6 staining in diaphragms of rats subjected to 6 h of inspiratory resistive loading. Membrane-associated (white arrows in A) and punctuate cytosolic (white arrows in B) positive IL-6 staining was evident inside small muscle fibers, whereas large muscle fibers showed no IL-6 staining (gray arrows). Blood vessels were negative for IL-6 protein (white arrow in C). Very weak IL-6 staining was detectable in the diaphragm of quietly breathing rats (D). [Adapted from Vassilakopoulos et al. (76).]

veals that myocytes are the main source of IL-6 production inside the diaphragm (76) (Fig. 2, *right*). Thus cytokines are constitutively expressed within the ventilatory muscles and are significantly upregulated during strenuous contractions, which are induced by inspiratory resistive loading. Moreover, there is indirect evidence that cytokines are upregulated in the diaphragm secondary to other forms of increased activation, such as the hyperventilation that accompanies exercise (48). In this study, rats were subjected to an acute bout of exercise (90 min of treadmill running). At the end of the exercise session, they were killed and their diaphragms were excised and homogenized. The diaphragmatic homogenate had the capacity to increase *in vitro* proliferation of thymocytes, suggesting, but not proving, that cytokine bioactivity inside the muscle rises significantly, since cytokines strongly stimulate thymocyte proliferation (48).

It has been suggested that cytokines produced within the diaphragm in response to inspiratory resistive loading might be released into the circulation and could partly account for the elevated cytokine plasma levels that are secondary to resistive breathing. This suggestion is based on the observations of Steensberg et al. (67), who reported a substantial rise in arteriovenous IL-6 differences across the contracting femoris muscle during leg extension exercise, suggesting that IL-6 production within the quadriceps femoris muscle accounts for the elevation in plasma IL-6 levels. It should be emphasized that the exact contribution of muscle fibers, endothelial and vascular smooth muscle cells, and resident neutrophils to enhanced IL-6 production was not determined in that study.

VENTILATORY MUSCLE ACTIVATION AND REACTIVE OXYGEN SPECIES PRODUCTION

Increased diaphragmatic activity leads to augmented reactive oxygen species (ROS) formation in a fashion similar to that described in other skeletal muscles (55). Under resting conditions *in vitro*, diaphragm fiber bundles produce relatively low levels of reactive oxygen intermediates, which are released into the extracellular space (56, 59). In unfatigued muscles, these relatively low levels of ROS are required for normal force generation (55). Strong ventilatory muscle contractions, such as those produced by *in vitro* and *in vivo* artificial nerve stimulation, result in significant augmentation of production and release of ROS by these muscles (32, 55, 56, 59, 68). Similarly, resistive breathing also increases the production of ROS inside the working ventilatory muscles *in vivo*. This was directly confirmed in the diaphragm using electron spin resonance spectroscopy (6). Furthermore, elevated ROS production inside ventilatory muscle fibers is associated with a significant decline in reduced glutathione levels, enhanced oxidized glutathione concentrations, and increased levels of thiobarbituric acid-reactive substances and 4-hydroxy-2-nonenal (HNE) protein adduct formation (indexes of lipid peroxidation) (1, 20, 69–71). Sources of increased ROS production in the ventilatory muscles, secondary to increased muscle activation, have not been completely elucidated but may involve xanthine oxidase pathways (68) and phospholipase A₂ activation (47). This occurs in the mitochondria to augment ROS generation by the mitochondrial electron transport chain (46) and/or on the inner surface of the sarcolemmal membrane to stimulate membrane-associated NADPH oxidase (24). In contrast to resting

muscles, enhanced ROS production during strong ventilatory muscle contraction contributes significantly to a decline in the contractile performance of these muscles.

VENTILATORY MUSCLE ACTIVATION AND NITRIC OXIDE PRODUCTION

Nitric oxide (NO) is synthesized inside normal skeletal muscle fibers by endothelial and neuronal NO synthases (eNOS and nNOS) (30, 31, 64). The eNOS isoform is localized inside the mitochondria (31) and in sarcolemmal caveolae (at least in cardiac myocytes) in association with caveolin-3 (15). The nNOS isoform directly associates with the dystrophin complex and is localized in close proximity to the sarcolemma of mainly type II fibers in rodents. More widespread nNOS expression across fiber types has been reported in human skeletal muscles (7). In contrast to abundant eNOS and nNOS expressions, very low levels of the inducible NOS (iNOS) isoform are expressed in skeletal muscles of normal mammals under resting conditions.

In the ventilatory muscles, the effects of muscle activation on NO production are time and load dependent. For instance, very short-term inspiratory resistive loading (<10 min) does not change NOS activity in the diaphragm (4), whereas 3 h of moderate inspiratory resistive loading results in a significant decline in ventilatory muscle NOS activity and no change in NOS protein expression (17). More severe inspiratory resistive loading elicits a significant decline in diaphragmatic NO production, despite a compensatory and time-dependent increase in eNOS, nNOS, and iNOS protein expression (77) (Fig. 3). The reduction in diaphragm NO level in response to acute increases in muscle activation is similar to that observed in the heart in response to acute bouts of exercise (21). In contrast to short-term increases in ventilatory muscle activation, long-term increases in diaphragmatic activity, elicited by the hyperventilation that accompanies chronic exercise training, evoke upregulation of NOS activity and eNOS and nNOS protein expression (74).

Responses of NO production and NOS expression to short-term activation in ventilatory muscles are clearly different from those in limb muscles. Electrical stimulation of the extensor digitorum longus or soleus muscle elicits an increase in muscle NO production (2, 72). Similarly, NOS activity increases by ~40% in the gastrocnemius muscle after 45 min of treadmill running (61). Moreover, an increase in nitrate concentrations can be detected in limb skeletal muscles after various treadmill-running protocols (53). The mechanisms responsible for these differences in NO production elicited by short-term activation in the ventilatory and limb muscles remain unclear. One mechanism might be alterations in NOS cofactor and substrate levels, which could be triggered by strenuous muscle activity. No information is available concerning whether such alterations occur in response to limb or ventilatory muscle activation. Another possible mechanism through which an acute increase in muscle activation may alter NO production in the ventilatory muscles is phosphorylation of nNOS at Ser⁸⁴⁷, which is known to cause significant inhibition of nNOS activity and attenuate NO production (33). Vassilakopoulos et al. (77) recently tested this possibility and reported that short-term inspiratory resistive loading in rats does not alter the degree of diaphragm nNOS phosphorylation at Ser⁸⁴⁷. Whether nNOS

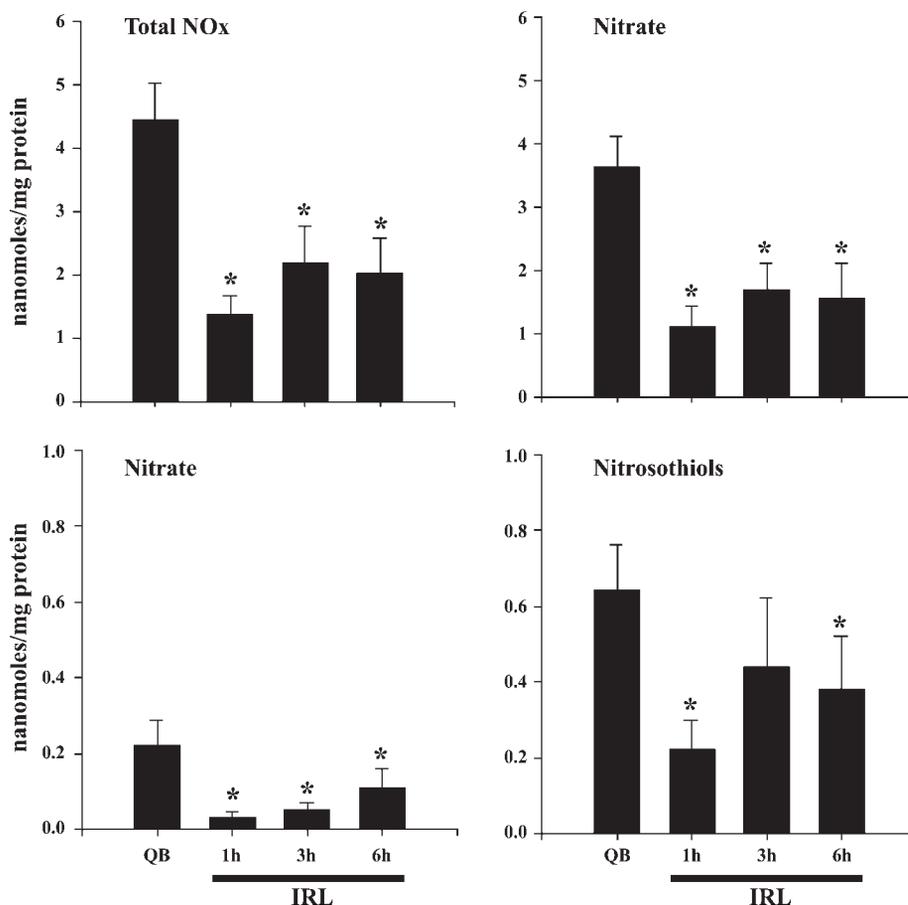


Fig. 3. Regulation of nitric oxide (NO) and NO derivatives inside the diaphragm in response to severe inspiratory resistive loading in rats. Total NO (NOx), nitrate, nitrite, and nitrosothiols (normalized per mg total protein) were measured in diaphragm homogenates from quietly breathing rats (QB) and rats exposed to 1, 3, and 6 h of inspiratory resistive loading. Values are means \pm SE. * $P < 0.05$ vs. QB. [Adapted from Vassilakopoulos et al. (77).]

phosphorylation at Ser⁸⁴⁷ is altered in response to limb muscle activation has not been determined.

Another likely mechanism behind the decline in ventilatory muscle NO production during short-term inspiratory resistive loading is augmentation of endogenous NOS inhibitors, such as the protein inhibitor of nNOS (PIN) and endothelins. PIN is an 89-amino acid protein that constitutes a subunit of the dynein complex, a microtubule-based molecular motor. Jaffrey and Snyder (23) reported that PIN physically interacts with and inhibits the activity of nNOS by preventing the dimerization of nNOS monomers. The existence of PIN inside skeletal muscle fibers has been verified, and the distribution of PIN protein expression inside skeletal muscle fibers is described as being similar to that of nNOS (19). The involvement of PIN in the regulation of ventilatory muscle NO production during short-term inspiratory resistive loading was recently evaluated by Vassilakopoulos et al. (77), who observed no significant alterations in diaphragmatic PIN mRNA expression in response to 1–6 h of severe inspiratory resistive loading. However, they did not evaluate PIN protein levels and the degree to which PIN associates with and inhibits nNOS protein activity inside the diaphragm. Hence, the exact contribution of PIN to inspiratory resistive loading-induced inhibition of diaphragm NO production remains unclear.

Other inhibitors of muscle NOS activity are endothelins, which are vasoactive peptides produced by vascular endothelial cells, as well as by skeletal muscle fibers (18, 45). Endothelins, particularly endothelin-1, act through endothelin type A receptors to inhibit NO synthesis, in part through a protein

kinase C-dependent pathway (22). Vassilakopoulos et al. (77) recently reported that short-term severe inspiratory resistive loading elicits a significant upregulation of endothelin-1 and endothelin-3 mRNA expression inside the diaphragm and that this response coincides with decreased NO levels in these muscles. These results suggest that upregulation of endothelin expression, in response to an acute increase in ventilatory muscle activation, may be involved in regulating muscle NO production.

Although the functional significance of decreased NO production in the ventilatory muscles during severe inspiratory resistive loading remains to be determined, one can predict several advantages of this response, such as removal of the inhibitory influence of NO on diaphragmatic contractility, an effect that is mediated through cGMP-dependent and cGMP-independent mechanisms regulating myosin ATPase activity, excitation-contraction coupling, and sarcoplasmic reticulum Ca²⁺ flux (54, 64). Another advantage of reducing muscle NO production is removal of the inhibitory influences of endogenous NO synthesis on the activity of mitochondrial enzymes, including cytochrome *c* oxidase (11). By attenuating NO production during inspiratory resistive loading, the ventilatory muscles are able to augment mitochondrial respiration and protect sarcoplasmic reticulum Ca²⁺ release in response to muscle membrane depolarization.

It should be emphasized that decreased NO levels inside the ventilatory muscles during severe inspiratory resistive loading are accompanied by a time-dependent augmentation of muscle NOS protein expression, particularly that of nNOS and, to a

lesser extent, eNOS (77). Interestingly, severe inspiratory loading also elicits significant induction of iNOS expression in the ventilatory muscles, which is clearly evident after 6 h of loading (77). Under normal conditions, the iNOS protein is expressed at very low levels inside skeletal muscle; however, this expression is significantly induced in response to proinflammatory stimuli, such as the development of severe sepsis in humans (40) or the administration of bacterial LPS in animals (5, 13).

The mechanisms responsible for inducing iNOS protein expression in the ventilatory muscles during severe inspiratory resistive loading are unknown. However, the fact that iNOS expression in skeletal myocytes is induced significantly in response to a mixture of IFN- γ , IL-1 β , and TNF- α (49, 86) and that inspiratory resistive loading elicits significant upregulation of these cytokines within the diaphragm (76) suggests that iNOS induction in the diaphragm, secondary to inspiratory resistive loading, might be the result of upregulation of local proinflammatory cytokines. A lack of diaphragmatic iNOS expression after only 3 h of resistive loading might be due to insufficient local levels of IFN- γ , IL-1 β , and TNF- α , since the expression of these cytokines within the diaphragm is significantly lower at 3 h than at 6 h of resistive loading. Expression of IFN- γ , a prerequisite cytokine for iNOS induction, is only upregulated after 6 h of resistive loading (76).

STIMULI RESPONSIBLE FOR MUSCLE ACTIVATION-INDUCED CYTOKINE PRODUCTION

The stimuli responsible for increased proinflammatory cytokine production in the ventilatory muscles during inspiratory resistive loading remain elusive. One likely stimulus is the enhanced generation of ROS. The involvement of ROS in regulating muscle IL-6 production has been tested in cultured skeletal satellite cells. Exposure of differentiated C₂C₁₂ murine satellite cells (myotubes) to ROS-producing agents such as pyrogallol, xanthine/xanthine oxidase, or H₂O₂ for 24 h results in a concentration-dependent increase in IL-6 production (34). This rise can be inhibited by the antioxidant enzymes superoxide dismutase and catalase (34). In addition, pretreatment of cells with *N*-acetylcysteine blocks TNF- α -induced IL-6 release, suggesting that endogenously produced ROS participate in IL-6 production. Myotubes stimulated with H₂O₂ exhibit increased I κ B α phosphorylation and degradation, and treatment of C₂C₁₂ myotubes with ROS-generating agents increases activator protein-1 and NF- κ B-dependent promoter activity. Moreover, preincubation of myotubes with the pharmacological inhibitor of NF- κ B diethylthiocarbamate or transient transfection with I κ B α mutant inhibits ROS-stimulated IL-6 release (34). These *in vitro* results indicate that ROS stimulate IL-6 production from skeletal muscle cells in a manner that involves transcriptional activation of the IL-6 gene through an NF- κ B-dependent pathway.

Oxidative stress is also a stimulus for increased plasma cytokine levels that are secondary to resistive breathing in humans. Indeed, Vassilakopoulos et al. (79) studied healthy subjects who performed two inspiratory resistive breathing sessions (45-min duration at 70% of maximum inspiratory pressure) before and after receiving a combination of antioxidants (vitamins E, A, and C, allopurinol, and *N*-acetylcysteine). Before antioxidant therapy, resistive breathing increased the

plasma levels of TNF- α , IL-1 β , and IL-6. After antioxidant therapy, plasma IL-1 β became undetectable, the TNF- α response was abolished, and the IL-6 response was significantly blunted (Fig. 1). Similarly, plasma levels of TNF- α , IL-1 β , and IL-6 increased significantly in response to whole body exercise (cycling) in healthy humans, and antioxidant therapy attenuated this response (78). Since a cocktail of ROS scavengers (vitamins A, E, and C and *N*-acetylcysteine) and inhibitors of ROS-producing enzymes (allopurinol) was used in these studies, the exact source of ROS responsible for the increase and, hence, enhanced cytokine production in response to exercise could not be determined. However, since there are multiple sources of ROS, including the mitochondrial electron transport chain, arachidonic acid metabolites, xanthine oxidase, and NADPH oxidase, in skeletal muscle fibers (24, 55), it is to be expected that a combination of scavengers and inhibitors would be more effective in lowering ROS levels than any single agent. In fact, defense against oxidative stress depends on an orchestrated synergism between several antioxidants.

Preliminary evidence suggests that, in addition to ROS, endogenous NO plays an important role in regulating cytokine production inside the diaphragm. When anesthetized rats are subjected to inspiratory resistive loading for 1 or 3 h in the presence of *N*^ω-nitro-L-arginine methyl ester-induced selective inhibition of NOS activity, proinflammatory cytokine production rises significantly higher than in animals that were not treated with *N*^ω-nitro-L-arginine methyl ester. This suggests that endogenous muscle NO production exerts a negative influence on intramuscular cytokine production (75). This effect might be mediated indirectly through scavenging effects of NO on ROS. On the basis of this possibility, one could also explain the association of reduced endogenous NO production inside the ventilatory muscles, during inspiratory resistive loading, with elevated ROS levels inside these muscles (77).

It should be emphasized that antioxidant therapy lessened, but did not completely abolish, exercise- or inspiratory resistive loading-induced IL-6 production by skeletal muscle fibers (79). It is possible that exogenous antioxidants were insufficient in concentration or compartmentalization to fully inhibit ROS-induced IL-6 production. Alternatively, this finding suggests that stimuli for IL-6 production during resistive breathing might be multiple. Although these stimuli have not been studied under resistive breathing conditions, results from whole body exercise studies suggest that carbohydrates attenuate the IL-6 response to exercise and glycogen depletion greatly augments it (52). Interestingly, significant glycogen depletion has been documented in the diaphragm, secondary to resistive breathing (10). Furthermore, total HNE-protein adduct formation inside the diaphragm (index of lipid peroxidation) rises significantly after inspiratory resistive loading. This response is attributed in part to increased HNE-derived modifications of the glycolysis enzymes enolase and aldolase (20). The fact that HNE-derived modifications of these enzymes, particularly enolase, results in direct inhibition of the activity of these enzymes (20) suggests that enhanced lipid peroxidation and HNE formation during short-term increases in ventilatory muscle activation, as occurs during inspiratory resistive loading, may result in inhibition of glycolysis enzymes. When coupled with increased muscle energy expenditure, this inhibition may lead to glycogen depletion and, consequently, increased IL-6 production by these muscles. Apart from the

case of IL-6, the mechanisms involved in muscle activation-induced proinflammatory cytokine production are not clearly understood. Nevertheless, the observation that pretreatment with antioxidants abolishes the resistive breathing-induced elevation of TNF- α and IL-1 β implicates ROS as major regulators of muscle-derived IL-1 β and TNF- α production (79).

FUNCTIONAL SIGNIFICANCE OF CYTOKINES IN THE VENTILATORY MUSCLES

The production of cytokines within the diaphragm may mediate the muscle fiber injury that occurs with strenuous contractions or contribute to the expected repair process. These cytokines may also compromise diaphragmatic contractility or contribute to the development of cytokinemia. They may also have systemic effects, mobilizing glucose from the liver and free fatty acids from the adipose tissue to the strenuously working respiratory muscles.

Muscle injury. Strenuous resistive breathing elicits significant diaphragmatic injury in animals and humans (25, 50, 60). It is likely that many factors, including increased activity of the proteolytic enzyme calpain (60) and augmented ROS production (25), are involved in this process. It is also possible that increased proinflammatory cytokines may be involved in diaphragmatic fiber injury that is secondary to inspiratory resistive loading (79). Indeed, proinflammatory cytokines and their receptors are upregulated in skeletal muscles in various forms of muscle injury (82) and in muscle diseases such as critical illness polyneuropathy and myopathy (12).

One possible mechanism through which cytokines such as IL-1 β contribute to the development of muscle fiber injury is upregulation of adhesion molecule expression on the surface of endothelial cells, which leads to augmentation of transendothelial migration of blood-derived inflammatory cells (9). This response eventually results in the recruitment, initially, of neutrophils and, later, of monocytes into muscle interstitial sites. This involvement of proinflammatory cytokines and adhesion molecules in ventilatory muscle fiber injury was assessed in rabbits exposed to 1.5 h of inspiratory resistive loading (81). Significant fiber injury was detected in the diaphragms of these animals 72 h after the termination of resistive loading. Fiber injury was associated with significant upregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in blood vessels that traversed the diaphragm as well as abundant infiltration of macrophages and neutrophils in the interstitium of the diaphragm (81). This response is similar to the eccentric exercise-induced intramuscular IL-1 expression and neutrophil infiltration associated with limb muscle damage in humans (16). Furthermore, proinflammatory cytokines such as TNF- α might augment oxidative stress in a paracrine fashion, which would also contribute to muscle injury (58). Increased oxidative stress has been documented in the diaphragm as long as 3 days after resistive loading (25) and could not be attributed simply to an acute rise in local cytokine production. It should also be emphasized that increased production of proinflammatory cytokines in the ventilatory muscles during acute inspiratory resistive loading is usually associated with compensatory upregulation of anti-inflammatory cytokines such as IL-4 and IL-10, a response designed to limit the harmful biological effects of proinflammatory cytokines (76).

Muscle regeneration. Although several studies have implicated proinflammatory cytokines in the development of skeletal muscle fiber injury, there is evidence that these cytokines may also be important in orchestrating muscle recovery after fiber injury. For instance, cytokines such as TNF- α , IL-6, leukemia inhibitory factor, IL-1 β , and their cognate receptors are upregulated in skeletal muscle several days after the development of fiber injury (8, 27, 28, 36, 82). These cytokines enhance proteolytic removal of damaged proteins and damaged cells through recruitment and activation of phagocytes (44, 73).

TNF- α and leukemia inhibitory factor are also important signaling proteins for the regeneration of muscle fibers after injury (35, 82). In TNF- α receptor double-knockout mice or mice receiving TNF- α -neutralizing antibodies, muscle strength recovery after injury was reduced compared with that in wild-type mice, and expression of the myogenic determination transcription factor MyoD was reduced as well (82). These results are consistent with an important role for TNF- α in the early phase of myoblast differentiation into myotubes. Indeed, Li and Schwartz (43) reported that TNF- α production increases significantly during the early stage of C₂C₁₂ myoblast differentiation into myotubes and that TNF- α then acts in an auto-crine fashion to stimulate differentiation and myosin expression in C₂C₁₂ myotubes. These results clearly suggest that TNF- α may activate satellite cells to enter the cell cycle from their normally quiescent state and enhances their proliferation once the cycle has been initiated (42). Satellite cells are quiescent cells of embryonic origin that reside in the muscle and are transformed into myocytes during normal muscle remodeling or replace damaged myocytes when the muscle becomes injured. Not all investigators agree on the above-described pro-myogenic roles for TNF- α (37–39). Clearly, further research is required to confirm whether TNF- α actually promotes or inhibits satellite cell differentiation into myotubes and whether it promotes or impedes the recovery of injured skeletal muscles.

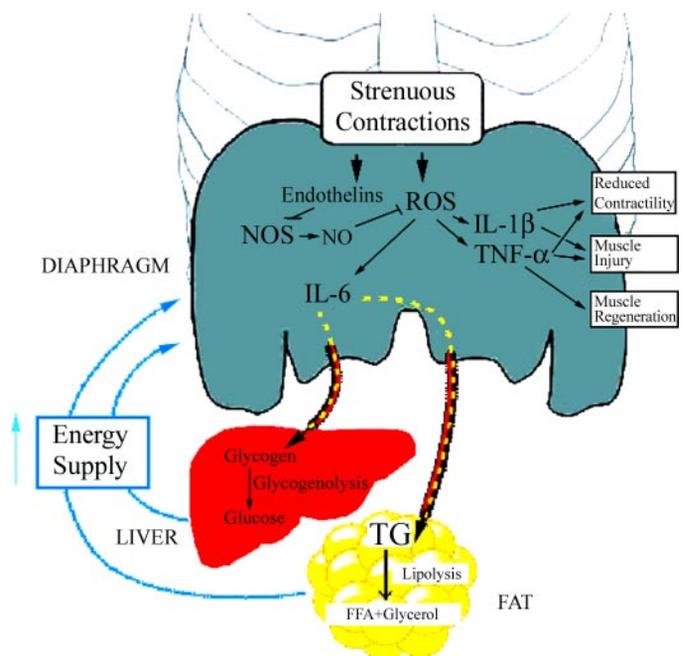


Fig. 4. Schematic presentation of the possible biological effects of muscle-derived cytokines, reactive oxygen species (ROS), and NO. TG, triglyceride; FFA, free fatty acid; NOS, NO synthase.

Muscle contractility. In addition to modulating muscle fiber injury and recovery, there is increasing evidence that proinflammatory cytokines exert important influences on ventilatory muscle contractile performance. An early study by Wilcox et al. (84) showed that secretory products derived from LPS-stimulated monocyte supernatants impair diaphragmatic contractility in vitro through a direct effect on muscle fibers. The most likely candidates for mediating the contractility-depressing effect are cytokines in the supernatant. Subsequent studies confirmed that in vitro exposure of diaphragm strips from hamsters or mice to TNF- α elicits a significant decline in muscle contractility (57, 83). Moreover, contractile dysfunction has been detected in diaphragm strips from mice with elevated circulating TNF- α levels (41). Further confirmation of the deleterious effects of TNF- α on ventilatory muscle contractility has been obtained in dogs receiving intravenous infusions of TNF- α (85).

The mechanisms through which TNF- α depresses diaphragmatic contractility have not been clearly elucidated; however, there is evidence that TNF- α signaling pathways act at the level of contractile proteins, since muscle fiber Ca²⁺ release and reuptake are not influenced by TNF- α exposure (57). It has been suggested, on the basis of recognition of the well-established inhibitory effects of TNF- α on muscle contractility, that increased production of TNF- α inside ventilatory muscle fibers may contribute to the development of fatigue of these muscles in response to inspiratory resistive loading (62). The intradiaphragmatic expression of cytokines, especially TNF- α , might also explain the observation that force decline after resistive loading is proportionally greater than muscle injury (26). Although force declines by as much as 30%, the degree of injury declines by only 9%, which suggests that other factors, in addition to fiber injury, are involved in reducing diaphragm contractility (26).

Effects on metabolism and endurance. Cytokines produced within the diaphragm, secondary to resistive breathing, may spill into the circulation, thereby explaining the cytokinemia observed after resistive loading in normal humans (79, 80). This rise in circulating levels of cytokines may serve several functions. Elevated IL-6 production within the diaphragm, secondary to resistive breathing, suggests that IL-6 may be involved in physiological muscle homeostasis, in addition to the regulation of injury-inflammation-repair processes (52). Strong diaphragmatic contractions, such as those generated during severe inspiratory loading, are likely to elicit muscle glycogen depletion (10), which is a strong stimulus for enhanced IL-6 production by skeletal muscle fibers (29, 66). In addition, IL-6 has a hormone-like role in carbohydrate metabolism: it signals that glycogen stores are reaching critically low levels in the contracting muscles and stimulates hepatic glucose output to maintain glucose homeostasis and muscle glucose supply (52) (Fig. 4). In addition, IL-6 also augments lipolysis and fat oxidation, which increases the energy available for the "exercising" ventilatory muscles (68). These findings suggest that IL-6 overproduction might be required to protect the endurance of the ventilatory muscles in conditions of increased respiratory load. This suggestion is based on the significantly reduced endurance and energy expenditure during whole body exercise in IL-6^{-/-} compared with wild-type mice, indicating that endogenously produced IL-6 is important for maintenance of exercise capacity (14). Finally, there is

evidence that IL-6 from the exercising limb or ventilatory muscles may exert an important anti-inflammatory effect by inhibiting the release of proinflammatory cytokines such as TNF- α . Indeed, LPS-induced TNF- α production in healthy humans is significantly attenuated when LPS is infused during exercise or during infusion of exogenous IL-6 (65). This finding suggests that physical activity mediates anti-inflammatory activity through IL-6 release from the exercising muscles. The greater induction of IL-6 within the diaphragm, secondary to resistive breathing, might represent an adaptive response designed to augment diaphragmatic endurance and attenuate the release of proinflammatory cytokines.

FUTURE PERSPECTIVES

Conventionally, cytokines have been considered mediators of inflammation during pathological conditions, such as infection or autoimmune diseases. Recently, however, accumulated evidence suggests that cytokines can be induced within the ventilatory muscles under physiological conditions, for example, during increased muscle activation. This knowledge provides a solid foundation for future investigations into the links between the mechanisms of cytokine upregulation, ventilatory muscle activation, and oxidative and nitrosative stresses. It also provides the experimental community with new concepts to address the interplay between these factors and inflammation, particularly the idea of resistive breathing as an immune challenge. Further research into the implications of the immune response in ventilatory muscle physiology and function will enhance our overall understanding of health and disease, inasmuch as improved methods of diagnosis and treatment of respiratory diseases such as asthma, chronic obstructive pulmonary disease, and upper airway obstruction require an understanding of the basic cellular mechanisms that contribute to the control of breathing.

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