Nitric oxide regulates cytokine induction in the diaphragm in response to inspiratory resistive breathing

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Nitric oxide regulates cytokine induction in the diaphragm in response to inspiratory resistive breathing. J Appl Physiol 113: 1594–1603, 2012. First published September 6, 2012; doi:10.1152/japplphysiol.00233.2012.---Respiratory breathing (encountered in chronic obstructive pulmonary disease and asthma) results in cytokine upregulation and decreased nitric oxide (NO) levels in the strenuously contracting diaphragm. NO can regulate gene expression. We hypothesized that endogenously produced NO downregulates cytokine production triggered by strenuous diaphragmatic contraction. Wistar rats treated with vehicle, the nonselective NO synthase inhibitor NG-nitro-L-arginine-methylester (L-NAME), or the NO donor diethylenetriamine-NONOate (DETA) were subjected to inspiratory resistive breathing (IRB; 50% of maximal inspiratory pressure) for 6 h or sham operation. Additional groups of rats were subjected to IRB for 6 h with concurrent administration of L-NAME and inhibitors of NFκB (BAY-11-7082, ERK1/2 (PD98059), or P38 (SB203580).

Inhibition of NO production (with L-NAME) resulted in upregulation of IRB-induced cytokine production, such as IL-6, IL-10, and IL-1β levels by 50%, 53%, 60%, 47%, and 45%, respectively. In contrast, the NO donor (DETA) attenuated the IRB-induced cytokine upregulation to levels characteristic of quietly breathing animals. L-NAME augmented IRB-induced activation of MAPKs (P38 and ERK1/2) and NFκB, whereas DETA triggered the opposite effect. NFκB and ERK1/2 inhibition in L-NAME-treated animals blunted the L-NAME-induced cytokine upregulation except IL-6, whereas P38 inhibition blunted all (including IL-6) cytokine upregulation. NO downregulates IRB-induced cytokine production in the strenuously contracting diaphragm through its action on MAPKs and NFκB function, although new studies point to their critical role on muscle regeneration after injury (52) and especially to IL-6 for its effects on glucose transport (42).

Skeletal muscle cells in their basal state continuously produce low levels of nitric oxide (NO) through the action of NO synthases (NOS) (50). From the three existing NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are consistently detected in skeletal muscles under basal conditions, whereas very low levels of inducible NOS can be detected in skeletal muscles of some mammals (22, 50). NO serves many physiological roles in skeletal muscle, such as regulation of muscle contraction, metabolism, blood flow, and myoblast differentiation (19, 20, 50). Furthermore, NO is an important signaling molecule that can regulate transcription factors and enzyme activity, affect mRNA stability or translation, and hence, regulate the expression of numerous genes (5). Accordingly, NO has been demonstrated to inhibit the expression of various cytokines in many cell types (12, 13, 17, 35). Our group has demonstrated that IRB results in decreased diaphragmatic NOS activity (15) and attenuation of end-products of NO metabolism (nitrite, nitrate, nitrosothiols) (56). On the basis of these studies, we propose that the decreased NO production inside muscle fibers of a strenuously contracting diaphragm contributes significantly to the concomitant intradiaphragmatic upregulation of cytokines. Recently, we reported that IRB-induced cytokine upregulation in the diaphragm is regulated by MAPKs and the transcription factor NFκB (49). NO can regulate NFκB and MAPK activity (5). We thus hypothesized that the effects of NO on the intradiaphragmatic cytokine expression are mediated through modulation of MAPKs and NFκB. To test our hypothesis, we conducted IRB in rats with and without pretreatment with the nonselective NOS inhibitor NG-nitro-L-arginine-methylester (L-NAME) and the NO donor diethylenetriamine-NONOate (DETA) to evaluate the effect of NO availability on IRB-induced cytokine upregulation. In a separate series of experiments, concurrent inhibitions of NO and NFκB, MAPK kinase 1/2 (MEK1/2)-ERK, and P38 were also carried out in rats subjected to IRB to evaluate the contribution of these pathways in the alteration of intradiaphragmatic cytokine expression mediated by NO.

METHODS

Animal preparation. All experimental procedures were approved by the Veterinary Administration of the Prefecture of Attica (Greece) and the hospital’s Ethics Committee. Wistar rats (200–250 g; Pasteur
Foundation, Athens, Greece) were anesthetized with ketamine and tracheostomized with polyethylene tubing connected to a two-way nonbreathing valve (Hans Rudolph, Kansas City, MO). After a short stabilization period, animals were randomly assigned to moderate IRB (peak inspiratory pressure of ~50% of maximum) for 6 h, according to the protocol described previously (49, 53, 56). In brief, the tracheal pressure was monitored continuously with the use of a pressure transducer (DirecWin, Raytech Instruments, Vancouver, Canada). The maximum inspiratory pressure (P_{\text{max}}) was measured during spontaneous breathing efforts through a totally occluded inspiratory port for 20 s. Then, the inspiratory line was connected to a tube of small diameter (resistance) adjusted to provide a peak tidal inspiratory pressure of ~50% of the measured P_{\text{max}} (49).

However, it should be mentioned that according to a recent study, transdiaphragmatic pressure (P_{\text{di}}) generated during inspiratory occlusion in rats is ~60% of maximum P_{\text{di}} (measured during bilateral phrenic nerve stimulation) (36). Thus, the way we adjusted the inspiratory resistance and consequently the load of the diaphragm in our study actually is an overestimation, given that the maximum pressure-generating capacity of the diaphragm is likely higher by ~40% (42). The inspiratory line was connected through the resistance to an oxygen reservoir that was supplied continuously with 100% oxygen. Animals subjected to IRB and quietly breathing animals received 100% oxygen throughout the 6 h of the experiment. Continuous oxygen supplementation prevented the development of hypoxemia in IRB rats (49, 55). We tested for the effect of hyperoxia by initially comparing two groups of quietly breathing animal rats inhaling either ambient air or 100% oxygen. As we did not find any differences in the parameters that we studied (signaling pathways, cytokines, etc.) between the two groups, quietly breathing animals received 100% oxygen to be in line with the IRB group. Throughout the 6 h of IRB protocol, all animals (controls and IRB group) were lightly sedated (but able to breath spontaneously) with periodic administration of ketamine/xylazine.

The study was conducted in two phases. 1) Animals (n = 10–15/group) were subjected to IRB for 6 h and treated with the nonselective NOS inhibitor l-NNAME (100 mg·kg\(^{-1}\)·h\(^{-1}\)) (4, 11), the NO donor DETA (10 mg/kg ip) (63), or vehicle (saline). 2) Animals (n = 10/group) were subjected to IRB for 6 h and pretreated with both l-NNAME and one of the following inhibitors: the NF-\kappaB inhibitor BAY-11-7082 (10 mg/kg ip) (30), the MEK1/2–ERK1/2 inhibitor PD98059 (0.5 mg/kg ip) (18), and the P38 inhibitor SB203580 (1 mg/kg ip) (18).

l-NNAME and DETA were purchased from Sigma-Aldrich (St. Louis, MO), whereas NF-\kappaB and MAPK inhibitors were from Calbiochem (La Jolla, CA). Sham-operated animals breathing against no load for 6 h and treated with vehicle or the above-mentioned compounds (n = 10/group) served as controls. All animals were killed at the end of the experiment, and the diaphragm and gastrocnemius muscle were excised quickly and frozen in liquid nitrogen. Blood was taken from the abdominal aorta just before animal death.

**Immunoblotting.** Crude tissue protein extracts (100 \(\mu\)g) (49) as well as 100 \(\mu\)g nuclear protein extracts (55) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed overnight with specific antibodies for phospho-NF-\kappaB/p65 (Ser 536), total and phosphorylated forms of P38 (Thr 180/Tyr 182) and ERK1/2 (Thr 202/Tyr 204, Cell Signaling Technology, Danvers, MA), and MKP-1 and b-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Specific proteins were detected with peroxidase-conjugated secondary antibodies and SuperSignal West Femto chemiluminescence kit (Pierce, Rockford, IL).

**ELISA for muscle cytokines.** IL-6, IL-2, IL-10, TNF-\alpha, and IL-1\(\beta\) protein levels in diaphragmatic crude extracts were evaluated by performing the appropriate ELISAs (DuoSet, R&D Systems, Abingdon, UK) according to the manufacturer’s instructions.

**Estimation of oxidative status.** Protein carbonyl content in diaphragm extracts and total nitrate/nitrite levels in serum were measured with the BioCell Protein Carbonyl Assay Kit (BioCell, New Zealand) and the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), respectively, according to the manufacturers’ instructions.

**Statistical analysis.** Statistical analysis was performed using STATISTICA 7 (StatSoft, Tulsa, OK). Statistical correlations were performed by ANOVA (with Tukey’s test for post hoc comparisons) and t-test by group analysis where appropriate. Data were expressed as mean ± SE. \(P \leq 0.05\) was considered statistically significant.

**RESULTS**

**Total nitrite–nitrate level.** To confirm that l-NNAME and DETA treatment successfully inhibited NO production and increased NO availability, respectively, total nitrite and nitrate levels (the stable end-products of NO metabolism) were measured in serum of quietly breathing animals and animals subjected to IRB for 6 h, treated with l-NNAME, DETA, or vehicle. l-NNAME treatment significantly reduced serum nitrite–nitrate content, whereas DETA increased it (Fig. 1).

**NOS inhibition increases IRB-induced cytokine production from the diaphragm.** Cytokine (IL-6, IL-10, IL-2, TNF-\alpha, and IL-1\(\beta\)) protein levels were estimated by ELISA in whole diaphragm homogenates. As shown previously (49), IRB for 6 h resulted in increased cytokine protein levels in the diaphragm. l-NNAME administration augmented this IRB-induced cytokine upregulation (Fig. 2). No difference was observed in cytokine protein levels in quietly breathing animals due to l-NNAME treatment (Fig. 2). Moreover, l-NNAME treatment had no effect on cytokine production from the noncontracting gastrocnemius muscle (Fig. 3).

**Effect of NO donor administration in the IRB-induced cytokine upregulation in the diaphragm.** The effect a NO donor (DETA) administration on cytokine production from the strenuously contracting diaphragm was evaluated in rats subjected to IRB for 6 h. DETA administration reduced the IRB-induced cytokine upregulation (for all studied cytokines) to control levels (Fig. 4). DETA had no effect on cytokine production from the diaphragm of quietly breathing animals (Fig. 4).

**Inhibition of NO production in the diaphragm increases IRB-induced MAPK and NF-\kappaB activation.** Recently, we demonstrated that the increased diaphragmatic cytokine production...
in response to IRB is under the regulatory control of MAPKs (P38 and ERK1/2) and NF-κB (49). To examine whether NO regulates this cytokine induction through modulations of these pathways, we examined the phosphorylation of MAPKs (P38 and ERK1/2) and that of the NF-κB/p65 subunit with immunoblotting in diaphragm extracts. IRB for 6 h resulted in increased phosphorylation of P38, ERK1/2, and NF-κB/p65 subunits, whereas NOS inhibition (with L-NAME) greatly augmented it (Fig. 5A). L-NAME treatment did not affect MAPK and NF-κB/p65 phosphorylation in the noncontracting gastrocnemius muscle (Fig. 5C).

**Effect of NO donor administration on the IRB-induced MAPK and NF-κB activation in the diaphragm.** The effect of NO donor–DETA administration on IRB-induced MAPK and NF-κB activation in the diaphragm was evaluated with immunoblotting. DETA administration attenuated P38 MAPK and ERK1/2 phosphorylation in the diaphragm of rats subjected to IRB for 6 h; treated or not with L-NAME. Quietly breathing animals served as control. Data are presented as pg/mg of total protein (TP), mean ± SE. *P < 0.05 vs. Ctr; #P < 0.05 vs. RB-NT animals.
6 h (Fig. 6A) to levels characteristic of quietly breathing animals. Similarly, IRB-induced NF-κB/p65 phosphorylation was decreased in DETA-treated animals (Fig. 6B).

Inhibition of NF-κB and MAPKs blunts the L-NAME effect on cytokine production from the diaphragm. To evaluate whether NO regulates cytokine induction through modulation of NF-κB and MAPKs, we conducted experiments in animals subjected to IRB for 6 h, pretreated with both L-NAME and inhibitors of NF-κB, ERK1/2, or P38 MAPK. As shown in Fig. 7, A–C, the NF-κB inhibitor BAY-11-7082, the MEK1/2–ERK1/2 inhibitor PD98059, and the P38 MAPK inhibitor SB203580 inhibited NF-κB/p65, ERK1/2, and P38 MAPK phosphorylation, respectively. Inhibition of NF-κB and ERK1/2 in L-NAME-treated animals reduced IL-10, IL-2, TNF-α, and IL-1β up-regulation but not that of IL-6 (Fig. 7, D and E). P38 MAPK inhibition blunted the upregulation of all examined cytokines (including IL-6) in the diaphragm of L-NAME-treated animals (Fig. 7, D and E). As shown previously (49), MAPK and NF-κB inhibitors did not affect cytokine production in the diaphragm of quietly breathing animals. Regarding MAPK inhibitors, the MEK1/2–ERK1/2 inhibitor did not affect P38 MAPK phosphorylation and expression, and the P38 MAPK inhibitor had no effect on ERK1/2 phosphorylation and expression, respectively (data not shown).

L-NAME affects NF-κB activation through its action on ERK1/2 and P38 MAPK. Inhibition of both P38 and ERK1/2 in L-NAME-treated animals subjected to IRB for 6 h blunted the upregulation of all NF-κB-dependent cytokines. To examine whether L-NAME affects NF-κB through its action on P38 and ERK1/2 (hence, P38 and ERK1/2 are upstream regulators of NF-κB), we tested NF-κB/p65 phosphorylation with immunoblotting in animals subjected to IRB and treated with L-NAME, with or without concurrent administration of P38 and ERK1/2 inhibitors. Inhibition of either P38 or ERK1/2 resulted in decreased NF-κB/p65 subunit phosphorylation (Fig. 8).

Effect of NO on MKP-1 expression in the diaphragm. One of the possible mechanisms through which L-NAME can affect the activation of MAPKs is through the regulation of MKP-1 (3), which dephosphorylates and hence, inactivates MAPKs. The expression of MKP-1 was evaluated with immunoblotting in the diaphragm of rats subjected to IRB for 6 h, treated with L-NAME, DETA, or vehicle. Neither L-NAME nor DETA treatment resulted in any difference of MKP-1 expression (Fig. 9).

Effect of NO blockade on diaphragm oxidative status. To examine whether the effect of NO blockade on cytokine production by the strenuously contracting diaphragm was due to modulations of diaphragmatic oxidative balance, protein carbonylation (an overall index of protein oxidative modifications) in the diaphragm was measured in quietly breathing animals and animals subjected to IRB for 6 h, treated with L-NAME, DETA, or vehicle. Neither L-NAME nor DETA treatment resulted in any difference of MKP-1 expression (Fig. 9).

Fig. 4. NO donor administration blunts IRB-induced cytokine production in the diaphragm. Protein levels (estimated by ELISA) of (A) IL-6, (B) IL-10, (C) IL-2, (D) TNF-α, and (E) IL-1β, respectively, in the diaphragm of rats subjected to IRB for 6 h and quietly breathing animals (control) treated with DETA or not treated. Data are presented as pg/mg of total protein, mean ± SE. *P < 0.05 vs. Ctr; #P < 0.05 vs. RB-NT animals.
DISCUSSION

The main findings of this study are that 1) blockade of NO production in rats subjected to IRB greatly augments IRB-induced cytokine production (IL-6, IL-10, IL-2, TNF-α, and IL-1β) and MAPK and NF-κB activation in the diaphragm, whereas administration of the NO donor triggers exactly the opposite effect; 2) NO regulates IL-6 induction through its action on P38 MAPK, whereas NO-mediated regulation of IL-10, IL-2, TNF-α, and IL-1β is mediated through ERK1/2, P38 MAPK, and NF-κB pathways; and 3) inhibition of NO production does not affect IRB-induced protein oxidation.

To our knowledge, this is the first study to establish a regulatory role for NO in cytokine expression inside diaphragmatic muscle fibers during IRB. IRB, a clinically relevant form of an acute increase of workload for the diaphragm, results in cytokine upregulation in the diaphragm (49, 55). This cytokine induction is a relatively rapid event (evidenced as early as the 1st h of IRB) and is sustained throughout the 6 h of resistive breathing protocol (49, 55). Previous work from our group demonstrated a decrease in NO derivatives in the diaphragm of rats subjected to IRB (56). NO is an important signaling molecule evidenced to regulate cytokine induction in many cell types (12, 13, 17, 27, 35). In this study, we demonstrate that IRB-induced cytokine upregulation in the diaphragm was augmented with blockade of NO production, whereas an increase in NO availability (with a NO donor administration) blunted it. This effect was specific to the diaphragm, as no difference was observed on cytokine induction from the noncontracting gastrocnemius muscle of RB and RB-LN rats.
represented as fold increase to control, mean ± SE. *P < 0.05 vs. C; #P < 0.05 vs. RB. p-P38 and p-ERK were normalized to respective total forms, whereas p-p65 to b-tubulin.

decline in NO production observed in the diaphragm in response to IRB contributes to the observed cytokine upregulation. Interestingly, NO had no effect on cytokine production from the diaphragm of quietly breathing animals. The level of cytokine expression in the resting diaphragm is quite low so that NO alteration by itself might be inadequate to produce significant, measurable changes in the level of the respective cytokines. We speculate that for NO to become a regulator of cytokine induction in the diaphragm, a level of diaphragm activation is required. The molecular pathways responsible for this requirement are not known.

Cytokine induction in response to acute increases of workload is a consistent finding in studies of exercising peripheral skeletal muscles (42). IL-6, which is the most abundant and most studied cytokine in skeletal muscles, is induced rapidly with exercise, reaching a peak at the end of the exercise protocol in different peripheral skeletal muscles submitted to various forms of exercise (in terms of protocol, duration, and intensity) (42). Previous work from our group and others provides evidence that cytokines originate from skeletal muscle themselves (21, 44, 55). However, the stimuli that regulate this cytokine induction, including endogenous NO release, are not yet defined. A recent exercise study on active leg muscles in humans demonstrated the positive regulatory role of NO on cytokine induction (51)—a finding that is opposite to the negative regulatory role of NO on diaphragmatic IRB-induced cytokine upregulation, which is demonstrated in our study. However, contrary to the decline in NO derivatives observed in the diaphragm in response to IRB, NO availability is increased in peripheral skeletal muscles in response to both acute (48) and chronic (54) increases in workload. Hence, it seems that the diaphragm and the peripheral skeletal muscles respond differently to acute increases of load in terms of NO production. A possible explanation for this could be the different functionality of the diaphragm—the only skeletal muscle along with the heart that contracts continuously throughout life, thus essentially lacking the opportunity to rest. In this perspective, the diaphragm and heart can be considered as physically “trained” skeletal muscles. Acute aerobic exercise results in a decrease of NO production in the heart (24), similarly to what happens in the diaphragm. Furthermore, training of peripheral skeletal muscles reduces the effect of an acute bout of exercise regarding NOS expression (33). It seems that peripheral skeletal muscles and the diaphragm exhibit the same response in terms of cytokine induction in response to increases of load, although the pathways that regulate this induction are probably not the same.

Skeletal muscles continuously produce low levels of NO in their basal state through the action of the two constitutive NO synthases: nNOS and eNOS (50). Low levels of NO are important for normal skeletal muscle function and oxidative balance (46, 50). Although NO levels decline in the diaphragm in response to IRB, the expression of both eNOS and nNOS is increased and evidenced already at 3 h of IRB (56), a response considered to be compensatory to the decline of NOS activity. L-NAME is a nonselective inhibitor of NOS synthases. Thus its administration in rats subjected to IRB provides evidence for the involvement of NO on the IRB-induced cytokine upregulation but cannot specify the specific isoform of NOS that is implicated in the cytokine response.

We have recently demonstrated that IRB results in activation of MAPKs (P38 and ERK1/2) and NF-κB in the diaphragm (49). This phenomenon was restricted to the diaphragm, as neither MAPKs nor NF-κB were activated in the noncontracting gastrocnemius muscle (49). The current study demonstrates that inhibition of NO production significantly augments IRB-induced ERK1/2 and P38 phosphorylation and NF-κB activation, whereas an increase in diaphragmatic NO availability (with NO donor administration) blunted both MAPK and NF-κB activation. It is noteworthy that NOS inhibition did not affect MAPK and NF-κB activation in the diaphragm of quietly breathing animals or in the noncontracting gastrocnemius muscle of animals subjected to IRB, suggesting a specific role of NO for the strenuously contracting diaphragm. However, systemic effects cannot be excluded. As the diaphragm differs from peripheral skeletal muscles in terms of its huge blood supply (microcirculation), modification in NO availability systemically—which can affect blood flow and blood pressure—is possible to affect shear stress, oxygenation, oxygen distribution, and mitochondrial function in the diaphragmatic tissue. Moreover, with the diaphragm having such a huge network of vasculature and lymphatics, we cannot totally exclude the contribution of diaphragmatic nonskeletal muscle cells or of inflammatory mediators transferred to the diaphragm from the peritoneum via lymphatics in the phenomenon, which we describe.

The ability of NO to regulate the activation of many transcription factors and signaling pathways, including NF-κB and MAPKs, is well established (5). NO has been shown to inhibit NF-κB activity, interfering negatively in many of the steps that are necessary for its activation. Specifically, NO stabilizes inhibitory NF-κB (I κB) (9, 28, 43), induces IκB mRNA (43), blocks the nuclear translocation of NF-κB (39), inhibits NF-κB DNA-binding capacity through S-nitrosylation of cys-
teine residues in the DNA-binding site of the Rel homology domain of the p50 (37) and p65 (29) subunits, and inactivates IKK again through S-nitrosylation (47). The mechanism of NO action on MAPKs is not characterized, as well as for NF-κB. Whereas inhibition of NO can suppress the activation of ERK1/2 and p38 MAPK, JNK pathways in many cell types (10, 25, 38, 60), the possible mechanisms are more speculative, mainly because of the many different pathways through which MAPKs can be activated. NO has been shown to exert inhibitory effects on MAPK activation either directly—JNK activation is inhibited by NO through S-nitrosylation of the cysteine residue Cys 116 (41)—or indirectly through downregulation of upstream-activating pathways (32, 38, 62) and modulation of the activity of MAPK phosphatases (3). Both cGMP-dependent and -independent mechanisms have been documented. Although our study provides evidence for NO-dependent regulation of MAPK and NF-κB activation, we cannot point to the exact sites of NO action. However, we did examine the possibility that NO regulates MAPK activation by affecting the expression of MKP-1, but we found no evidence to support this hypothesis. Of course, this does not exclude possible NO-mediated effects on MKP-1 activation at the post-translational level, as MKP-1 is extremely redox sensitive, with the existing evidence supporting that both reactive oxygen species (ROS) and nitrosylation can affect its activity (16, 34). Furthermore, we provide evidence that MAPKs are upstream regulators of NF-κB activation, although this does not exclude an immediate effect of NO on NF-κB activation.

This NO-mediated activation of MAPKs and NF-κB regulates IRB-induced cytokine production. Of course, this does not exclude possible NO-mediated effects on MKP-1 activation at the post-translational level, as MKP-1 is extremely redox sensitive, with the existing evidence supporting that both reactive oxygen species (ROS) and nitrosylation can affect its activity (16, 34). Furthermore, we provide evidence that MAPKs are upstream regulators of NF-κB activation, although this does not exclude an immediate effect of NO on NF-κB activation.

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Fig. 7. Effect of concurrent inhibition of NO and NF-κB or MAPKs on diaphragmatic cytokine production. A–C: the NF-κB inhibitor BAY-11-7082 (BAY; A), the MEK1/2–ERK1/2 inhibitor PD98059 (PD; B), and the P38 MAPK inhibitor SB203580 (SB; C) successfully blunted the NF-κB/p65 subunit (p-p65), ERK1/2 (p-ERK), and P38 (p-P38) phosphorylation, respectively. Representative results of immunoblotting analysis from the diaphragm of rats subjected to IRB for 6 h, treated with l-NAME and either BAY-11-7082 (RB-LN-BAY), PD98059 (RB-LN-PD), SB203580 (RB-LN-SB), or nothing (RB-LN). Results are presented as percent of RB-LN, mean ± SE. *P < 0.05 vs. RB-LN. p-P38 and p-ERK were normalized to respective total forms, whereas p-p65 was to b-tubulin. D and E: protein levels (estimated with ELISA) of (D) IL-6 and IL-10 and (E) IL-2, TNF-α, and IL-1β from the diaphragm of rats subjected to IRB for 6 h, treated with l-NAME and either BAY (RB-LN-BAY), PD98059 (RB-LN-PD), SB203580 (RB-LN-SB), or nothing (RB-LN). Results are presented as fold increase to control, mean ± SE. *P < 0.05 vs. RB-LN.
for all studied cytokines with the exception of IL-6, which is regulated by a P38-dependent pathway. The current findings are in agreement with our previous study, which established that IRB-induced cytokine upregulation is differentially regulated by these pathways (49).

Skeletal muscles in their quiescent state produce relatively low levels of ROS as well (46). Both exercise and IRB induce ROS formation in skeletal muscles and diaphragm, respectively (45, 57). ROS are able to activate both NF-κB and MAPKs (1), and indeed, N-acetyl cysteine (an antioxidant) administration is able to attenuate IRB-induced cytokine upregulation as well as MAPK and NF-κB activation (49). NO is a free radical that can downregulate ROS levels, either through its direct scavenging action on superoxide anions or through inhibition of superoxide-generating enzymes, such as xanthine oxidoreductase (23, 31) and NADPH oxidase (14, 26). Therefore, it is important to determine whether the effect of NO in the induction of cytokines in the diaphragm was through its direct action on the signaling pathways or via modulation of its oxidative balance. This study demonstrates that IRB increases protein carbonyl content (a ROS-induced protein modification), but treatment with L-NAME or DETA had no effect on that. Moreover, tyrosine nitration of proteins was reduced in the diaphragm of rats subjected to IRB (56), further supporting that the observed decline in NO derivatives is not due to the scavenging effect of NO on ROS. Collectively, our results point toward a direct action of NO in the regulation of signaling pathways and cytokines in the diaphragm rather than an indirect one through the modulation of its oxidative balance. In this setting, we propose that IRB-induced cytokine upregulation in the diaphragm of rats is regulated independently by both ROS and NO.

**Implications.** Data about cytokine expression in the human diaphragm after acute increases of load are lacking. However, COPD patients, who chronically have to overcome increased respiratory muscle load, demonstrate increased cytokine expression (TNF-α, IL-1β, and IL-6) in their external intercostal muscles, and at least TNF-α levels correlate negatively with respiratory muscle strength (8). Preliminary data suggest that cytokine upregulation is evidenced in the diaphragm of patients with severe COPD (48a). Additionally, nitrotyrosine formation (an index of NO availability) was decreased in the diaphragm of severe, stable COPD patients correlating negatively with lung volume (index of hyperinflation) (61). Treatment with substrates that can increase NO availability is tested widely in exercise physiology, where recent studies support a decrease in the oxygen cost of exercise and an increase in the time to task failure (2). Our study provides new insights into the mechanisms of regulation of cytokine expression in the diaphragm secondary to increased activation, showing that the cytokine...
induction can be modulated with NO-modifying agents that are already in clinical use.

In conclusion, this study provides evidence regarding the regulatory role of NO during IRB-induced cytokine upregulation in the diaphragm. Reduction of NO in the diaphragm activates MAPKs (ERK1/2 and P38), NF-κB, and cytokines, whereas NO supplementation downregulates this response. Cytokines are differentially regulated by these pathways in a way that involves MAPKs and NF-κB for all cytokines (IL-10, IL-2, TNF-α, and IL-1β), except IL-6, which is regulated by a P38-dependent but NF-κB-independent pathway. These effects of NO cannot be attributed to alterations in the oxidative status of the diaphragm.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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