MAPKs and NF-κB differentially regulate cytokine expression in the diaphragm in response to resistive breathing: the role of oxidative stress

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RESISTIVE BREATHING IS ENCOUNTERED in many disease states such as acute asthma attacks, upper airway obstruction, sleep apnea–hypopnea syndrome, and exacerbations of chronic obstructive pulmonary disease (COPD). Resistive breathing induces cytokine upregulation in the diaphragm, a response that is quite immediate (within 1 h) and time dependent (55), and results in elevated levels of circulating cytokines in humans (58, 60). This early response, before any inflammatory cell influx in the diaphragm (61), suggests that these cytokines might serve physiological roles rather than being nonspecific markers of inflammation.

Cytokines are molecules with many attributed functions that can potentially both harm and benefit the strenuously contracting muscle. The initial observation that cytokines promote skeletal muscle injury and impair contractile performance (8) are supplemented by later studies demonstrating the beneficial role of cytokines on muscle regeneration after injury, their role in the adaptation to increased load, their hormonal and metabolic effects, and their ability to propagate muscle-to-central nervous system communication via nonmyelinated (small) afferent fiber stimulation (6, 7, 33, 49, 57).

The stimuli for cytokine upregulation in the diaphragm in response to inspiratory resistive breathing (IRB) as well as the signaling pathways and transcription factors involved are not yet established. Reactive oxygen species (ROS) are continuously produced from the quietly contracting diaphragm and regulate force production (38). An increase in muscle activity is accompanied by accelerated ROS production. Accordingly, moderate or severe IRB promotes oxidative stress in the diaphragm as evidenced by the increased ROS-induced modifications of proteins and lipids (5, 21). ROS can activate important signaling molecules such as MAPKs and transcription factors such as NF-κB and hence regulate the expression of numerous genes (1). In cultured skeletal muscle cells, ROS induced IL-6 production through activation of P38 MAPK and NF-κB (26).

MAPKs are activated in peripheral skeletal muscles in response to contractile activity in both in vivo models of exercise (16) and in response to artificial stimulation (41). Exercise models in both animals and humans implicate P38 MAPK as a possible regulator of IL-6 (3, 10). The regulation of other cytokines such as TNF-α, IL-1β, IL-2, and IL-10 has not been studied in models of strenuous muscle contraction.

Both ROS and MAPKs can regulate the expression of many genes through their action on transcription factors such as NF-κB (1, 53) and activator protein-1 (AP-1). However, the effects of skeletal muscle contraction on NF-κB activity is a matter of controversy with some studies demonstrating increased NF-κB binding activity in response to exercise (16, 19, 22), whereas other studies failed to confirm NF-κB activation (10, 44). In the diaphragm, NF-κB activation has been shown to increase with passive stretching (27) and to decrease with artificial ex vivo electrical stimulation (15). Myocyte culture experiments have also suggested that NF-κB regulates IL-6 induction secondary to stimulation with ROS (26) and cytokines such as IL-1β and TNF-α (12, 26, 30). MAPKs and
NF-κB have rather synergistic or complementary roles in IL-6 induction in these in vitro studies. However, a regulatory role of NF-κB in cytokine induction has not been established until now either in peripheral skeletal muscles or in the diaphragm secondary to increased contractile activity.

In this study, we examined the role of MAPKs, ROS, and NF-κB in the cytokine upregulation in the diaphragm of rats subjected to resistive breathing (RB rats). We hypothesized that both MAPKs and NF-κB are activated in response to the augmented diaphragmatic contractile activity induced by resistive breathing and upregulate the intradiaphragmatic cytokine expression in a ROS-dependent manner.

**MATERIALS AND METHODS**

**Animal preparation.** All experimental procedures were approved by the Veterinary Administration of the Prefecture of Attica, Greece and the hospital ethics committee. Wistar rats (200–250 g) were anesthetized with ketamine (100 mg/kg) and tracheostomized with polyethylene tubing connected to a two-way nonrebreathing valve (Hans Rudolph, Kansas City, MO). After a short stabilization period, animals were subjected to IRB for up to 6 h (peak inspiratory pressure of ~50% of maximum), according to the protocol previously described (51, 55, 56) (RB rats). Supplemental doses of ketamine 50 mg/kg were administered by intraperitoneal injection whenever needed (on the basis of presence or absence of a reaction to pain stimulus).

In brief, with the use of a pressure transducer, the tracheal pressure was monitored and the maximum inspiratory pressure (Pimax) was measured during spontaneous breathing efforts through a totally occluded inspiratory port for 10 s (model Direcwin; Raytech Instruments). The inspiratory port was then connected to a tube of small diameter (resistance), and the diameter was adjusted to provide a peak tidal Pi of 50% of maximum IRB (Fig. 1) (51). The inspiratory line supplied 100% oxygen throughout the period of RB so as to prevent the development of hypoxemia. For this, the inspiratory line was connected through the “resistance” to a 5-liter plastic bag that was continuously supplied with 100% oxygen (51). No positive pressure was applied to the system. The following seven groups of animals were studied 1) IRB group: animals (n = 10) were subjected to the above described IRB protocol (RB rats) for 1, 3, or 6 h, respectively. 2) Control group: sham-operated animals breathing against no load for 1, 3, or 6 h (n = 8 per group) while receiving 100% oxygen. An additional group of sham-operated animal breathing against no load for 1, 3, or 6 h but receiving ambient air instead of 100% oxygen was studied as well to control for the effects of hyperoxia per se. As we did not find any difference in any of the parameters studied (cytokines, MAPKs, NF-κB) between the animals receiving 100% oxygen and the animals receiving ambient air, the control animals presented in the result section of the study have all received 100% oxygen to be similar to the IRB animals. 3) NF-κB inhibition group: animals (n = 10) subjected to IRB for 6 h pretreated with the NF-κB inhibitor BAY-11–7082 (10 mg/kg) (25) (also called RB-BAY group). 4) ERK1/2 inhibition group: animals (n = 10) subjected to IRB for 6 h pretreated with the ERK1/2 inhibitor PD98059 (0.5 mg/kg) (17) (also called RB-PD group). 5) P38 MAPK inhibition group: animals (n = 10) subjected to IRB for 6 h pretreated with the P38 MAPK inhibitor SB203580 (1 mg/kg) (17) (also called RB-SB group). 6) NAC group: animals (n = 10) subjected to IRB for 6 h pretreated with N-acetyl-cysteine (NAC, 1 g/kg) (also called RB-NAC group). 7) Treated control groups: sham-operated animals breathing against no load for 6 h (n = 7 per group) while receiving 100% oxygen pretreated with one of the above mentioned inhibitors or NAC served as respective controls (Ctr-BAY, Ctr-PD, Ctr-SB, Ctr-NAC, respectively).

All three inhibitors were purchased from Calbiochem (La Jolla, CA), while NAC was from Sigma-Aldrich (St. Louis, MO), and were administered by intraperitoneal injection 30 min before the beginning of the IRB protocol. Animals not treated with an inhibitor or NAC received the same amount of vehicle by intraperitoneal injection. At the end of the experiments animals were killed, and the diaphragm and gastrocnemius muscles were quickly excised and frozen in liquid nitrogen.

**Gas exchange.** In a subgroup of animals, an arterial catheter was placed into the femoral artery for sampling of arterial blood. Samples were collected at the beginning (quietly breathing) and at 3 and 6 h of resistive breathing. Arterial blood gases were analyzed by means of an automatic blood-gas analyzer (Radiometer ABL 700 Series).

**Whole protein extraction.** Frozen tissue (100 g of skeletal muscle) were homogenized in 500 μl of protein extraction buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail (Complete Mini; Roche)] and then phosphatase inhibitors cocktail (PhosSTOP; Roche). Samples were incubated on ice for 30 min before being centrifuged at 12,000 rpm, 15 min, at 4°C. Supernatant (whole protein extract) was collected, and the total protein concentration (in mg/ml) was estimated spectrophotometrically by using DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

**Nuclear extracts from diaphragm tissue.** Mechanically homogenized tissue (100 mg) were resuspended in 500 μl of hypotonic buffer: 25 mM Tris-Cl pH 7.5, 5 mM KCl, 0.5 mM MgCl₂, 0.5% Triton X-100, protease inhibitor cocktail (Complete Mini; Roche). Cytoplasmic proteins were collected on the supernatant of a 10-min 4°C centrifugation at 5,000 g following a 40-min incubation on ice. The pellet was washed two times with isotonic buffer: 25 mM Tris-Cl pH 7.5, 5 mM KCl, 0.5 mM MgCl₂, 0.2 M sucrose, protease inhibitor cocktail (Complete Mini; Roche) and then resuspended in 500 μl of isotonic buffer. Afterward, to collect the nuclei, a 10-min centrifugation at 5,000 g through a 100-μm filter (BD Biosciences) was followed by a new 10-min microcentrifugation at 15,000 g. Finally, nuclear extracts were prepared after a 40-min incubation in 150 μl nuclear extraction buffer (25 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1% Igepal, 0.5% SDS, 0.5 mM PMSF, 0.5 mM DTT) on ice, followed by a microcentrifugation at 15,000 g for 40 min at 4°C to pellet down the nuclear debris. Total protein concentration (in mg/ml) was estimated spectrophotometrically by using DC Protein Assay (Bio-Rad Laboratories).

**ELISA for muscle cytokines.** IL-6, IL-1β, IL-2, IL-10, and TNF-α protein levels were determined by performing the appropriate developmental ELISAs (model DuoSet; R&D Systems, Minneapolis, MN).
R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Each experiment was carried out three times. In each experiment, undiluted samples (in duplicate) from control and treated animals were included. Cytokine concentration was calculated in picograms per milliliter of protein extract and normalized using total protein concentrations in picograms per milligram of total protein.

**Immunoblotting.** Crude diaphragmatic muscle homogenates (100 μg of total protein per sample) were mixed with sample buffer, boiled for 15 min at 95°C, and then loaded onto 10% Tris-glycine SDS polyacrylamide gels and separated by electrophoresis. Proteins were then transferred to methanol-presoaked polyvinylidene difluoride membranes (Millipore, Bedford, PA), blocked with 5% nonfat dry milk for 1 h, and subsequently incubated overnight at 4°C with specific primary antibodies (1/1,000 dilution). Antibodies for phospho NF-κB p65 (Ser 536), phospho-IκBα (Ser 32), IκBα, phospho-P38 MAPK (Thr 180/Tyr 182), P38 MAPK, phospho-ERK1/2 (Thr 202/Tyr 204), ERK1/2 MAPK, phospho-MEK1/2 (Ser 217/221), MEK1/2, phospho-JNK (Thr 183/Tyr 185), JNK, phospho-c-Fos (Ser 32), c-Fos, phospho-c-Jun (Ser 63 and Ser 73), c-Jun, and Pan-actin were purchased from Cell Signaling Technology (Danvers, MA), whereas phospho-ERK5 (Thr 218/Tyr 220) and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Specific proteins were detected with peroxidase-conjugated secondary antibodies (1/5,000 dilution) (Jackson ImmunoResearch, Suffolk, UK) and SuperSignal-WestFemto Chemiluminescence Kit (Pierce, Rockford, IL).

**Myeloperoxidase activity assay.** Myeloperoxidase activity was measured in crude diaphragmatic homogenates as previously described (55). Activity was expressed in the units of change in absorbance per minute per gram protein.

**Protein carbonyl content measurement.** Protein carbonyl content of the diaphragm was measured on diaphragm homogenates using the Protein Carbonyl Assay Kit (Biocell, New Zealand) and following the manufacturer’s instructions.

**Statistical analysis.** After testing for normal distribution, all statistical comparisons were performed by ANOVA (with Tukey’s test for post hoc comparisons) or Student’s t-test where appropriate, using STATISTICA 7 (StatSoft, Tulsa, OK). Cytokine protein levels were always compared in absolute values (pg/mg). Data were expressed as means ± SE. Comparisons were considered statistically significant at P ≤ 0.05.

**RESULTS**

**Tracheal Pressure and Arterial Blood Gases During IRB**

In absolute values, Pimax in our experiment was −48.7 ± 2.36 cmH2O (means ± SE, n = 8) and didn’t change during the course of IRB. Pi was set at 50% of Pimax and remained unchanged during the IRB session (−25.6 ± 1.12, −26.2 ± 2.6, and −27.5 ± 1.5 cmH2O; values measured at 1, 3, and 6 h of IRB, respectively, n = 8) (Fig. 1). Consistent with previous results from our group (55), IRB resulted in a time-dependent hypercapnia and respiratory acidosis without hypoxemia due to oxygen supplementation. The arterial partial pressure of oxygen declined from 372 ± 32.45 mmHg (quietly breathing animals) to 175.6 ± 15.17 mmHg at 3 h of IRB and 138 ± 16.03 mmHg at 6 h of IRB, respectively. The carbon dioxide partial pressure increased from 43.32 ± 1.27 mmHg (quietly breathing animals) to 98 ± 8.2 mmHg at 3 h of IRB and 108.58 ± 9.33 mmHg at 6 h of IRB, respectively, This was accompanied by a decrease on pH value from 7.4 ± 0.01 (quietly breathing animals) to 7.04 ± 0.1 and 7 ± 0.02 at 3 and 6 h of IRB, respectively.

**Effect of IRB on MAPKs, NF-κB, and AP-1**

The activation of P38, ERK1/2, MEK1/2 (the upstream kinase of ERK1/2), and JNK was evaluated by examining the level of phosphorylated P38, ERK1/2, MEK1/2, and JNK with immunoblotting in crude diaphragm homogenates (whole cell extracts) from control and rats subjected to IRB for 1, 3, and 6 h (RB1h, RB3h, RB6h, respectively) (Figs. 2, A–B). Our results show that P38 is phosphorylated after 1 h of IRB and remains phosphorylated thereafter throughout the 6 h of IRB (Fig. 2A), whereas ERK1/2 phosphorylation is evidenced later (at 6 h of IRB) (Fig. 2B). In accordance with this, phosphorylation of MEK1/2 (the upstream kinase of ERK1/2) is evident earlier, i.e., at 3 h of IRB and thereafter (Fig. 2B). No difference was observed on JNK phosphorylation between control and IRB-subjected animals at the time points studied (Fig. 2A). No difference was observed on P38, ERK1/2, MEK1/2, and JNK phosphorylation and expression with time in the quietly breathing animals. Additionally, the effect of IRB on ERK5 phosphorylation was examined, but no difference between control and RB animals was detected (data not shown).

Activation of NF-κB through its classical pathway was evaluated by determining the expression and phosphorylation of both its inhibitory protein IκBα and the NF-κB/p65 subunit (in whole cell extracts) and the translocation of phosphorylated NF-κB/p65 subunit to the nucleus (in nuclear extracts) with immunoblotting in the diaphragms of control rats and rats subjected to IRB for 1, 3, and 6 h. IκBα phosphorylation is already evident after 1 h of IRB and remains elevated thereafter, whereas IκBα protein levels decrease only after 6 h of IRB (Fig. 3A). p65 Phosphorylation is evidenced only at 6 h of IRB in whole cell extracts (Fig. 3B). However, an increase in the nuclear localization of the phosphorylated NF-κB/p65 subunit is observed from the first hour of IRB and is main-
Effect of IRB on Cytokine Protein Levels

Cytokine protein levels were measured in the diaphragm of quietly breathing rats and rats subjected to IRB for 1, 3, and 6 h with ELISA (Fig. 4, A–E). IRB resulted in an upregulation of IL-6, IL-2, IL-10, and IL-1β in response to IRB was studied by evaluating the expression of immunoblotting analysis for the phosphorylated (p-) and total (T-) form of NF-κB/p65 subunit (p-p65) in nuclear extracts. For A–C, results are presented as fold increase to control (means ± SE, n = 10 animals per study group). β-tubulin and MyoD were used to test for equal loading in A and C, respectively.

Effect of IRB on Cytokine Protein Levels

Effect of 6-h IRB on Cytokine Expression and MAPKs-NF-κB Activation in the Gastrocnemius

The observed effects of IRB on cytokine production, MAPKs, and NF-κB activation can be either attributed to a systemic response or considered a local phenomenon in the strenuously contracting diaphragm. To exclude any major systemic effects, we measured cytokine production in the gastrocnemius muscle (noncontracting muscle) excised from rats subjected to IRB for 6 h and quietly breathing animals with ELISA. Cytokine protein levels did not differ between control and RB6h animals (Fig. 5A). Similarly, no difference was observed on MAPKs (P38, ERK1/2, and JNK) and NF-κB (IkBα and p65 phosphorylation) activation on gastrocnemius muscle between control and RB6h rats (Fig. 5, B–C).

Inhibition of NF-κB, ERK1/2, and P38 and Cytokine Expression

To evaluate the potential role of NF-κB on cytokine upregulation in the diaphragm secondary to RB, we conducted experiments with the NF-κB inhibitor BAY-11–7082 (25). BAY at the dose used (10 mg/kg ip) successfully inhibited p65 phosphorylation (Fig. 6A). IL-6 protein levels were increased (compared to RB6h animals) in response to NF-κB inhibition (Fig. 7A). In contrast, NF-κB inhibition blunted the TNF-α, IL-2, IL-10, and IL-1β protein upregulation (Fig. 7, B–E). NF-κB inhibitor didn’t alter cytokine protein levels in quietly breathing animals (Fig. 7, A–E).

Interaction Among P38, ERK1/2, and NF-κB

MAPKs can influence the activation of many transcription factors including NF-κB (53). To evaluate whether P38 and ERK1/2 are upstream regulators of NF-κB, the phosphorylated status of p65 was determined with immunoblotting in the diaphragm of RB animals with or without pretreatment with SB203580 or PD98059. Both inhibition of P38 and ERK1/2 reduced the levels of RB-induced p65 phosphorylation in the diaphragm (Fig. 8, A and B).

Myeloperoxidase Activity

No difference was observed on the myeloperoxidase activity (an index of neutrophil activation) in the diaphragm of either control or rats subjected to IRB for 6 h (76.4 ± 7.2 and 82.3 ± 6.7 units, respectively, P = not significant).

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significant increase in the phosphorylation of P38 in the diaphragm of RB rats (Fig. 8C). Similarly, ERK1/2 inhibition resulted in augmented P38 phosphorylation (Fig. 8D). NF-κB inhibition augmented ERK1/2 phosphorylation (Fig. 8E), as contrasted to P38 inhibition, which did not affect ERK1/2 phosphorylation (Fig. 8F).

Role of Oxidative Stress on IRB-Induced Cytokine Upregulation in the Diaphragm

Increased skeletal muscle activity is accompanied by elevated ROS production. To examine whether ROS can be the stimulus for cytokine upregulation in the diaphragm in response to IRB, rats were pretreated with NAC and subjected to IRB for 6 h with concurrent measurement of carbonyl protein. IRB for 6 h resulted in increased protein carbonyl formation in the diaphragm (0.11 ± 0.005 and 0.06 ± 0.007 nmol/mg total protein for RB6h and control animals, respectively, P = 0.0015), whereas NAC administration decreased it (0.07 ± 0.013 nmol/mg total protein for RB-NAC animals, P = 0.024 compared with RB6h). NAC pretreatment blunted the IRB-induced increase on cytokine protein levels (Fig. 9A). No difference on cytokine production was observed in quietly breathing animals (Fig. 9B). Moreover NAC pretreated animals exhibited decreased phosphorylation of P38, ERK1/2, and NF-κB/p65 compared with RB6h animals (Fig. 9, C and D).

**DISCUSSION**

The main findings of this study are 1) IRB activates MAPKs (P38 and ERK1/2, but not JNK) and NF-κB (but not AP-1) in the diaphragm; 2) TNF-α, IL-1β, IL-2, and IL-10 upregulation is NF-κB dependent; 3) inhibition of both P38 and ERK1/2 reversed the upregulation of NF-κB-dependent cytokines; 4) IL-6 induction involves the activation of P38, whereas NF-κB exerts a negative regulatory effect; and 5) antioxidant (NAC) administration blunted the IRB-induced cytokine upregulation in the diaphragm and reduced P38, ERK1/2, and NF-κB/p65 subunit phosphorylation.

To our knowledge this is the first study demonstrating a relationship between oxidative stress, signaling pathways, and induction of cytokines within the diaphragm in response to IRB. Resistive breathing induces cytokine upregulation within the diaphragm of rats (55). This is not a nonspecific response to stress, since no induction was elicited in the noncontracting gastrocnemius. The cytokine induction in response to strenuous contraction takes place within the skeletal muscle cells (18, 34). This is further supported by the fact that we found no difference in the myeloperoxidase activity (an index of neutrophil activation) in the diaphragm of control and RB animals.

We used a model that applies moderate load during inspiration for a prolonged period of time. This is relevant to the load the respiratory muscles of COPD patients are facing.
during an exacerbation (59, 63). Most studies in the literature have used a high inspiratory load, demonstrating a time-to-task failure that ranged from few minutes to 1 h maximum (2, 47). However, recent studies have shown that IRB with a moderate load differs from that with a high load (21, 42, 43, 51, 55, 56). Specifically, compared with the rapid development of diaphragmatic fatigue that led to pump failure observed with high inspiratory loads (42), a moderate load can be sustained for a long time, tracheal pressure does remain constant during the course of IRB, and hypercapnia development precedes that of diaphragmatic fatigue that is a late event (43). Hypercapnia was observed in our animals, whereas tidal inspiratory pressure did not change until the end of the protocol at 6 h. The most probable reason for the development of hypercapnia is a change in the breathing pattern (rapid shallow breathing) that increases the dead space-to-tidal volume ratio.

Increased MAPKs and NF-κB Activation in the Diaphragm in Response to Strenuous Contraction

Activation of MAPKs in skeletal muscles in response to exercise is well documented (16, 19). However, the diaphragm and the peripheral skeletal muscles may respond differently. For instance, IRB leads to decreased nitric oxide production in the diaphragm (56) as contrasted to increased nitric oxide production in peripheral skeletal muscles in response to exercise (40). Thus, the results obtained in peripheral skeletal muscles are not directly applicable to the diaphragm. Our study shows for the first time that IRB results in the activation of the MAPKs signaling pathways. The time course of their activation, however, is different; P38 phosphorylation is an early event that lasts throughout the 6 h of IRB, whereas ERK1/2 phosphorylation is evidenced later in the course of IRB. Interestingly, there was no evidence of JNK activation at least at the time points studied. JNK correlates better with the tension of muscle contraction rather than the duration (31). Our model of IRB applies moderate tension in the diaphragm for a prolonged period of time, providing a possible explanation for the lack of JNK activation. Possible stimuli for MAPK activation include, among others, increased muscle activity per se, oxidative stress, hypoxia, acidosis, and the release of secondary mediators, including cytokines themselves (37). In our study, the lack of any evidence of MAPKs phosphorylation in the noncontracting gastrocnemius muscle points toward a local rather than a systemic factor that regulates MAPKs activation in the diaphragm in response to IRB. Moreover, hypoxia as stimulus can be safely excluded, as none of our animals were hypoxic. The possible involvement of oxidative stress on MAPKs activation in our study is supported by the decrease in both P38 and ERK1/2 phosphorylation following NAC administration. However, both the prolonged activation of P38 and the late ERK1/2 phosphorylation cannot exclude the involvement of other possible stimuli on MAPK activation, such as cytokines themselves or of hypercapnia and respiratory acidosis.

Fig. 5. IRB does not affect cytokine expression, MAPKs, and NF-κB activation in the noncontracting gastrocnemius muscle. A: protein levels (estimated with ELISA) of IL-6, TNF-α, IL-2, IL-10, and IL-1β, respectively, in the gastrocnemius of quietly breathing rats (control) and rats subjected to IRB for 6 h (RB6h). Data are means ± SE μmol/mg total protein concentration (TP). B–C: representative results of immunoblotting analysis for the phosphorylated (p-) and total (T) forms of P38, ERK1/2 and JNK (B) and the phosphorylated form (p-) of IkBα and NF-κB/p65 (C), in the gastrocnemius muscle of control and RB6h rats. Pan-actin (actin) is presented to test for equal loading (C).

Fig. 6. Evaluation of the effect of inhibitors on the corresponding pathways: the NF-κB inhibitor BAY-11–7082 successfully inhibits NF-κB/p65 subunit phosphorylation (p-p65) (A); and the MEK1/2-ERK1/2 inhibitor PD98059 (B) and the P38 MAPK inhibitor SB203580 (C) inhibit ERK1/2 and P38 phosphorylation (p-ERK1/2 and p-P38, respectively), without affecting their expression (T-ERK1/2 and T-P38, respectively). Representative blots from the diaphragm of rats subjected to IRB for 6 h without (RB6h) or with pretreatment with inhibitor of NF-κB (RB-BAY), MEK1/2-ERK1/2 (RB-PD), or P38 (RB-SB). Data are means ± SE % of RB6h. (*P < 0.05 vs. RB6h).
Both ROS and MAPKs can regulate the transcription of many genes through their action on transcription factors such as NF-κB and AP-1. The role of NF-κB in the contracting skeletal muscle is still controversial. NF-κB has been shown to increase (16, 19, 22), decrease (15), or remain unchanged (10, 44) in peripheral skeletal muscles in response to exercise. Passive stretching of the diaphragm resulted in increased NF-κB activation (27), whereas in vitro electrical stimulation decreased it (15). Our study demonstrates that IRB results in increased NF-κB phosphorylation and degradation, increased phosphorylation of NF-κB/p65 subunit, and mobilization of p65 to the nucleus, suggesting that the classical NF-κB pathway is activated.

The activation of AP-1 with increased activity of skeletal muscles is much less studied. Both exercise and electrical stimulation of peripheral skeletal muscles (36, 54), as well as mechanical stretch of the diaphragm (28) activate AP-1. In our model of IRB, we didn’t find any increase of c-Fos and c-Jun protein levels or any difference on their phosphorylation. These findings suggest that AP-1 is not activated in the diaphragm during moderate IRB.

Regulation of Cytokine Expression in the Strenuously Contracting Diaphragm

The molecular events that orchestrate cytokine upregulation in the working skeletal muscles are barely known. Our study demonstrates that all cytokines are not regulated in the same way, as NF-κB is the transcription factor responsible for the increased expression of TNF-α, IL-2, IL-10, IL-1β, but not of IL-6. Although, earlier studies in striated muscle cells (24) had suggested that TNF-α and IL-6 are differentially regulated, no in vivo study until now has confirmed that. NF-κB is an important transcription factor that is implicated in the transcription of many genes. It can be activated by a variety of stimuli including oxidative stress (26) and elevations of intracellular calcium (20) both of which occur during muscular contraction. The regulatory role of NF-κB in cytokine induction in the diaphragm secondary to LPS is well established (13). Our study for the first time implicates NF-κB in the induction of cytokines in the normally strenuously contracting diaphragm.

Differential Regulation of IL-6 (from the Other Cytokines) in the Diaphragm

IL-6 is the most abundant cytokine in the muscle that is consistently found to increase in response to skeletal muscle contraction and is released into the circulation where it exerts systemic effects (metabolic, hormonal, immunological) (33). The different regulation of IL-6 compared with all other (studied) cytokines may reflect the unique role of IL-6 in muscle metabolism (33, 57). The molecular events that regulate IL-6 induction are not yet fully clarified. Contrary to cultured skeletal muscle cell studies that demonstrated a clear relationship between IL-6 induction and NF-κB (12, 26, 30),
no in vivo study has confirmed this relationship. Our study shows that NF-κB inhibits IL-6 induction in the diaphragm during strenuous contraction, as its inhibition not only failed to decrease IL-6 levels but, contrary to this, augmented them. Accumulating evidence in the literature connects exercise-induced IL-6 production in skeletal muscles with decreased energy substrate availability in the muscle (10), increased intracellular calcium (3), and increased oxidative stress (26). Interestingly, all of these stimuli directly or indirectly implicate the P38 MAPK as the key signaling molecule for the transcriptional induction of IL-6 (3, 10, 26). However, direct in vivo evidence is lacking. Our study is the first to establish a causative role for P38 in the signaling events that control IL-6 expression secondary to muscular contraction in vivo since P38 inhibition downregulated intradiaphragmatic IL-6 expression.

MAPKs and NF-κB Interaction

Inhibition of both P38 and ERK1/2 in rats subjected to IRB blunted the upregulation of the NF-κB-dependent cytokines, implicating the existence of a cross-talk between MAPKs and NF-κB that regulate cytokine expression in the diaphragm. Earlier studies in striated muscle cells (12, 30) and animals (19) have placed P38 and ERK1/2 upstream in the NF-κB activation pathway. In our study, inhibition of both P38 and ERK1/2 decreased p65 phosphorylation, suggesting that both regulate NF-κB activation, yet in a different time frame in the strenuously contracting diaphragm. The observed effect of ERK1/2 inhibition of decreasing NF-κB/p65 subunit phosphorylation and downregulating NF-κB dependent cytokines cannot be attributed to an effect on P38 MAPK, as ERK1/2 inhibition resulted in increased P38 MAPK phosphorylation. Whether the effects of MAPKs on NF-κB activation are direct or indirect cannot be established from the present study. Moreover, since our study relied on pharmacological inhibitors to modulate MAPK and NF-κB activation we cannot exclude unintended effects of these inhibitors, despite their widespread use in the literature.

Interestingly, inhibition of NF-κB increased phosphorylation of both P38 and ERK1/2, suggesting that MAPKs activation may be under the regulatory control of NF-κB as well, in a negative feedback loop in the contracting diaphragm. This increased activation of both ERK1/2 and P38 was not accompanied by any induction of NF-κB-dependent cytokines, further supports that both P38 and ERK1/2 affect the induction of these cytokines through their action on NF-κB and, accordingly, should be regarded as upstream regulators. It is noteworthy that both NF-κB and ERK1/2 inhibition resulted in increased P38 phosphorylation and IL-6 expression despite the resulting decrease of NF-κB phosphorylation and their oppos-

Fig. 8. Interaction between P38, ERK1/2, and NF-κB in the diaphragm in response to IRB. A–B: inhibition of both P38 (A) and ERK1/2 (B) in rats imposed to IRB for 6 h blocks the phosphorylation of the p65 subunit of NF-κB. Representative results of immunoblotting analysis for the phosphorylated form of p65 (p-p65) on diaphragm of rats subjected to IRB for 6 h pretreated with the P38 MAPK inhibitor SB203580 (RB-SB), the ERK1/2 inhibitor PD98059 (RB-PD), or none (RB6h). C–D: inhibition of both NF-κB (C) and ERK1/2 (D) upregulates P38 MAPK phosphorylation in the diaphragm of rats subjected to IRB for 6 h. Representative results of immunoblotting analysis for the phosphorylated form of P38 (p-P38) on diaphragm of rats subjected to IRB for 6 h pretreated with the NF-κB inhibitor BAY-11–7082 (RB-BAY), the ERK1/2 inhibitor PD98059 (RB-PD), or none (RB6h). No change was observed on the levels of total P38 (T-P38). The effect of NF-κB (E) and P38 MAPK (F) inhibition on ERK1/2 phosphorylation is shown. Representative results of immunoblotting analysis for the phosphorylated form of ERK1/2 (p-ERK) on diaphragm of rats subjected to IRB for 6 h pretreated with the NF-κB inhibitor BAY-11–7082 (RB-BAY), the P38 MAPK inhibitor SB203580 (RB-SB), or none (RB6h). No change was observed on the levels of total ERK1/2 (T-ERK). Results in A–F are means ± SE in % of RB6h (*P < 0.05 vs. RB, n = 8 animals per study group). For A–B β-tubulin was used to test for equal loading.
Pimax increased lipid peroxidation (21) and protein carbonylation (35). IRB for 6 h leads to the development of mitochondrial function, which impairs muscle contractile performance and the antioxidant defense systems of the cell, oxidative stress, and increased production of reactive oxygen species (ROS). Increased ROS production, which cannot be balanced by increased activation of skeletal muscle IL-10, IL-2, and IL-1β, whereas P38 MAPK is responsible for IL-6.

Role of Oxidative Stress

Skeletal muscle cells in their basal state generate ROS continuously at a low rate, a phenomenon essential for normal force generation and cell signaling (38). Changes in ROS production rate can activate signaling pathways, including MAPKs and NF-κB (1, 26), and hence regulate the expression of many antioxidant (16, 64) and cytokine (26) genes. In our model, NAC administration blunts protein carbonyl formation in the diaphragm, decreased IRB-induced cytokine upregulation, and in parallel decreased P38, ERK1/2, and NF-κB/p65 subunit phosphorylation, suggesting that ROS is a stimulus for MAPKs and NF-κB activation and hence cytokine production in response to IRB.

Based on our findings, we may speculate on a mechanism that regulates cytokine induction in the diaphragm (Fig. 10) in response to IRB. IRB increases ROS production in the diaphragm. ROS can activate MAPKs and NF-κB. NF-κB activation is responsible for the upregulation of TNF-α, IL-10, IL-2, and IL-1β, whereas P38 MAPK is responsible for IL-6.

Cytokines have diverse roles in the contracting skeletal muscle that can be both beneficial and damaging. TNF-α and IL-1β are implicated in muscle injury secondary to strenuous contraction, as both facilitate the accumulation of neutrophils and monocytes that can damage the muscle (8, 32, 48). Moreover, TNF-α (39, 57, 62), IL-1β (29), and IL-2 can impair striated muscle function (50). However, apart from damaging the muscle, cytokines can augment the repair process (after injury) by enhancing the activation of macrophages (48, 57), through promoting myoblast differentiation (TNF-α and IL-10) (11, 46, 49) or promoting myoblast proliferation on the initial phase of muscle regeneration (IL-6) (49). Moreover, IL-10 attenuates cytokine upregulation within the diaphragm of mice with Pseudomonas aeruginosa lung infection (14). IL-6 is a cytokine with hormonal and metabolic effects, which may facilitate diaphragmatic energy supplementation (33, 57).

![Fig. 9. N-acetyl-cysteine (NAC) inhibits IRB-induced cytokine upregulation and MAPKs-NFκB activation in the diaphragm. A–B: protein levels (estimated with ELISA) of IL-6, TNF-α, IL-2, IL-10, and IL-1β, respectively, in the diaphragm of rats subjected to IRB for 6 h treated (RB-NAC) or not (RB6h) with NAC (A) and quietly breathing animals treated (Ctr-NAC) or not (CTR) with NAC (B). Data are presented as fold increase to control (means ± SE; *P < 0.05 vs. RB6h, n = 10 animals per study group). Control and RB6h animals are the same presented on Fig. 4. C–D: NAC pretreatment blunts IRB-induced P38, ERK1/2, and NF-κB/p65 subunit phosphorylation in the diaphragm. C: representative blots for the total (T-) and phosphorylated (p-) forms of ERK1/2 and P38 and the phosphorylated form of NF-κB/p65 from (p-p65) in the diaphragm of RB6h and RB-NAC animals. D: graph presentation of C. p-P38 and p-ERK1/2 are normalized vs. the respective total forms, whereas p-p65 vs. β-tubulin. Data are means ± SE in % of RB6h (*P < 0.05 vs. RB6h, n = 8 animals per study group).

![Fig. 10. Proposed mechanism of MAPKs and NF-κB interaction on cytokine induction in the diaphragm in response to IRB. IRB results in increased ROS production in the diaphragm, increased activation of P38, ERK1/2, and NF-κB/p65 subunit phosphorylation, suggesting that ROS is a stimulus for MAPKs and NF-κB activation and hence cytokine production in response to IRB.

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Role of Cytokines

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nally, cytokines, such as TNF-α and IL-6, can stimulate non-myelinated (small) afferent nerve fibers (6, 7), propagating diaphragm-to-central nervous system communication.

**Perspectives and Significance**

IRB induces cytokine upregulation in the diaphragm, in the lung (51), and systemically (58). IRB, when severe or prolonged enough, can promote diaphragm injury. In this model of IRB we used a load that has clinical impact as it is relevant to the load observed during COPD exacerbations (59, 63) or asthma attacks (45). Our study provides evidence that clinically relevant inspiratory loading induces oxidative stress in the diaphragm that is linked with cytokine upregulation and can be modified with NAC administration (5). Cytokine upregulation and oxidative stress are observed in the respiratory muscles of patients with severe COPD, correlating inversely with respiratory muscle function (4, 9). Our study describes mechanisms that regulate cytokine induction in the diaphragm secondary to acute increases of load. It is noteworthy that IL-6, a cytokine with potentially beneficial metabolic and regenerative functions for the diaphragm, is regulated in a different way from the rest of upregulated cytokines, raising the possibility of differential modification of diaphragmatic cytokine response secondary to resistive breathing.

In conclusion, IRB induces ROS formation and cytokine upregulation in the diaphragm. The cytokine response is under the regulatory control of NF-κB for TNF-α, IL-2, IL-10, and IL-1β. The induction of IL-6 expression involves p38 MAPK activation, whereas ERK1/2 and NF-κB downregulate IL-6 expression. MAPKs are upstream activators of the NF-κB pathway, whereas NF-κB exerts a negative feedback effect on MAPKs activation. Modification of the oxidative status of the diaphragm with NAC administration decreased the MAPKs upregulation in the diaphragm. The cytokine response is under the regulatory control of NF-κB.

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