ABNORMALITIES OF THE DIAPHRAGM MUSCLE contribute to the pathophysiology of chronic heart failure (CHF) (10, 46). The degree of diaphragm dysfunction depends on the stage of the disease, where patients with severe CHF (class III or IV) are weaker than patients with mild CHF (class I), as shown by studies using volitional tests (3, 24, 28) or direct measurement of diaphragm strength with phrenic nerve stimulation (31). Importantly, left ventricular ejection fraction is not correlated with maximal inspiratory pressure (43), and decreased inspiratory muscle endurance is not reversed by heart transplant (41).

Diaphragm biopsies from patients with severe CHF undergoing heart transplant show ultrastructural abnormalities (38), and studies in animal models have shown that CHF leads to impairments in diaphragm contractile function (12, 29, 55, 59). Oxidants impair diaphragm contractile function (11, 49), and CHF increases oxidants in the diaphragm (12, 55). In CHF rats, systemic antioxidant administration increases submaximal diaphragm strength, whereas oxidants impair diaphragm contractile function (12, 55). In CHF, the specific source of oxidants and its relevance to diaphragm weakness in CHF remain to be elucidated.

NAD(P)H oxidase subunit p47phox is elevated, and p47phox knockout prevents diaphragm contractile dysfunction in heart failure

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Ahn B, Beharry AW, Frye GS, Judge AR, Ferreira LF. NAD(P)H oxidase subunit p47phox is elevated, and p47phox knockout prevents diaphragm contractile dysfunction in heart failure. Am J Physiol Lung Cell Mol Physiol 309: L497–L505, 2015.—Patients with chronic heart failure (CHF) have dyspnea and exercise intolerance, which are caused in part by diaphragm abnormalities. Oxidants impair diaphragm contractile function, and CHF increases diaphragm oxidants. However, the specific source of oxidants and its relevance to diaphragm abnormalities in CHF is unclear. The p47phox-dependent oxidants are involved in the increased oxidants and contractile dysfunction in CHF. These findings suggest that a p47phox-dependent NAD(P)H oxidase mediates the increase in diaphragm oxidants and contractile dysfunction in CHF.

Oxidative stress; respiratory muscle; myocardial infarction

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anesthesia (2% isoflurane), we exposed the heart via a left-sided thoracotomy in the fourth or fifth intercostal space, removed the pericardium, and ligated the left anterior descending coronary artery near the left atrium using a 6-0 PGA suture (Demesorb; Demetech). After the ligation, we hyperinflated the lungs, approximated the ribs using a 6-0 PGA suture, and closed the skin incision with 3-0 suture (Demelon; Demetech). Once extubated, the animals were transferred to a heated pad for recovery. Sham surgeries were similar to the MI procedure, except that we skipped the ligation of the coronary artery. All surgical procedures were performed according to aseptic conditions. The animals received topical bupivacaine injection immediately after the skin was closed. In addition, we injected buprenorphine (20–40 μg/kg sc) during surgery and every 8–12 h for 3 days postsurgery. Experiments were performed ~14 wk post-MI surgeries, as a study in rats showed decreases in maximal and submaximal diaphragm force at this time (59). A small subgroup of WT mice (n = 5 sham and 4 CHF) was studied 4 wk postsurgery for measurements of Nox2 subunit mRNA levels, as increases in diaphragm oxidants with CHF have been shown within 6 wk postsurgery (55).

Echocardiography. We used transthoracic echocardiography (Vevo 770; Visual Sonics, Toronto, ON, Canada) with a 30-MHz probe to obtain parasternal two-dimensional views of the left ventricle (LV). We placed the anesthetized mouse (1–2% isoflurane delivered via a nose cone) in the supine position on a heated pad. Heart rates of the animals ranged from 400 to 500 beats/min during the imaging. We used M-mode tracings of the parasternal short-axis view to determine LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) at approximately the midpapillary level over three cardiac cycles to calculate LV fractional shortening (%) as (LVEDD – LVESD)/LVEDD × 100. Our measurements were consistent with the recommendations of the American Society of Echocardiography (35).

Tissue harvesting and infarct size. We isolated the diaphragm and heart with the animals in the surgical plane of anesthesia. Fresh diaphragms were used for contractile functions and Amplex Red assay, while portions of the costal diaphragm were snap-frozen in liquid nitrogen and later processed for measurement of mRNA and protein expressions. We further dissected the right (RV) and left ventricles (LV) for measurements of weight and infarct area. To determine infarct area, we cut the interventricular septum from the base to the apex of the LV and acquired a digital photograph using a stereozoom microscope. The transmural infarct area was determined by planimetry (25). Based on previous studies in rodents (25, 59, 60) and the more pronounced diaphragm weakness in patients with severe CHF (24), we included only mice with infarct area ≥ 20% of LV + septum in the study. Sixteen (out of 27) WT MI mice that survived met the inclusion criteria for infarct area. Among knockouts, 12 (out of 19) survivors met the inclusion criteria for infarct area. Thus, the number of animals used for data analysis in the 14-wk studies was 18 sham and 16 CHF for WT and 14 sham and 12 CHF for p47phox−/−.

Diaphragm contractile properties in vitro. The assessment of diaphragm isometric and isotonic contractile properties was consistent with previous studies (2, 50). We dissected a diaphragm strip from the left hemidiaphragm along with the rib and central tendon in bicarbonate-buffered solution (in mmol/l: 137 NaCl, 5 KCl, 1 MgSO4, 1 NaH2PO4, 24 NaHCO3, and 2 CaCl2) gassed with a mixture of 95% O2 and 5% CO2 at room temperature. We used a 4.0 braided silk length (determined the length that elicited maximal twitch force [optimal length (Lo)]). To find Lo, we preloaded the muscle with 25–30 mN and stimulated at 1 Hz (600-mA current, 0.25-ms pulse). The stimulations were repeated in 0.2- to 0.3-mm shortening steps until reaching maximal twitch force. We then placed the muscle at Lo, increased the temperature of the organ bath to 37°C, and added d-tubocurarine (25 μM) to the buffer. After 20 min of thermoequilibration, we started our force-frequency protocol. The isometric force-frequency protocol consisted of stimulus frequencies of 1–300 Hz interspersed by 1-min intervals. The stimulation protocol consisted of supramaximal electrical current (600 mA) with pulse duration of 0.25- and 300-ms train duration delivered through platinum electrodes using a biphasic high-power stimulator (701C; Aurora Scientific). Isometric force was normalized for bundle cross-sectional area (CSA; N/cm²). After the protocol, we measured the bundle’s Lo and weight. To estimate the bundle CSA, we divided the diaphragm bundle weight (g) by Lo and multiplied to muscle-specific density (1.056 g/cm³). We used the sigmoidal Hill equation to analyze the force-frequency relationship and determine the frequency that elicits 50% maximal force and the slope of the relationship.

To test isometric properties of the diaphragm, we used afterload contracted employing a protocol similar to previous studies (9, 59). The bundle was dissected and placed at Lo, as described above. After 20 min of thermoequilibration, we stimulated the muscle supramaximally (600 mA, 300 Hz, 0.25-pulse, 200-ms train) and allowed it to shorten against an external load corresponding to 2–80% of the maximal isometric tension. Each step of the protocol was done with 2-min intervals between stimulations. Force and length data were sampled at 1,000 Hz. After the protocol, we measured Lo and bundle weight to estimate bundle CSA. We analyzed shortening velocity ≥ 10 ms after the initial change in length and within the linear portion of the tracing (DMA software; Aurora Scientific). The force-velocity curve was plotted and fitted to the hyperbolic Hill equation. We determined maximal shortening velocity (Vmax) as the velocity at zero force in the force-velocity relationship. We multiplied force and velocity to calculate power and used the curve fit of the force-velocity relationship to determine peak power (W). Shortening velocity was normalized to Lo, and peak power was normalized to bundle weight (kg).

Diaphragm oxidants. We used an Amplex Red assay to measure extracellular oxidants from intact tissue, following established procedures (16) with slight adaptation to the diaphragm muscle. Specifically, we dissected a diaphragm bundle and clamped the muscle and central tendon using tissue ring supports (Radnoti). We then placed the muscle in an organ bath containing bicarbonate-buffered solution (see above) at room temperature, attached the bundle to a glass rod and lever system, and adjusted muscle length to ~ 10 mm (average Lo in our preparations), and increased the temperature of the organ bath to 37°C, allowing 10 min for thermoequilibration. After thermoequilibration, we exposed the muscle to the buffer solution with 20 μM Amplex Red, 0.4 U/ml horseradish peroxidase, and 35 U/ml superoxide dismutase (SOD) at 37°C for 30 min under quiescent (unstimulated) conditions. We measured Amplex Red fluorescence (excitation = 530 nm, emission = 590 nm) in a standard cuvette using a spectrofluorometer (SpectraMax M5; Molecular Devices) and normalized the signal to diaphragm bundle wet weight. The assay reagents were prepared fresh daily from frozen or refrigerated stock solutions. Amplex Red is a membrane-impermeable probe specific for measurement of hydrogen peroxide. We included SOD in our preparation to convert superoxide to hydrogen peroxide and obtain a global measure of extracellular oxidants.

To avoid problems introduced by day-to-day and time-of-day variability in the preparation and fluorescence measurements, we performed experiments involving Amplex Red fluorescence in matched pairs of sham and CHF mice within strains. The assay reagents were prepared fresh from frozen or refrigerated stock solutions for each paired set of experiments. This approach is consistent with that used by other groups in fluorescence assays of oxidants in the diaphragm (37, 53).

Gelelectrophoresis and immunoblotting. We homogenized diaphragm samples on ice in a protein extraction buffer consisting of 20 mmol/l HEPES, 2 mmol/l EGTA, 1% Triton X-100, and 50 mmol/l β-glycerophosphate, pH 7.4, with protease and phosphatase inhibitor cocktails. We rotated the homogenates end over end for 1 h at ~4°C, sonicated once for ~3 s, and centrifuged at 1,500 g for 2 min at room
temperature. We isolated the supernatant and determined its protein contents using the DC protein assay (Bio-Rad Laboratories). Homogenates were mixed with Laemmli sample buffer and heat-denatured for SDS-PAGE.

We loaded 10–30 μg of proteins into 4–20% stain-free TGX gels (Bio-Rad Laboratories) and performed electrophoresis at 200 V for 50 min on ice. We scanned the gel to quantify total proteins (Gel Doc EZ Imager; Bio-Rad Laboratories) and transferred the proteins to a nitrocellulose membrane at 100 mA overnight at 4°C. We blocked the membrane using Li-COR Blocking Buffer (Li-COR, Lincoln, NE) for 1 h at room temperature and subsequently probed with primary antibodies. We used primary antibodies targeting the following proteins: p47phox (SAB2500674; Sigma-Aldrich), Rac1 (05-389; Millipore), gp91phox (611414; BD Transduction Laboratories), p22phox (FL-195; Santa Cruz Biotechnology), p67phox (07-502; Millipore), and 4-hydroxynonenol (4-HNE; ab46544; Abcam). We diluted the primary antibodies in Li-COR Blocking Buffer at a 1:1,000 ratio, except for p22phox (1:250). Primary antibody incubations were done at room temperature for either 1 (p22phox, 4-HNE) or 4 h (p47phox, Rac1, gp91phox, p67phox). After primary antibody incubation, we washed the membranes in TBS-T (4 × 5 min), incubated in secondary antibody (IR Dye, 1:10,000; LI-COR) for 1 h at room temperature, washed again (TBS-T, 4 × 5 min), and rinsed in 1× TBS. We dried the membranes (37°C, 15 min) and scanned using an Odyssey Infrared Imaging system (LI-COR). The immunoblot signal of each target protein was normalized to the total protein signal measured in the corresponding stain-free gel lanes, as described in our recent study (1). Stain-free gels provide a total protein signal that is conceptually similar to gel staining with Comassie Blue (57). These procedures are consistent with recent recommendations for data analysis of Western blots using fluorescence methods and stain-free gels (20, 44).

Oxyblot. We measured protein carbonyls in whole diaphragm homogenates using the OxyBlot Protein Oxidation Detection Kit (ST150; Millipore), following the manufacturer’s instructions with minor modifications. Briefly, we denatured 10 μg of proteins using 6% SDS and derivatized the sample by adding 10 μl of 2,4 dinitrophenylhydrazine or derivatization-control solution (negative control). We incubated the samples at room temperature for 15 min before adding 15 μl of neutralization solution to stop the derivatization reaction. We immunoblotted (see above) using anti-DNP primary antibody (diluted at 1:150 ratio) at room temperature for 1 h. We then incubated the membrane for 1 h at room temperature in anti-rabbit secondary antibody (IRDye 800CW: 1:10,000). We scanned the membrane, quantified integrated intensity in each lane, and determined total protein in the gels, as explained above.

Immunoprecipitation. We homogenized diaphragm bundles in 1× RIPA buffer (in mmol/l: 20 Tris-HCl, 150 NaCl, 1 Na2EDTA, 1 EGTA, 2.5 Na2O-P2, 1 C2H2Na2O4P, 1 Na2VO4, 1 μg/ml leupeptin, 1% NP-40, and 1% C2H2Na2O2; Cell Signaling Technology) using a Kontes Duall Homogenizer centrifuged for 2 min at 1,500 g (4°C), saved the supernatant, and measured protein content using DC protein assay (Bio-Rad Laboratories). We diluted each sample as needed to obtain a final protein content of 2.5 μg/ml and used a commercial kit for immunoprecipitation (Catch-and-Release version 2.0 kit, cat. no. 17-500; Millipore), following the manufacturer’s recommendations with optimizations for our experiment. Specifically, we centrifuged spin columns for 30 s at 2,000 g to remove resin buffer and washed twice in 1× wash buffer (400 μl). For immunoprecipitation reaction, we added to the spin column 1× wash buffer (370 μl), tissue lysate (250 μg of protein), anti-p47phox monoclonal antibody (Ab; 4 μg of Ab, sc-17845; Santa Cruz Biotechnology), and Ab capture affinity ligand (10 μl). We then incubated samples using a 360° rotator for ~20 h at 4°C. After the incubation, we centrifuged the spin columns for 30 s at 2,000 g, followed by three washes with 1× wash buffer (2,000 g, 30 s each time), added 70 μl of 1× denaturing elution buffer with 5% vol/vol β-mercaptoethanol, incubated on a vortex shaker for 45 min, and centrifuged for 1 min at 5,000 g to collect the eluent. We heat-denatured (5 min, 98°C) the eluent and stored at −20°C until use for gel electrophoresis and immunoblotting. We used a phosphoepitope antibody (Clone 7F12; Invitrogen) at 1:1,000 and total p47phox antibody (SAB2500674; Sigma-Aldrich) to calculate the phosphorylated-to-total p47phox ratio. This is a standard approach to examine p47phox phosphorylation in animal tissue (32).

Quantitative PCR. We isolated total RNA from diaphragm tissue with Trizol reagent. We then used an Ambion RETROscript First Strand Synthesis Kit (Life Technologies, Carlsbad, CA) to generate cDNA from 1 μg of RNA. The cDNA was then used as template for qRT-PCR (7300 real-time PCR system; Applied Biosystems, Austin, TX). We used TaqMan PCR assay primers (Life Technologies) targeting the following genes: p47phox (Ncf1; GeneBank NM_001286307.1), Rac1 (GeneBank NM_009007.2), gp91phox (Cybb; GeneBank NM_007807.5), p22phox (Cyba; GeneBank NM_007806.3), p67phox (Ncf2; GeneBank NM_010877.4), and p40phox (Ncf4; GeneBank NM_008677.2). Gene expression quantification was performed using the relative standard curve method, and all data were normalized to the absolute control group and subsequently normalized to the gene expression of 18S rRNA.

Statistical analysis. We performed statistical analysis using SigmaPlot version 12.5 (Systat Software, San Jose, CA). For specific comparisons, we used paired and unpaired Student’s t-test, one-way ANOVA, and repeated-measures two-way ANOVA. Post hoc comparisons were done with Dunnett’s test. These data are given as means ± SE. Data that failed the normality (Shapiro-Willk) or equal variance tests were compared using the Mann-Whitney rank sum test. Nonparametric data are presented as median (interquartile range) and shown in box and whisker plots. We declared statistical significance when P < 0.05.

RESULTS

Mouse model of CHF. Echocardiography and morphological cardiac measurements showed signs of LV dilation and hypertrophy and decreased fractional shortening that are consistent with CHF postinfarct (Table 1).

Nox2 subunit mRNA, protein level, and p47phox phosphorylation. CHF increased diaphragm mRNA levels of all Nox2-related subunits within 4 wk (Fig. 1A). At ~14 wk postsurgery, the mRNA levels of p47phox and p40phox remained significantly elevated in CHF (Fig. 1B). Similarly, CHF increased the protein level of p47phox, with no effect on other Nox2-related subunits (Fig. 2, A and B). Considering that p47phox phosphorylation regulates activation of Nox2, we immunoprecipitated p47phox and immunoblotted for serine phosphorylation. This approach revealed that CHF increased p47phox phosphorylation in the mouse diaphragm (Fig. 2, C and D). Immunoblotting confirmed the absence of p47phox in the diaphragm of p47phox−/− mice (data not shown). Based on these observations, we examined the role of p47phox on diaphragm oxidants and contractile dysfunction in CHF.

Extracellular oxidants and Nox activity. Nox is localized in cell membranes and can produce oxidants in the extracellular space (36). Our Amplex Red assay included SOD to convert superoxide to hydrogen peroxide and provide a global measure of extracellular oxidants. We observed that CHF increased diaphragm extracellular oxidants in WT mice, whereas deficiency in p47phox prevented the increase in extracellular oxidants elicited by CHF (Fig. 3). However, we were not able to detect changes in Nox activity measured by NADH consumption from cytosolic plus membrane fractions (in μM·min−1·mg protein−1: sham 4.42 ± 0.7, CHF 5.4 ± 0.50; n = 4/group).
4-HNE adducts were not increased in diaphragm of WT mice, and 4-HNE adducts (7, 12). However, protein carbonyls and associated with markers of protein oxidation such as carbonyls (Fig. 5). Abnormalities in contractile function are typically force were unchanged in CHF compared with sham WT mice Twitch kinetics and the frequency that elicits 50% maximal function induced by CHF (Fig. 4, A and B, and 5). These data are consistent with a decrease in diaphragm force in all stimulus frequencies. Iso- tonic contractile properties of the diaphragm were also im- paired by CHF. Specifically, CHF slowed twitch kinetics and diminished peak power by 50% compared with sham (Fig. 4, E and F). To our knowledge, these are the first data to show diaphragm contractile dysfunction in a mouse model of CHF. However, our most important findings were that p47phox mice were fully protected from isometric and isometric contractile dysfunction induced by CHF (Fig. 4, A–F). Twitch kinetics and the frequency that elicits 50% maximal force were unchanged in CHF compared with sham WT mice (Fig. 5). Abnormalities in contractile function are typically associated with markers of protein oxidation such as carbonyls and 4-HNE adducts (7, 12). However, protein carbonyls and 4-HNE adducts were not increased in diaphragm of WT mice, as shown in Fig. 6.

<table>
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<th>Animal characteristics</th>
<th>Wild Type</th>
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<th>p47phox /−/−</th>
<th>Wild Type</th>
<th>CHF</th>
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<td>Body weight presurgery, g</td>
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<td>28 ± 1</td>
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<td>92 ± 4</td>
<td>148 ± 7*</td>
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<td>32 ± 1*</td>
<td>18 ± 1</td>
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<td>Infarcted area, %</td>
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<td>26 ± 1</td>
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<td>Heart rate, beats/min</td>
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<td>418 ± 33</td>
<td>490 ± 15</td>
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<td>LVEDD, mm</td>
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<td>LVESD, mm</td>
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<td>Fractional shortening, %</td>
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<td>40 ± 10</td>
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Data are means ± SE; n = 16–18/group (wild type) and 8–10/group (p47phox /−/−). CHF, chronic heart failure; LV, left ventricle; RV, right ventricle; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter. Heart rate was measured during echocardiography. *P < 0.05 vs. sham within strain; †P < 0.05 vs. WT CHF.

**Diaphragm contractile function and markers of protein oxidation.** We examined isometric and isometric contractile properties of mouse diaphragm bundles in vitro. CHF decreased maximal isometric specific force and peak twitch force (Figs. 4, A and B, and 5). These data are consistent with a decrease in diaphragm force in all stimulus frequencies. Iso- tonic contractile properties of the diaphragm were also im- paired by CHF. Specifically, CHF slowed Vmax by 40% (Fig. 4, C and D) and diminished peak power by 50% compared with sham (Fig. 4, E and F). To our knowledge, these are the first data to show diaphragm contractile dysfunction in a mouse model of CHF. However, our most important findings were that p47phox mice were fully protected from isometric and isometric contractile dysfunction induced by CHF (Fig. 4, A–F). Twitch kinetics and the frequency that elicits 50% maximal force were unchanged in CHF compared with sham WT mice (Fig. 5). Abnormalities in contractile function are typically associated with markers of protein oxidation such as carbonyls and 4-HNE adducts (7, 12). However, protein carbonyls and 4-HNE adducts were not increased in diaphragm of WT mice, as shown in Fig. 6.

**DISCUSSION**

Ventilatory abnormalities play an important role in the prognosis, diminished physical capacity, and dyspnea of CHF patients (10, 14, 40). The diaphragm is the main inspiratory muscle, and our study suggests that the p47phox subunit of NAD(P)H oxidase is involved in the diaphragm dysfunction elicited by CHF. The diaphragm of CHF mice showed increases in gene and protein levels of Nox2 subunits and phosphorylation of p47phox. In this setting, deficiency in p47phox prevented the increase in diaphragm oxidants and contractile dysfunction induced by CHF.

**Mouse model of CHF.** As expected (18), MI caused CHF in mice at 14 wk postsurgery. Infarct area was greater in p47phox−/− mice, likely because WT mice with infarcts as large as those of p47phox−/− had lower survival rates (18). The changes in RV and LV weights were greater in p47phox−/− compared with WT, with similar decreases in fractional shortening in both strains. These data suggest that the p47phox−/− mice included in our study had similar or even worse degrees of CHF than the WT group due to the larger infarct area in...
p47phox knockout mice. Thus, it is unlikely that our results from p47phox−/− mice would be explained by attenuated LV dysfunction post-MI (18). This is important because diaphragm weakness in CHF depends on the severity of the disease (3, 24, 31).

**Diaphragm Nox2 mRNA and protein levels.** Diaphragm Nox2 has a subunit composition and subcellular localization generally similar to the isoform found in heart and vasculature (33, 36), where p47phox is required for enzyme activation. CHF increased diaphragm mRNA levels of p47phox and p40phox, whereas the increase in other subunits was close ($P < 0.06$) to the α-level of 0.05 declared a priori for significance at the 14-wk time point. There was also a less prominent but uniform increase in mRNA levels of all Nox2-related subunits in the diaphragm at an earlier stage of the disease (4 wk; Fig. 1A). At this time point, the increase in diaphragm mRNA level of Nox2 subunits is similar to that seen in the heart (18, 39). Overall, our data also suggest that CHF’s effect on the Nox2 subunit mRNA level is time/disease dependent, being increased in the more severe and advanced stage of the disease.

**Phosphorylation of p47phox.** Receptor-mediated activation of Nox2 by endocrine mediators such as angiotensin II, cytokines, and adrenergic agonists that are elevated in CHF occurs via phosphorylation of p47phox (13, 32, 34, 36). CHF increased p47phox phosphorylation in the diaphragm. We did not determine the specific residues, as site-specific mouse antibodies are not available, and immunoprecipitation followed by immunoblot with phosphoserine specific antibody is the standard approach to detect p47phox phosphorylation in animals (e.g., see Ref. 32). However, site-specific modification studies in white blood cells have established that phosphorylation of serine residues between amino acids 303 and 379 releases autoinhibition and is required for full enzyme activation (21, 26). Thus, the increase in p47phox phosphorylation in CHF suggests elevated Nox2 activity and oxidant production in the diaphragm.

**Diaphragm oxidants.** CHF increased NAD(P)H oxidase activity and extracellular oxidants in the diaphragm, which is in agreement with previous studies (7, 12, 55). To date, the specific source of CHF-induced heightened oxidants in diaphragm has been unclear. A recent study showed increased diaphragm Nox activity during early-stage (72 h) post-MI (7). We used a similar approach but observed no significant change in Nox activity, which may reflect a limitation of the assay or the number of animals per group that we tested. However, we found that deficiency in p47phox prevented the increase in diaphragm extracellular oxidants stimulated by CHF. This is
evidence in support of the involvement of p47phox, presumably via Nox2 action, on heightened oxidants in the CHF dia-
phragm. Mitochondria isolated from the diaphragms of CHF rats also show excess oxidant emission in the CHF dia-
phragm (55), and xanthine oxidase could also be involved, as seen in the heart (18). At first glance, our findings and those of
Supinski and Callahan (55) may appear conflicting, but mito-
chondrial oxidants could be upstream or downstream of

![Diaphragm isometric (A and B) and isotonic (C–F) contractile properties. A and B: absolute force (N) is normalized for bundle cross-sectional area (cm²) and shown as specific force in N/cm² (n = 7–10 mice/group). Wild type (WT): sham (○) and CHF (●). p47phox−/−: sham (light gray squares) and CHF (dark gray squares). Maximal shortening velocity (V_max; D) and peak power (F) are determined from force-velocity (C) and force-power relationship (E) (n = 5–7 mice/group). *P < 0.05 vs. other groups by Dunnett’s test.](image)

Fig. 4. Diaphragm twitch and force-frequency characteristics. A: peak twitch force. B: time to peak tension (TPT). C: stimulus frequency that elicits 50% of maximal force (F₅₀). D: one-half (½) relaxation time (RT). *P < 0.05 vs. other groups by Dunnett’s test. (55)
p47phox signaling. Indeed, a cross-talk between Nox2 and mitochondria oxidants has been reported in vascular smooth muscle (15, 17). Overall, our data show that whole body p47phox knockout modulates CHF-induced accumulation of oxidants in the diaphragm.

Diaphragm contractile function. Patients with CHF have decreased inspiratory muscle pressure during static and dynamic maneuvers (31, 40), which suggests impairments in diaphragm function that worsen as the disease progresses. Consistent with this notion, diaphragm contractile dysfunction has been shown in several animal models of CHF (8, 12, 54, 59). Assessment of isometric function gives insights into the force-generating capacity of the diaphragm. As the diaphragm exerts inspiratory function primarily by shortening, isotonic contractile properties are the most relevant for ventilation. We show herein that diaphragms from WT mice with CHF induced by MI have isometric and isotonic contractile dysfunction, as seen in rats (12, 22, 59). Our findings in mice set the stage for specific hypothesis testing using genetically modified animals.

The increase in oxidants that we observed in CHF is a putative mechanism for impaired diaphragm contractile function. Direct exposure of skeletal muscle cells to excess oxidants impairs contractile function, with decreases in maximal force (11), shortening velocity (12), actomyosin ATPase activity (49), and calcium sensitivity of the contractile apparatus (4). Importantly, systemic treatment of CHF rats with a membrane-permeable SOD prevents the decrease in submaximal diaphragm isometric force (55). Thus, we reason that the lack of increase in diaphragm oxidants in p47phox−/− mice conferred protection against depression of force, slowing of maximal shortening velocity, and reduction in peak power induced by CHF.

Protein oxidation. The exact molecular mechanisms underlying the depression in diaphragm contractile function in CHF is less clear. Our data cannot distinguish between impairments in excitation-contraction coupling or myofibrillar proteins, both of which could be impaired by protein oxidation (12, 58). In an acute model of heart failure in mice (72 h post-MI), protein carbonyls were increased in the diaphragm (7). In our study, carbonyls were not changed in a chronic model of heart failure (~14 wk post-MI). The discrepancy between our findings and those of Bowen et al. (7) may be related to the time course or the severity of the disease. A potential cause of loss of diaphragm force, shortening velocity, and power that we observed in CHF mice is the oxidation of thiol groups in the ryanodine-receptor channel (58), myosin heavy chain (27, 49), or actin (23). The resolution of specific thiol modifications underlying the CHF-induced diaphragm contractile dysfunction requires the use of more sophisticated and sensitive techniques (e.g., see Refs. 23 and 42). Alternatively, posttranslational modifications such as ubiquitination and (de)phosphorylation could be triggered by oxidants and impair contractile function.

Methodological considerations. We cannot attribute our findings to p47phox or NAD(P)H oxidase within diaphragm muscle cells per se. In addition to muscle fibers, several other cell types within the diaphragm express p47phox, e.g., endothelium, smooth muscle, and macrophages. Experiments using cell type-specific interventions and inducible knockouts will be required to resolve the source of p47phox and NAD(P)H oxidase responsible for diaphragm abnormalities in CHF.

Conclusion. To sum up, oxidants are known to cause abnormalities in diaphragm muscles during chronic diseases. However, the specific sources of oxidants and their clinical relevance are not well defined. Our study establishes a critical role for the p47phox subunit of NAD(P)H oxidase on the pathophysiology of diaphragm dysfunction in CHF. In this setting, Nox2 is presumably an important source of increased diaphragm oxidants that causes weakness in heart failure. Thus, targeting p47phox signaling should prevent the increase in diaphragm oxidants that causes weakness and loss of power that contributes to dyspnea and exercise intolerance in CHF.

REFERENCES

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