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

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ABSTRACT

Patients with chronic heart failure (CHF) have dyspnea and exercise intolerance that 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 Nox2 isoform of NAD(P)H oxidase is a putative source of diaphragm oxidants. Thus, we conducted our study with the goal of determining the effects of CHF on diaphragm level of Nox2 complex subunits and test the hypothesis that p47phox knockout prevents diaphragm contractile dysfunction elicited by CHF. CHF caused a two- to six-fold increase (p < 0.05) in diaphragm mRNA and protein levels of several Nox2 subunits, with p47phox being upregulated and hyperphosphorylated. CHF increased diaphragm extracellular oxidant emission in wild-type but not p47phox knockout mice. Diaphragm isometric force, shortening velocity, and peak power were decreased by 20-50% in CHF wild type mice (p < 0.05), whereas p47phox knockout mice were protected from impairments in diaphragm contractile function elicited by CHF. Our experiments show that p47phox is upregulated and involved in the increased oxidants and contractile dysfunction in CHF diaphragm. These findings suggest that a p47phox-dependent NAD(P)H oxidase mediates the increase in diaphragm oxidants and contractile dysfunction in CHF.

Keywords: Oxidative stress, respiratory muscle, myocardial infarction
INTRODUCTION

Abnormalities of the diaphragm muscle contribute to the pathophysiology of chronic heart failure (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 force to values seen in control animals (55). However, the specific source of oxidants and its relevance to diaphragm weakness in CHF remain to be elucidated.

NAD(P)H oxidases (Nox) have emerged as an important source of oxidants in skeletal muscle (47, 51) and diaphragm (6, 48). The Nox2 isoform of NAD(P)H oxidase is present in the diaphragm and, like in heart and vessels, the functionally active enzyme consists of phagocyte oxidase (phox) subunits gp91phox (Nox2), p22phox, p67phox, p40phox, and p47phox (33, 36). Nox2 is activated by factors that are increased in the serum of CHF patients such as angiotensin II and cytokines (19, 45), and indirect evidence supports a role for NAD(P)H oxidase in the pathophysiology of limb muscle abnormalities in CHF (5, 52). It is conceivable that CHF triggers an endocrine-mediated activation of Nox2 in the diaphragm that increases oxidants and impairs
contractile function. Receptor-mediated activation of Nox2 involves phosphorylation of p47\textsubscript{phox} (34, 36). Mice deficient in p47\textsubscript{phox} lack NAD(P)H oxidase activity in skeletal muscle (47, 52), are protected from increases in oxidants and diaphragm weakness in vitro (6), and have reduced pathophysiological left ventricular remodeling and dysfunction after myocardial infarction (18).

Our goal in the current study was to examine the role of NAD(P)H oxidase on diaphragm abnormalities in CHF. Initially, we characterized the effects of CHF on the mRNA and protein level profile of Nox2-related subunits in the diaphragm. Based on our initial findings, we tested the hypothesis that p47\textsubscript{phox} knockout prevents the increases in diaphragm oxidants and contractile dysfunction in CHF.

METHODS

Animals: Our study involved a total of sixty five male adult wild-type (WT, C57BL6) and fifty nine B6(Cg)-Ncf1\textsuperscript{m1J/J} mice (Jackson Laboratories) that are deficient in p47\textsubscript{phox} (p47\textsubscript{phox}-/-) due to a spontaneous mutation in the Ncf1 gene (30). Eighteen wild-type and fourteen p47\textsubscript{phox}-/- mice underwent sham surgery. The remainder (WT, n = 47 and p47\textsubscript{phox}-/-, n = 45) underwent myocardial infarction surgery described below. Breeding pairs of p47\textsubscript{phox}-/- mice were purchased from Jax labs and a colony was established at the University of Florida and maintained by the institutional animal care services breeding personnel. Mice were individually caged and exposed to standard dark-light cycles with free access to food and water throughout the study. We confirmed deficiency of p47\textsubscript{phox} in the diaphragm using immunoblotting as described below. The Institutional Animal Care and Use Committee of the University of Florida approved all procedures performed in our study.

Surgical preparation and coronary artery ligation: We caused myocardial infarction via ligation of the left anterior descending (LAD) coronary artery to induce CHF (56). We shaved the
left side of the thorax and cleaned the surgical area with 4% chlorhexidine and sterile saline. Thereafter, we performed orotracheal intubation using a PE tube (O.D. 1.27 mm) and connected the animal to a mechanical ventilator (Model 683, Harvard Apparatus). While the animal was in the surgical plane of 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 LAD coronary artery near the left atrium using 6-0 PGA suture (Demersorb™, Demetech, Florida, USA). After the ligation, we hyperinflated the lungs, approximated the ribs using 6-0 PGA suture, and closed the skin incision with 3-0 suture (Demelon™, Demetech, USA). 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 maintaining aseptic conditions. The animals received topical bupivacaine injection immediately after closing the skin. In addition, we injected buprenorphine (20-40 µg/kg, subcutaneous) during surgery and every 8-12 hrs for 3 days post-surgery. Experiments were performed approximately 14 weeks post-myocardial infarction (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 weeks post-surgery for measurements of Nox2 subunit mRNA levels as increases in diaphragm oxidants with CHF have been shown within six weeks post-surgery (55).

**Echocardiography:** We used transthoracic echocardiography (Vevo 770, Visual Sonics, Toronto, Canada) with a 30 MHz probe to obtain parasternal 2D 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 400-500 bpm 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 mid-papillary 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 only included 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 weeks studies was WT (Sham = 18, CHF = 16) and p47phox-/- (Sham = 14, CHF = 12).

**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 rib and central tendon in bicarbonate-buffered solution (in mmol/L: 137 NaCl, 5 KCl, 1 MgSO4, 1 NaH2PO4, 24 NaHCO3, 2 CaCl2) gassed with a mixture of 95% O2 and 5% CO2 at room temperature. We used 4.0 braided silk suture to tie the rib to a glass rod and attach the central tendon to a Dual-Mode Muscle Lever System (300C-LR, Aurora Scientific Inc., Aurora, Canada). We placed the diaphragm strip in an organ bath containing bicarbonate-buffered solution at room temperature and determined the length that elicited maximal twitch force (optimal length, $L_0$). To find $L_0$, 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 – 0.3 mm shortening steps until reaching maximal twitch force. We then placed the muscle at $L_0$, increased the temperature of the organ bath to 37°C, and added D-tubocurarine (25 μM) to the buffer. After 20 min of thermo-equilibration, 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 ms and 300 ms train duration delivered through platinum electrodes using a biphasic high-power stimulator (701C, Aurora Scientific Inc.). Isometric force was normalized for bundle cross-sectional area (N/cm²). After the protocol, we measured bundle’s $L_0$ and weight. To estimate the bundle cross-sectional area (CSA), we divided the diaphragm bundle weight (g) by $L_0$ 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 ($F_{50}$), and the slope of the relationship.

To test isotonic properties of the diaphragm, we used afterloaded contractions employing a protocol similar to previous studies (9, 59). The bundle was dissected and placed at $L_0$ as described above. After 20 min of thermos-equilibration, 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 $L_0$ and bundle weight to estimate bundle CSA. We analyzed shortening velocity at least 10 ms after the initial change in length and within the linear portion of the tracing (DMA software, Aurora Scientific Inc, Aurora, Canada). The force-velocity curve was plotted and fitted to the hyperbolic Hill equation. We determined maximal shortening velocity ($V_{max}$) 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 \( L_0 \), 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, California, USA). 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, adjusted muscle length to approximately 10 mm (average \( L_0 \) in our preparation), and increased the temperature of the organ bath to 37°C allowing 10 min for thermo-equilibration. After thermo-equilibration, 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, USA) 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).
**Gel electrophoresis 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, 50 mmol/L β-Glycerophosphate, pH 7.4 with protease and phosphatase inhibitor cocktails. We rotated the homogenates end over end for 1 hour at ~4°C, sonicated once for ~3 seconds, and centrifuged at 1,500 g for 2 minutes at room temp. We isolated 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 hour at room temperature and subsequently probed with primary antibodies. We used primary antibodies targeting the following proteins: p47(phox) (SAB2500674, Sigma-Aldrich), Rac1 (05-389, Millipore), gp91(phox) (611414, BD Transduction Laboratories), p22(phox) (FL-195, Santa Cruz), p67(phox) (07-502, Millipore), and 4-hydroxynonenol (4-HNE; ab46544, Abcam). We diluted the primary antibodies in Li-COR Blocking Buffer at 1:1,000 ratio, except for p22(phox) (1:250). Primary antibody incubations were done at room temp for either 1 hr (p22(phox), 4-HNE) or 4 hrs (p47(phox), Rac1, gp91(phox), p67(phox)). After primary antibody incubation, we washed the membranes in TBS-T (4× 5 min), incubated in secondary antibody (IR Dye, Li-COR; 1:10,000) for 1 hour at room temperature, washed again (TBS-T, 4× 5 min), and rinsed in 1x TBS. We dried the membranes (37°C, 15 min) and scanned using an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE). 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 (S7150, 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 (DNPH) or derivatization-control solution
(negative control). We incubated the samples at room temp 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 1:150 ratio) at room temp for 1 hour. We then incubated the
membrane for 1 hour 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 1x RIPA buffer (in mmol/L: 20
Tris-HCl, 150 NaCl, 1 Na2EDTA, 1 EGTA, 2.5 Na4O7P2, 1 C3H7Na2O6P, 1 Na3VO4; 1 µg/mL
leupeptin, 1% NP-40, 1% C24H39NaO4; Cell Signaling technologies) 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/µl and used a commercial kit for
immunoprecipitation (Catch-and-Release v2.0 kit; Cat. No. 17-500, Milipore) following the
manufacturer’s recommendations with optimizations for our experiment. Specifically, we
centrifuged spin columns for 30 s at 2,000g to remove resin buffer and washed twice in 1x
Wash Buffer (400 μL). For immunoprecipitation reaction, we added to the spin column 1x Wash
Buffer (370 μL), tissue lysate (250 μg protein), anti-p47 phox mAb (4 μg Ab; sc-17845, Santa Cruz
Biotechnology), and Ab capture affinity ligand (10 µL). We then incubated samples using a 360° rotator for approximately 20 hrs at 4°C. After the incubation, we centrifuged the spin columns for 30 s at 2,000 g, followed by three washes with 1x Wash Buffer (2,000 g; 30 s each time), added 70 µL of 1x denaturing elution buffer with 5% v/v 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 phosphoserine antibody (Clone 7F12, Invitrogen) at 1:1,000 and total p47\textsuperscript{phox} antibody (SAB2500674, Sigma-Aldrich) to calculate the phospho-to-total p47\textsuperscript{phox} ratio. This is a standard approach to examine p47\textsuperscript{phox} phosphorylation in animal tissue (32).

**qPCR:** We isolated total RNA from diaphragm tissue with Trizol reagent. We then used Ambion RETROscript First Strand Synthesis Kit (Life Technologies, Carlsbad, CA, USA) 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: p47\textsuperscript{phox} (Ncf1, GeneBank NM_001286037.1), Rac1 (GeneBank NM_009007.2), gp91\textsuperscript{phox} (Cybb, GeneBank NM_007807.5), p22\textsuperscript{phox} (Cyba, GeneBank NM_007806.3), p67\textsuperscript{phox} (Ncf2, GeneBank NM_010877.4), and p40\textsuperscript{phox} (Ncf4, GeneBank NM_008677.2). Gene expression quantification was performed using the relative standard-curve method, and all data was normalized to the absolute control group and subsequently normalized to the gene expression of 18S rRNA.

**Statistical analysis:** We performed statistical analysis using SigmaPlot v.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 Dunnet's test. These data are given as mean ± SE. Data that failed the normality (Shapiro-Wilk) or equal variance tests were compared using Mann-Whitney rank sum test. Non-parametric
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 measures showed signs of LV dilation and hypertrophy and decreased fractional shortening that are consistent with CHF post-infarct (Table 1).

Nox2 subunits mRNA and protein level, and p47phox phosphorylation: CHF increased diaphragm mRNA levels of all Nox2-related subunits within 4 weeks (Fig. 1A). At approximately 14 weeks post-surgery, 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. 2A-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. 2C-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, while 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/mg protein: Sham 4.42 ± 0.7, CHF 5.4 ± 0.50; n = 4/group).
Diaphragm contractile function and markers of protein oxidation: We examined isometric and isotonic contractile properties of mouse diaphragm bundles in vitro. CHF decreased maximal isometric specific force and peak twitch force (Fig. 4A, B and Fig. 5). These data are consistent with a decrease in diaphragm force in all stimulus frequencies. Isotonic contractile properties of the diaphragm were also impaired by CHF. Specifically, CHF slowed maximal shortening velocity ($V_{max}$) by 40% (Fig. 4C, D) and diminished peak power by 50% compared to sham (Fig. 4E, F). To our knowledge, this is the first data to show diaphragm contractile dysfunction in a mouse model of CHF. However, our most important findings were that p47<sup>phox</sup>-/- mice were fully protected from isometric and isotonic contractile dysfunction induced by CHF (Fig. 4A-F). Twitch kinetics and the frequency that elicits 50% maximal force were unchanged in CHF compared to 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 suggest that the p47<sup>phox</sup> 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 level of Nox2 subunits and phosphorylation of p47<sup>phox</sup>. In this setting, deficiency in p47<sup>phox</sup> prevented the increase in diaphragm oxidants and contractile dysfunction induced by CHF.

**Mouse model of CHF.** As expected (18), myocardial infarction caused CHF in mice 14 weeks post-surgery. Infarct area was greater in p47<sup>phox</sup>-/- mice, likely because WT mice with infarcts as large as those of p47<sup>phox</sup>-/- had lower survival rate (18). The changes in RV and LV
weights were greater in p47<sup>phox</sup>-/- compared with WT, with similar decrease in fractional shortening in both strains. These data suggest that p47<sup>phox</sup>-/- mice included in our study had similar or even worse degree of CHF than the WT group due to the larger infarct area in p47<sup>phox</sup> knockout mice. Thus, it is unlikely that our results from p47<sup>phox</sup>-/- 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 p47<sup>phox</sup> is required for enzyme activation. CHF increased diaphragm mRNA level of p47<sup>phox</sup> and p40<sup>phox</sup>, whereas the increase in other subunits was close (P < 0.06) to the α-level of 0.05 declared <em>a priori</em> for significance at the 14-week 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 weeks; 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 effects on Nox2 subunit mRNA level is time/disease dependent, being increased in more severe and advanced stage of the disease.

Phosphorylation of p47<sup>phox</sup>. 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 p47<sup>phox</sup> (13, 32, 34, 36). CHF increased p47<sup>phox</sup> 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 phospho-serine specific antibody is the standard approach to detect p47<sup>phox</sup> phosphorylation in animals, e.g., ref. (32). However, site-specific modification studies in white blood cells have established that phosphorylation of serine residues between amino acids 303-379 release auto-inhibition and
are required for full enzyme activation (21, 26). Thus, the increase in p47\textsuperscript{phox} 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 stages (72 hrs) post-myocardial infarction (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 that we tested per group. However, we found that deficiency in p47\textsuperscript{phox} prevented the increase in diaphragm extracellular oxidants stimulated by CHF. This is evidence in support of the involvement of p47\textsuperscript{phox}, presumably via Nox2 action, on heightened oxidants in CHF diaphragm. Mitochondria isolated from diaphragm of CHF rats also show excess oxidant emission in CHF diaphragm (55) and xanthine oxidase could also be involved as seen in the heart (18). At a first glance, our findings and those of Supinski & Callahan (55) may appear conflicting, but mitochondrial oxidants could be upstream or downstream of p47\textsuperscript{phox} 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 p47\textsuperscript{phox} 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 worsens 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 diaphragm from WT mice with CHF induced by myocardial infarction 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. In this regard, the absence of p47\(^{phox}\) subunit of NAD(P)H oxidase prevented both isometric and isotonic diaphragm contractile impairments caused by CHF (see Fig. 5).

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 impair 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 p47\(^{phox-/-}\) 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 (E-C) coupling or myofibrillar proteins, which could both be impaired by protein oxidation (12, 58). In an acute model of heart failure in mice (72 hrs 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 weeks 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., (23, 42). Alternatively, post-translational modifications such as ubiquitination and (de)phosphorylation could be triggered by oxidants and impair contractile function.

**Methodological considerations.** We cannot attribute our findings to p47<sub>phox</sub> or NAD(P)H oxidase within diaphragm muscle cells *per se*. In addition to muscle fibers, several other cell types within the diaphragm express p47<sub>phox</sub>, e.g., endothelium, smooth muscle, and macrophages. Experiments using cell-type specific interventions and inducible knockouts will be required to resolve the source of p47<sub>phox</sub> 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 p47<sub>phox</sub> 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 p47<sub>phox</sub> signaling should prevent the increase in diaphragm oxidants that causes weakness and loss of power that contributes to dyspnea and exercise intolerance in CHF.

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References


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Table 1. Animal Characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>p47&lt;sup&gt;phox&lt;/sup&gt;/-</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>Body weight (g), pre-surgery</td>
<td>30 ± 1</td>
<td>28 ± 1</td>
<td>22 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Body weight (g), post-surgery</td>
<td>35 ± 1</td>
<td>35 ± 1</td>
<td>25 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>112 ± 2</td>
<td>143 ± 4*</td>
<td>92 ± 4</td>
<td>148 ± 7*</td>
</tr>
<tr>
<td>RV weight (mg)</td>
<td>25 ± 1</td>
<td>32 ± 1*</td>
<td>18 ± 1</td>
<td>26 ± 1*</td>
</tr>
<tr>
<td>LV wt/Tibial length (mg/mm)</td>
<td>5.8 ± 0.4</td>
<td>7.8 ± 0.2*</td>
<td>5.3 ± 0.3</td>
<td>8.3 ± 1.2*</td>
</tr>
<tr>
<td>RV wt/Tibial length (mg/mm)</td>
<td>1.4 ± 0.4</td>
<td>1.8 ± 0.1*</td>
<td>1.0 ± 0.04</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Infarcted area (%)</td>
<td>-</td>
<td>26 ± 1</td>
<td>-</td>
<td>40 ± 5†</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>441 ± 18</td>
<td>418 ± 33</td>
<td>490 ± 15</td>
<td>475 ± 15</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.76 ± 0.15</td>
<td>4.41 ± 0.11*</td>
<td>3.25 ± 0.12</td>
<td>4.60 ± 0.39*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.29 ± 0.07</td>
<td>3.45 ± 0.08*</td>
<td>1.97 ± 0.20</td>
<td>3.47 ± 0.38*</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>39 ± 2</td>
<td>21.6 ± 4*</td>
<td>40 ± 10</td>
<td>25 ± 6*</td>
</tr>
</tbody>
</table>

CHF, chronic heart failure; LV, left ventricle; RV, right ventricle; wt, weight; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter. Heart rate was measured during echocardiography. Data are mean ± SE. N = 16-18/group (Wild type) and 8-10/group (p47<sup>phox</sup>/-). * P < 0.05 vs Sham within strain. † P < 0.05 vs WT CHF.
Figure legends

**Figure 1.** Diaphragm mRNA levels of NAD(P)H oxidase isoform 2 (Nox2) subunits 4 weeks (A) and approximately 14 weeks post-surgery (B). Note that panels have different scale on y-axis. * P < 0.05 by unpaired t-test or Mann-Whitney rank sum test. Non-parametric data are shown by box and whisker plots.

**Figure 2.** Mouse diaphragm Nox2 subunit protein level and serine phosphorylation of p47\textsubscript{phox}. A, C) representative images of immunoblots (IB). IP, immunoprecipitation. B, D) quantification of protein level and serine phosphorylation (P-Ser) in sham (open boxes; n = 4-5) and CHF mice (gray boxes; n = 4-6). * P < 0.05 by t-test.

**Figure 3.** Extracellular oxidants in diaphragm bundles. Amplex Red fluorescence was divided by diaphragm bundle weight and value shown is relative to pair-matched Sham within strains. * P < 0.05 by paired Student t-test. See methods for rationale on paired experiments and statistical analysis.

**Figure 4.** Diaphragm isometric (A, B) and isotonic (C-F) contractile properties. A, B) Absolute force (N) is normalized for bundle cross-sectional area (cm\(^2\)) and shown as specific force in N/cm\(^2\) (n = 7-10 mice per group). Circles, WT: Sham (open) and CHF (closed). Squares, p47\textsubscript{phox-/-}: Sham (light gray) and CHF (dark gray). Maximal shortening velocity (V\textsubscript{max}, Panel D) and peak power (Panel F) are determined from force-velocity (Panel C) and force-power relationship (Panel E) (n = 5-7 mice per group). * P < 0.05 vs. other groups by Dunnet’s test.
Figure 5. Diaphragm twitch and force-frequency characteristics. A) Peak twitch force, B) one-half relaxation time (RT), C) $F_{50}$ is stimulus frequency that elicits 50% of maximal force, and D) TPT, time to peak tension. * $P < 0.05$ vs. other groups by Dunnet’s test.

Figure 6. Protein carbonyls and 4-hydroxynonenal (HNE) adducts in mouse diaphragm. Western blot data are from $n = 5-6$ mice/group. The total sum of carbonyls (A) or HNE (B) signal in each lane was normalized to total protein of respective lanes.
A

Sham | CHF

p47^{phox} |  | 
Rac1 |  | 
gp91^{phox} |  | 
p22^{phox} |  | 
p67^{phox} |  |
protein gel (40 kDa)

B

Protein level (Fold control)

\[
\begin{array}{c|c|c|c|c}
& p47^{phox} & Rac1 & gp91^{phox} & p22^{phox} & p67^{phox} \\
\hline
\text{Sham} & 0.0 & 1.0 & 0.5 & 1.2 & 0.8 \\
\text{CHF} & 1.5 & 1.1 & 1.0 & 1.3 & 1.0 \\
\end{array}
\]

C

IP: p47^{phox}

IB: P-Ser

50 kDa

IB: p47^{phox}

Sham | CHF

D

Phospho/total p47^{phox} (fold control)

\[
\begin{array}{c|c}
& \text{Sham} & \text{CHF} \\
\hline
\text{Phospho/total} & 0.8 & 1.5 \\
\end{array}
\]
Amplex Red Fluorescence (Relative to pair-matched sham)

Wild Type

p47^{phox-/-}