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Nox-derived ROS are acutely activated in pressure overload pulmonary hypertension: indications for a seminal role for mitochondrial Nox4

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Frazziano G, Al Ghoulhe I, Baust J, Shiva S, Champion HC, Pagano PJ. Nox-derived ROS are acutely activated in pressure overload pulmonary hypertension: indications for a seminal role for mitochondrial Nox4. Am J Physiol Heart Circ Physiol 306: H197–H205, 2014. First published November 8, 2013; doi:10.1152/ajpheart.00977.2012.—Pulmonary arterial hypertension is a severe progressive disease with marked morbidity and high mortality in which right ventricular (RV) failure is the major cause of death. Thus knowledge of the mechanisms underlying RV failure is an area of active interest. Previous studies suggest a role of NADPH oxidase in cardiomyocyte dysfunction in the left heart. Here we postulate that acute pressure overload induced by pulmonary artery banding (PAB) leads to a Nox4-initiated increase in reactive oxygen species (ROS) in mouse RV that may lead to feed-forward induction of Nox2. To test our hypothesis, ROS production was measured in RV and left ventricle homogenates. The data show that hydrogen peroxide (H2O2), but not superoxide anion (O2·−), was increased in the early phases (within 6 h) of PAB in RV and that this increase was diminished by catalase and diphénylepsilonodium chloride but not by SOD, N°-nitro-L-arginin methyl ester, febuxostat, or indomethacin. H2O2 production in RV was not attenuated in Nox2 null mice subjected to 6 h PAB. Moreover, we observed an upregulation of Nox4 mRNA after 1 h of PAB and an increase in mitochondrial Nox4 protein 6 h post-PAB. In contrast, we observed an increase in Nox2 mRNA 1 day post-PAB. Expression of antioxidant enzymes SOD, catalase, and glutathione peroxidase did not change, but catalase activity increased 6 h post-PAB. Taken together, these findings show a role of mitochondria-localized Nox4 in the early phase of PAB and suggest an involvement of this isoform in early ROS generation possibly contributing to progression of RV dysfunction and failure.

NADPH oxidase; reactive oxygen species; pulmonary artery banding; right ventricle

PULMONARY ARTERIAL HYPERTENSION (PAH) is a severe progressive disease characterized by a sustained increase in the pulmonary vascular resistance leading to right ventricular (RV) failure and death (13, 17). Although many drugs are available for the treatment of this disease, mortality remains high principally due to the RV failure, whose mechanisms are not fully known. Emerging evidence reveals that reactive oxygen species (ROS), generated in cardiomyocytes by different sources among which the most important are mitochondria, NADPH oxidase (Nox), and nitric oxide synthases (4), may be a driver of cardiac disease progression.

Many studies showed the importance of Nox-derived ROS in the progression of cardiac hypertrophy (8, 12, 21, 28, 29). The two Nox isoforms reported to be most expressed and have functional effects in left heart cardiac myocytes have been Nox2 and Nox4 (36). In general, Nox2 is normally quiescent and is acutely activated by stimuli such as G protein-coupled receptor agonists (e.g., angiotensin II, endothelin-1), growth factors, and cytokines (39) to generate superoxide (O2·−), which, in turn, is rapidly dismuted to hydrogen peroxide (H2O2) through the activity of SOD (7, 25). In contrast, Nox4 is constitutively active, and seems to be regulated largely by changes in transcription and translation and/or post-translational modifications (7, 10, 25, 34, 40). In this respect, Nox4 may be regarded as an inducible isoform (39). Additionally, Nox4 appears to generate predominantly H2O2 rather than O2·−, although this is still somewhat controversial and may involve close association of the isoform with SOD (4, 24). Moreover, the intracellular locations of the two isoforms in cardiomyocytes are distinct (39). There is broad consensus that activated Nox2 is found predominantly on the plasma membrane, whereas Nox4 is found in multiple organelles intracellularly (7, 24). Nox2 may be activated by several different cardiac stressors, and an increasing number of studies suggest important pathophysiological roles for this isoform in cardiac disease (8, 12, 31, 39). Nox4 expression increases during stress such as hypoxia (30, 39), mitochondrial dysfunction (18, 41), and in vivo left ventricle (LV) pressure overload (12, 24, 42), and recent studies have shown both a deleterious and a protective role of Nox4 in response to chronic LV pressure overload (33, 42).

Much less is known about the role of Nox2 and Nox4 in the RV. In this study, we investigate the role of Nox isoforms using pulmonary artery (PA) banding (PAB), a surgical model of RV pressure overload, and hypothesize that PAB results in an increase in ROS production due to Nox4, which is responsible for cardiac dysfunction and RV failure. Our findings show that PAB, within the first 6 h, induces a primary increase in Nox4-derived ROS, and an upregulation of mitochondria-localized Nox4.

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MATERIAL AND METHODS

Pulmonary artery banding. Male C57BL/6J wild-type, Nox2 null, and p47phox null mice, 6–8 wk old, were obtained from Jackson Laboratory (Bar Harbor, ME). PAB was performed as previously described (20). Briefly, mice were anesthetized with an intraperitoneal injection of etomidate (5–10 mg/kg-i), intubated, and placed on a ventilator (Type845; Harvard Apparatus, Holliston, MA) using a tidal volume of ~225 μl and a respiratory rate of ~200 breaths/min. Mice were then placed supine on a heated pad to maintain a body temperature of 37°–39°C. Body temperature was monitored via rectal probe thermometer (THM100; Indus Instruments, Houston, TX). A left thoracotomy was performed and the PA carefully dissected free from the aorta. A surgical clip (Weck 005200; Research Triangle Park, North Carolina, NC) that had been calibrated to a 27-gauge diameter was placed around the PA to create pressure afterload on the RV. After banding, the thoracic cavity was closed in layers, and the mice were placed on a heating pad until they regained their righting reflex and became ambulatory. This banding produces a pressure of 20 /H9262 g/cm² of the RV with an RV end-systolic pressure of ~35 mmHg (data not shown). Sham-operated animals underwent the same procedure except for the banding of the PA. Animals were euthanized at the indicated times and hearts collected. The RV was carefully separated from the LV and septum and Fulton Index (RV wet weight/LV + septum wet weight) determined. For all assays the tissues were homogenized using a scinted glass mortar on glass pestle connected to an electric motor. An appropriate buffer of homogenization for the assay employed was used and maintained at 4°C during homogenization. All animal studies were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

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\text{H}_2\text{O}_2 \text{ production (Amplex Red assay). } H_2O_2 \text{ production was measured in tissue homogenates using Amplex Red. Frozen RV or LV from sham or mice subjected to PAB were homogenized in PBS containing protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany), Phospho-Stop cocktail (Roche Diagnostics GmbH, Mannheim, Germany), and 0.1 mM PMSF. The homogenates were centrifuged at 1,000 g for 10 min at 4°C to eliminate unbroken tissues, and an appropriate amount of supernatant (equivalent to 50 μg of protein) was loaded into black 96-well plates in assay buffer (25 mM Hepes, pH 7.4, containing 0.12 M NaCl, 1 mM MgCl\(_2\), 0.1 mM Amplex Red, and 0.32 U/ml horseradish peroxidase) in the presence or absence of different inhibitors: 3,000 U/mL catalase, 20 μM diphenylene iodonium (DPI), 200 U/ml SOD, 10 μM NADPH reductase, 10 μM indomethacin, and 100 μM N\textsuperscript{a}m-nitro-l-arginin methyl ester (l-NAME). Fluorescence was measured using a Biotek Synergy 4 hybrid multimode microplate reader with a 530/25-excitation and a 590/35-emission filter, for 1 h and 30 min at 37°C.
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\text{Superoxide production (cytochrome c assay). } \text{Frozen RV or LV from sham mice and mice subjected to PAB were homogenized in lysis buffer composed of 8 mM potassium, sodium phosphate buffer (pH 7.0), 131 mM NaCl, 340 mM sucrose, 2 mM NaN\textsubscript{3}, 5 mM MgCl\(_2\), 1 mM EGTA, 1 mM EDTA, protease inhibitor cocktail, and 1 mM dithiothreitol and then subjected to 5 freeze/thaw cycles and lysis buffer composed of 8 mM potassium, sodium phosphate buffer (pH 7.0) containing H\textsubscript{2}O\textsubscript{2} (10 mM). H\textsubscript{2}O\textsubscript{2} absorbance was measured at 240 nm to obtain the rate of H\textsubscript{2}O\textsubscript{2} decomposition as absorbance per minute. Catalase activity was calculated in terms of units per milligram of protein where 1 unit is defined as the quantity of catalase that scavenges 1.0 μmol of H\textsubscript{2}O\textsubscript{2} per min at pH = 7.0 and 25°C.
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\text{Real-time quantitative PCR. } \text{Total RNA was extracted from tissues using RNeasy Plus Kit (Qiagen) as per the manufacturer’s protocol. All RNA samples were quantified by spectrophotometry, and 1 μg of total RNA was used in reverse transcription to obtain cDNA. Commercially available primers used in quantitative PCR were Nox2 Catalog No. 4331182 Mm01287742_m1 TaqMan Gene Expression assay; Nox4 Catalog No. 4331182 Mm00479236_m1 TaqMan Gene Expression assay; and HPRT1 Catalog No. 4331182 Mm03024075_m1 TaqMan Gene Expression assay (Applied Biosystems). HPRT1 was used as a housekeeping gene. Real-time quantitative PCR was performed with the ABI 7700 detection system (Applied Biosystems). The reaction was carried out using TaqMan Gene expression Assays (TaqMan MGB probes, FAM dye-labeled): 5 μl of TaqMan Universal PCR Master Mix, No Amperase UNG (2×) fast reaction, 0.5 μl 20× TaqMan Gene expression Assay Mix (primers), and 4.5 μl of the proper dilution of cDNA for a final volume of 10 μl per reaction (assays were performed in 384-well plates). The thermal cycling program consisted of an initial denaturation for 20 s at 95°C, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. All analyses were done in triplicate. All results are normalized for the housekeeping gene HPRT1, and relative quantification was obtained using the ΔΔC\textsubscript{t} (threshold cycle) method; relative expression was calculated as 2\textsuperscript{−ΔΔC\textsubscript{t}}.
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\text{Statistical analysis. } \text{All results are expressed as means ± SE. Paired Student’s t-test for single-paired point comparisons and a two-way ANOVA with replication followed by a Tukey’s post hoc modified t-test for group comparisons were used. } P < 0.05 \text{ was considered statistically significant. The number of animals used in }
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each experiment is represented by $n$ values. For each animal, experiments were performed in triplicate with the exception of Western blots, which were performed in singlicate.

RESULTS

Increased RV and LV ROS production after PAB. For a comprehensive overview of the production of ROS in RV and LV, we began by measuring H$_2$O$_2$ production, a more general indicator of oxidase activity, at different time points after PAB. In the RV of mice subjected to PAB, an increase in fluorescence, expressed as relative fluorescence units (RFU), compared with its respective sham time control (assigned a value of 100%) is shown. There was an 18.00 ± 12.00%, 39.17 ± 19.86%, and 49.85 ± 10.96% increase over sham at 1, 3, and 6 h after PAB, respectively ($n$ = 3–9; $P < 0.05$ vs. sham at 6 h). No increase was observed at 1 day after PAB (96.87 ± 16.39% of sham; $n$ = 3; Fig. 1A). In contrast, there was no change in H$_2$O$_2$ production in the LV of PAB mice at any time point ($87.84 ± 11.27%$, $86.56 ± 7.47%$, $86.55 ± 11.36%$, and 95.02 ± 3.25% vs. sham at 1, 3, and 6 h and 1 day after PAB, respectively; Fig. 1B).

RV and LV O$_2^-$ production after PAB. O$_2^-$ production was measured by SOD-inhibitable cytochrome $c$ reduction in RV (Fig. 2A) and LV (Fig. 2B). In the RV, O$_2^-$ production was measured as picomoles O$_2^-$ per minute per milligram protein (prot) as means ± SE; $n$ = 3–5 animals. *$P < 0.05$ indicates significant difference vs. sham.

Effect of ROS inhibitors on H$_2$O$_2$ production in RV after PAB. Pharmacological inhibitors were used to explore whether RV Nox could be involved in whether RV Nox could be involved in H$_2$O$_2$ production, to verify that H$_2$O$_2$ measured was not due to the dismutation of O$_2^-$, and to verify the identity of H$_2$O$_2$. RV homogenates from 1-, 3-, and 6 h and 1 day PAB or their respective sham samples were treated with DPI (20 μM), l-NAME (100 μM), febuxostat (10 nM), indomethacin (10 μM), SOD (200 U/ml), and catalase (3,000 U/ml). DPI significantly decreased activity in sham and PAB at all time points (1, 3, and 6 h and 1 day; Fig. 2B).
production is primary and immediate and not due to dismutation of O$_2$$. L-NAME, febuxostat, and indomethacin inhibited H$_2$O$_2$ production at 1 and 3 h, suggesting a contribution of nitric oxide synthase, xanthine oxidase, and cyclooxygenase, respectively, on H$_2$O$_2$ generation at these time points. Importantly, there was no significant inhibition by L-NAME, febuxostat, and indomethacin at 6 h of PAB, supporting a primarily Nox-derived H$_2$O$_2$ production at this time point.

mRNA expression of Nox4 and Nox2 at early time points after PAB in the RV. To confirm the role of Nox in PAB, we analyzed RV tissue for mRNA expression of the two most prevalent Nox isoforms reportedly expressed in mouse hearts, Nox2 and Nox4. We observed an upregulation of Nox4 mRNA expression in the early phase of PAB (significant after 1 h vs. sham, $n = 3$; Fig. 4) and a later more modest upregulation of Nox2 mRNA expression (statistically significant after 1 day, $n = 5$; Fig. 4). These findings suggested an early activation of Nox4 in PAB followed by induction of Nox2 expression.

Nox4 protein expression in mitochondria and in total homogenates from RV. Nox4 has been reported to be expressed in the mitochondria of various cell types (9, 24). Inasmuch as the mitochondria are expected to be an abundant and significant source of ROS in the RV, we isolated RV mitochondria from sham- and PAB-operated mice at 6 h and measured Nox4 protein levels. We found a nearly twofold increase in Nox4 protein expression in mitochondria from PAB versus sham mice (Fig. 5, A and B). To test whether the increase in mitochondrial Nox4 protein expression in response to PAB also coincided with global increase in Nox4 in RV cardiomyocytes, we analyzed Nox4 expression in RV homogenates in wild-type
and Nox2 null mice subjected to PAB. Our data show that Nox4 in total homogenates did not change in response to PAB compared with sham-operated controls (Fig. 6). Moreover, Nox4 expression was similar in PAB Nox2 null mice compared with PAB wild-type mice, suggesting that the absence of Nox2 did not affect Nox4 protein expression under PAB (Fig. 6).

Increased RV H$_2$O$_2$ production at early time points of PAB in Nox2 null mice. We measured H$_2$O$_2$ production in Nox2 null mice to test whether the absence of Nox2 negates RV H$_2$O$_2$ production after PAB as a result of Nox2 activity or Nox2-induced Nox4 activity (15, 19). Nox2-derived O$_2^-$ is typically dismutated to H$_2$O$_2$, and Nox4 appears to directly generate H$_2$O$_2$. Thus Amplex Red fluorescence is a measure of ROS production from both Nox isoforms. We detected an increase in signal comparable with the increase shown in wild-type mice (see Fig. 3C) after 6 h PAB (n = 3, P < 0.05; Fig. 7), an effect abolished by catalase and DPI but unaltered by SOD. These findings suggest that Nox4 rather than Nox2 is responsible for ROS after 6 h PAB and that Nox2 deletion is inconsequential at early time points of pressure overload-induced ROS production.

Protein expression of RV antioxidant enzymes (catalase, SOD, and GPx) does not change in response to PAB. An increase of oxidative stress is usually followed by an increase in antioxidant enzymes expression and/or activity. Therefore, we analyzed the protein expression of the major antioxidant systems present in the heart, catalase, SOD, and GPx (Fig. 8). We did not find a difference in catalase (Fig. 8B), SOD (Fig. 8C), or GPx (Fig. 8D) expression between sham-operated and PAB mice at any time point. This suggested that alterations in ROS levels were not a consequence of changes in the expression of major antioxidant systems controlling ROS.

Catalase activity increase after 6 h of PAB. Since expression of antioxidants was not altered in response to PAB, we tested whether activity of antioxidants increased. Because H$_2$O$_2$ was the major ROS observed to increase in response to PAB, we measured catalase activity. There was a twofold increase in catalase activity after 6 h of PAB compared with sham-operated mice (Fig. 9). In contrast, catalase activity was unaltered between PAB and sham at 1 and 3 h.

**DISCUSSION**

In this study, we show for the first time a rapid induction in RV Nox expression and H$_2$O$_2$ levels within the first 6 h (acute
phase) of pressure overload in PA-banded mice, a surgical model capable of inducing pure hemodynamic stress in the RV and maladaptive remodeling and failure within 2 to 3 wk (chronic phase) of sustained pressure overload (20). This effect was specific for the RV, because no increase of H$_2$O$_2$ was shown in LV, suggesting that this model does not affect generalized heart ROS production. In contrast, at all time points there was no difference in RV O$_2$.H$_2$O$_2$ levels between sham and PAB mice, suggesting that O$_2$.H$_2$O$_2$ is not the major ROS produced following acute PAB. In contrast, pharmacological experiments supported that H$_2$O$_2$ is the major ROS in the RV post-PAB and suggested that NADPH oxidase is the likely source of the H$_2$O$_2$ at 6 h post-PAB. Nox isofrm expression profile revealed an early fourfold increase in Nox4 expression 1 h post-PAB followed by a twofold increase in Nox2 expression at 1 day post-PAB. Western blot analysis demonstrated an early approximately twofold increase in mitochondrial Nox4 expression in response to 6 h PAB, supporting a role for mitochondrial Nox4 in this process. This finding was supported by data in which PAB-induced ROS was not attenuated in RV from Nox2 null mice. Finally, our data demonstrated that changes in H$_2$O$_2$ production in response to PAB coincided with an increase in catalase activity at 6 h of PAB but not with changes in expression of catalase or the other ROS scavenging enzymes SOD, and GPx.

Fig. 8. Catalase, SOD, and glutathione peroxidase (GPx) protein expression do not change after PAB. Catalase, SOD, and GPx protein expression were measured in RV after PAB by Western blot. β-Actin was used as a loading control. A: representative blots of protein expression in sham and PAB at 3 h. B–D: quantification of catalase (B), SOD (C), and GPx (D) protein expression in response to PAB for the indicated times. Results are expressed as means ± SE of fold change from the respective sham for each time point; n = 3–6 animals. Norm, normalized.

Fig. 9. Catalase activity increases after 6 h of PAB. Catalase activity was measured 1, 3, and 6 h after PAB in RV. Results are expressed as means ± SE of fold changes from sham; n = 3 animals. *P < 0.05 indicates significant difference between PAB and sham.
result of pressure overload was H$_2$O$_2$ and not O$_2^-$·. Incubation with catalase abolished PAB-induced ROS elevation in RV at every time point, supporting that the observed Amplex Red fluorescence was H$_2$O$_2$ specific and not due to other sources, such as autofluorescence of the tissue homogenates for example. The lack of effect of added SOD confirmed that the observed H$_2$O$_2$ is not a consequence of O$_2^-$· production and subsequent dismutation at the time points analyzed. In fact, O$_2^-$· levels were significantly reduced 3 h postbanding. These data are different from previous findings which implicated mainly O$_2^-$· in the dysfunctional heart (8, 24). Inasmuch as the current study explored the effects of pressure overload on the right ventricle per se, this discrepancy may be due to multiple factors including 1) differences in the cellular Nox composition in those studies which were performed in the LV (vs. the RV), 2) the particular model used and differences in the level and type of hemodynamic stress, and 3) unique cellular signaling within the two tissue types. Assessing distinctions in signaling upstream and downstream of Nox between the right and left ventricle is an area of current investigation.

The Nox family is a major source of ROS and consists of seven members, Nox1, Nox2, Nox3, Nox4, Nox5, and dual oxidase 1 and 2, that display distinct subcellular localizations, tissue distributions, mechanisms of regulation and activities, and (patho)physiological functions (11). In general, the literature reports that Nox2 and Nox4 are major cardiomyocyte Nox isoforms. However, most, if not all, of these data are derived from studies of the LV. Thus, little to no information is available on the role of Nox in the RV. Our data suggest that Nox4 is the source of the increase in H$_2$O$_2$ observed following PAB. That is, PAB-induced H$_2$O$_2$ increase (i) was attenuated by incubation of RV homogenates with the iodonium compound DPI, an inhibitor of flavin-containing enzymes including Nox at all time points but not by incubation with L-NAME, indomethacin, or febuxostat; 2) exhibited an early increase in RV Nox4 and not Nox2 mRNA as well as an increase in mitochondrial Nox4 protein; and 3) was unaffected by deletion of Nox2. The data are in agreement with mounting evidence that the primary product of Nox4 is H$_2$O$_2$ (4, 42) and is supported by findings of attenuated H$_2$O$_2$ generation in heart tissue from Nox4 null mice (42). Our data are also in agreement with studies by Byrne et al. (12) who demonstrated that the rise in ROS levels in the LV in response to pressure overload was not attenuated in Nox2 null hearts. Moreover, Looi et al. (31) observed a similar lack of effect of Nox2 deletion on ROS in a model of myocardial infarction. Our data are unique in that they are, to our knowledge, the first to implicate Nox4-derived H$_2$O$_2$ in RV pressure overload and are supported by studies showing that Nox4 is involved in LV pressure overload-induced heart failure (12, 24) and that Nox4 worsens heart function (24).

mRNA expression profiles demonstrated an increase in Nox4 mRNA 1 h post-PAB followed by an increase in Nox2 mRNA 1 day post-PAB. Thus the data appear to suggest a positive feed-forward mechanism whereby Nox4-derived H$_2$O$_2$ during the early phase of pressure overload leads to subsequent upregulation of Nox2-derived ROS in response to sustained chronic pressure overload and a chain reaction, which ultimately leads to RV dysfunction. Nox2 activation (i.e., as a result of post-translational modification and/or recruitment of cytosolic activating subunits) did not likely play a role in the early phase as evidenced by sustained (<1 day) PAB-induced ROS in Nox2 null mice. Indeed, pressure overload leads to RV dysfunction in this model, and deletion of Nox2 oxidase essential cytosolic subunit p47phox ameliorates the dysfunction (Fig. 10). Thus both Nox4 and Nox2 play critical roles in pressure overload-induced RV dysfunction. This is in close agreement with reports suggesting that Nox enzymes can feed-forward activate other Nox proteins and oxidases via ROS production (4, 15, 19). The factors and mechanisms mediating this feed-forward chain of Nox activation leading to RV dysfunction require deeper investigation and are currently part of a much larger study.

Our data demonstrate that mitochondrial protein expression of Nox4 was increased approximately twofold 6 h post-PAB compared with sham-operated mice. To date, Nox4 has been localized to multiple subcellular organelles, and in cardiomyocytes, it appears to be expressed primarily in the mitochondria (9, 24), although this remains controversial (33). Our data showing the presence of Nox4 in isolated mitochondria appear to go some way to solve this controversy. The mitochondria are an important source of ROS in any cell, but in cardiomyocytes evidence suggests that they are the major source of ROS involved in heart failure (2, 4, 24). Thus the upregulation of Nox4 we observe herein in the mitochondria is likely to play a key role in the dysfunction following PAB. Indeed, previous studies demonstrate that Nox4 mediates mitochondrial and cardiac dysfunction in a model of LV pressure overload (24). It is also supported by data in other cells in which mitochondrial Nox4 is a major source of oxidative stress (23). Nox4 has also been found in the endoplasmic reticulum (ER) in vascular smooth muscle endothelium (5, 14) and in focal adhesions, plasma membrane and nucleus in different cell types (6, 22, 27). Mitochondrial preparations used for this set of studies did not contain ER-specific protein disulfide isomerase activity (data not shown). Thus the possibility of contamination of mitochondrial preparations by ER membranes (and thus ER-localized Nox4) was ruled out. Taken together, our data suggest the potential for a feed-forward mechanism in which an increase in mitochondrial Nox4-derived ROS leads to mitochondrial dysfunction and subsequent electron leak from mitochondria.

Fig. 10. RV hypertrophy is attenuated in p47phox null mice after PAB. Fulton Index [RV wet weight/LV + septum (S) wet weight] was determined in hearts from sham or 3-wk PAB wild-type mice (WT) vs. 3-wk PAB p47phox nulls. n = 4–6 animals. *P < 0.005 indicates significance between PAB in wild type mice; ***P < 0.005 indicates significance between PAB in wild-type (WT) and p47phox null mice.
the mitochondrial respiratory chain. This would undoubtedly result in further ROS production, exacerbating cardiomyocyte dysfunction and ultimately RV failure.

Finally, we assessed the potential regulation of ROS scavenging enzymes SOD, catalase, and GPx in response to pressure overload in this model to determine whether they could be playing a role in an elevated ROS steady state. Our data reveal that there is no difference in SOD, catalase, or GPx protein expression in RV between PAB- and sham-operated animals at any of the time points in this study. We did observe, however, a twofold increase in catalase activity at 6 h of PAB, which may explain the observation that H$_2$O$_2$ levels reached after 3 h of PAB are similar to those at 6 h. That is, the increase in catalase activity at 6 h PAB may suggest a steady state between H$_2$O$_2$ production and H$_2$O$_2$ scavenging at this time point. Our findings with respect to SOD expression are in contrast with studies demonstrating an amplification of left heart dysfunction in response to pressure overload in mice deficient in extracellular SOD (32). Consistent with our findings, however, other studies showed a failure to activate ROS, scavenging enzymes in response to pressure overload in the RV (16). These discrepancies in the literature and from our study are likely to be the result of differential responses in antioxidant activation mechanisms between the RV and LV.

Limitations of the study. A number of limitations in the current study warrant discussion. First, pharmacological inhibitors under some conditions display off-target effects and thus remain limited in their ability to identify the enzymatic source of ROS in tissues. This limitation was addressed by the use of a spectrum of ROS source inhibitors and a process of elimination to implicate a Nox source of PAB-induced ROS in the RV. Second, specificity of Nox antibodies remains a challenge for the field. To address this issue, we tested the specificity of the Nox antibodies in heterologous COS-7 cell systems expressing one particular Nox isoform at a time (data not shown) and confirmed that the immunoblot bands being detected were indeed Nox isoform specific. Our findings using Western blot were also supported by mRNA expression profiles using quantitative PCR. Third, our current study investigated ROS production largely in an acute model of pressure overload, thus the result of differential responses in antioxidant activation mechanisms between the RV and LV.

Conclusion

This study sheds important light on the early cellular mechanisms of pressure overload injury in the RV. Our data suggest that mitochondrial Nox4-derived ROS are involved in propagating cardiomyocyte phenotypic changes in PAH that ultimately lead to RV failure. The use of the PAB model afforded us the opportunity to separate RV responses from changes that occur in the pulmonary circulation and thus focus on the cardiomyocyte. There are only a handful of studies investigating the RV in pressure overload and ROS production, and no studies are available on the role of Nox enzymes in this model. Thus the current findings identify an important gap in knowledge and spotlight a novel role for Nox4 in RV dysfunction. Future studies are planned interrogating the mechanisms by which PAB leads, respectively, to Nox4- and Nox2-mediated hypertrophy and RV dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


