NFAT is required for spontaneous pulmonary hypertension in superoxide dismutase 1 knockout mice

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Submitted 10 December 2012; accepted in final form 4 March 2013


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ROS include, but are not limited to, superoxide anion and hydrogen peroxide. Increases in lung superoxide levels have been demonstrated in several animal models of PH, including chronic hypoxia (CH) (11, 71), monocrotaline (74), and the transgenic Ren2 rat, which overexpresses the mouse renin gene (30). Additionally, our group has demonstrated that increased superoxide levels are implicated in CH-induced pulmonary vasoconstriction in rats (11, 49, 71). However, conflicting findings are reported regarding the levels of hydrogen peroxide in PH. Several reports suggest that elevated pulmonary arterial hydrogen peroxide levels contribute to the development of PH (38, 77, 98). On the contrary, a reduction in hydrogen peroxide has been implicated in hypoxic pulmonary vasoconstriction (67, 99), CH (84), experimental pulmonary arterial hypertension (PAH) (65), and pulmonary arterial hypertensive Fawn-Hooded rats (3, 88).

We have demonstrated that the Ca2+-regulated transcription factor nuclear factor of activated T cells isoform c3 (NFATc3) is required for CH-induced PH in mice (6, 28). NFATc3 activation leads to an immediate proliferative response followed by recovery of the contractile phenotype and hypertension of pulmonary arterial smooth muscle cells (PASMC) (6, 26, 28). Superoxide and hydrogen peroxide have opposite effects on crystalline silica-induced NFAT activation in epidermal cells (53). However, the molecular mechanism that mediates NFAT activation under those conditions is unknown. Increased superoxide and decreased hydrogen peroxide resulting from decreased antioxidant capacity and increased superoxide generation may also occur in several animal models of PH (15, 23, 29–31, 35, 37, 47, 57, 72, 73, 96) as well as in human PH (10, 62, 63); however, this superoxide/hydrogen peroxide imbalance is not well established.

Superoxide dismutases are the major antioxidant defense systems against superoxide. There are three superoxide dismutase (SOD) isoforms expressed in the vasculature (1): CuZnSOD (SOD1 and SOD3) and MnSOD (SOD2), with SOD3 being extracellular and SOD1 being the predominant cytosolic isoform (43, 52, 55). SOD2 deficiency initiates and sustains a proliferative, apoptosis-resistant PASMC (3). However, the role of NFAT in this phenotype has not been explored. Dennis et al. (31) reported that the NADPH oxidase homolog family member 1 (NOX1) is increased, and expression and activity of SOD1 are diminished in pulmonary arteries of piglets exposed to 3 and 10 days of CH. This is associated with increased superoxide and decreased hydrogen peroxide. In addition, SOD1 expression and activity are decreased in rats with monocrotaline-induced PH, which is associated with increased markers of oxidative stress and decreased total antioxidant capacity despite enhanced SOD2 expression (103). Loss of function of SOD3 exacerbated monocrotaline-induced oxidative stress and PAH (103). Despite the availability of SOD1 knockout (KO) mice, no studies have examined whether these mice develop PH spontaneously or are more sensitive to...
hypoxia, as previously observed in SOD3 KO mice and SOD3 loss-of-function rats (103).

The goal of this study was to determine the role that an imbalance in superoxide/hydrogen peroxide plays in NFATc3 activation in PASMC and in PH pathogenesis. On the basis of findings that ROS mediate NFAT activation in T cells (51), mouse epidermal (53), human lung bronchoepithelial (53), and cardiac cells (42), we hypothesized that SOD1 KO mice develop NFAT-dependent PH due to an imbalance of superoxide/hydrogen peroxide. Consistent with our hypothesis, we found that SOD1 KO mice display elevated pulmonary arterial superoxide and decreased hydrogen peroxide levels, increased PASMC NFATc3 activity and have signs of PH, which were all prevented by selective NFAT inhibition.

MATERIALS AND METHODS

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (Albuquerque, NM). Animals. All experiments used male and female SOD1 KO and wild-type (WT) mice that were backcrossed with NFAT-luciferase reporter (NFAT-luc) mice (at least 8 generations). SOD1 KO mice were obtained from Jackson Laboratory (Sod1 tm1Leb/J, no. 002972). NFAT-luc mice were provided by Dr. Jeffery D. Molkentin (Department of Pediatrics, Children’s Health Medical Center, Cincinnati, OH). Heterozygous SOD1/NFAT-luciferase crossed mice were bred to obtain age-matched WT and KO mice. All animals were maintained on a 12-h:12-h light/dark cycle. SOD1 KO mice develop normally up to 6 mo of age when they show signs of motor axonopathy (83). No compensatory upregulation of SOD2 and/or SOD3 in the brain and kidney was found in this mouse model (13, 83). Ho et al. (46) reported that Cu/Zn-SOD (SOD1 and SOD3) activity was absent in brain and liver of SOD1 KO and significantly reduced in SOD1+/− mice. However, a very low level of Cu/Zn-SOD activity was present in lung. This activity presumably represents the activity of SOD3, as expression of this SOD isoform is relatively high in the lungs compared with other tissues. SOD1 KO mice had no differences in the activity of other cellular antioxidant enzymes such as SOD2, catalase, glutathione peroxidase, and the enzymes that participate in the recycling of oxidized glutathione including glutathione reductase and glucose-6-phosphate dehydrogenase in brain, liver, and lung compared with WT and SOD1+/− mice (46).

Animal treatments. Animals were treated with vehicle [drinking water or 1:2,000 i.p. dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO)], tempol [SOD mimetic (19, 30, 35, 79, 95) in drinking water (1 mmol/l, 20 mg/kg per day; 3 wk)], or A-285222 (0.16 mg/kg/day in MO), tempol [SOD mimetic (19, 30, 35, 79, 95) in drinking water (1 mmol/l, 20 mg/kg per day; 3 wk)], or A-285222 [NFAT selective inhibitor (7, 70, 94)] was kindly provided by Abbott Laboratories (Abbott Park, IL). Heterozygous SOD1/NFAT-luciferase crossed mice were bred to obtain age-matched WT and KO mice. All animals were maintained on a 12-h:12-h light/dark cycle. SOD1 KO mice develop normally up to 6 mo of age when they show signs of motor axonopathy (83). No compensatory upregulation of SOD2 and/or SOD3 in the brain and kidney was found in this mouse model (13, 83). Ho et al. (46) reported that Cu/Zn-SOD (SOD1 and SOD3) activity was absent in brain and liver of SOD1 KO and significantly reduced in SOD1+/− mice. However, a very low level of Cu/Zn-SOD activity was present in lung. This activity presumably represents the activity of SOD3, as expression of this SOD isoform is relatively high in the lungs compared with other tissues. SOD1 KO mice had no differences in the activity of other cellular antioxidant enzymes such as SOD2, catalase, glutathione peroxidase, and the enzymes that participate in the recycling of oxidized glutathione including glutathione reductase and glucose-6-phosphate dehydrogenase in brain, liver, and lung compared with WT and SOD1+/− mice (46).

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Chronic hypoxia exposure. Animals designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 Torr for 5 or 21 days. Control animals were housed at ambient barometric pressure (normoxia, ~630 Torr). All animals were maintained on a 12-h:12-h light/dark cycle.

In vivo assessment of right ventricular systolic pressure and right ventricular hypertrophy. Adult mice were anesthetized with 2% halothane (balance O2). A 23-gauge needle attached to a pressure transducer was inserted into the abdomen below the xiphoid process and directed into the thoracic cavity toward the right ventricle (RV) of the heart (64). Entry into the RV was confirmed by monitoring the pressure waveform. Peak RV systolic pressures (RVSP) and heart rate (HR) were obtained using Windaq data acquisition software (Dataga Instruments, Akron, OH).

After we collected hemodynamic data, the heart was isolated, and the atria and major vessels were removed. The RV was dissected from the left ventricle (LV) and septum (S). The degree of RV hypertrophy was expressed as the percentage ratio of RV to LV+S weight and RV to body weight (BW).

Mean arterial blood pressure measurement. Telemeter catheters were implanted as previously described (24). Mice were given buprenex (0.05 mg/kg sc) 20 min before surgery and anesthetized using isoflurane (2%, balance O2). The catheter tip was inserted and secured in the carotid artery, and the transmitter body (PA-C20; Data Sciences International, New Brighton, MN) was secured subcutaneously above the right flank. Warmed sterile 0.9% NaCl solution (0.5 ml sc) was given after surgery, and mice recovered 5–7 days before recording was started. Mean arterial pressure (MAP), HR and activity were recorded daily for up to 4 days.

Vascular morphometry. Animals were anesthetized with 2% isoflurane (balance O2) and perfused via the right ventricle with ~5 ml of modified physiological saline solution (HEPES-PSS, 134 mM NaCl, 6 mM KCl, 1 mM MgCl2, 10 mM HEPES, 2 mM CaCl2, 0.026 mM EDTA, and 10 mM glucose) containing heparin, 4% albumin (Sigma) and 10−4 M papaverine (Sigma), at 20 mmHg to maximally dilate and flush the circulation of blood. Mice were then perfused with 4% paraformaldehyde (Polyscience, Warrington, PA) in PBS at the same pressure. Following fixation, the lungs were inflated with fixative via the trachea to maximal capacity. The tissue was then dehydrated in increasing concentrations of ethanol, with a final dehydration in xylene, and then embedded in paraffin. Lung sections (5 μm) were stained with rabbit anti-smooth muscle α-actin (Abcam, Cambridge, MA) antibody followed by immunohistochemistry detection with anti-rabbit secondary antibody labeled with horseradish peroxidase and contained with hematoxylin. Vessels were examined with a ×40 objective with an Eclipse E400 scope, and images were acquired with DS-Fi1 camera using NIS-Elements F 3.0 software. Images were analyzed with Image J (NIH, Bethesda, MD). Vessels sectioned at oblique angles were excluded from analysis. Medial wall was calculated and compared between groups using the following equation: external area – luminal area. The analysis was performed in arteries from three different diameter ranges <50, 50–100, and 100–300 μm. Approximately 10 arteries/animal were analyzed.

Wall thickness was determined as outer – inner diameter (μm)) in cannulated pressurized intrapulmonary arteries maximally dilated with Ca2+-free PSS [plus 10 μM tetrodotoxin (Sigma), 2 μM cytochalasin B (Sigma), and 1 μM Myosin light chain kinase inhibitor peptide (EMD Millipore, Billerica, MA)]. These arteries had an outer diameter that ranged from 120–150 μm.

Superoxide levels. Lung cryostat sections were stained with the superoxide-sensitive fluorescent dye dihydroethidium (DHE) as previously described (20, 95, 104). Specific DHE fluorescence intensity was analyzed in the arterial wall using Image J software. Fluorescence intensity of four arteries per lung section per animal was averaged.

Hydrogen peroxide levels. Hydrogen peroxide production was determined by the Amplex Red/Peroxidase Assay (Life Technologies, Gaithersburg, MD) (3) in one branch of intrapulmonary arteries (second and third order arteries, ~4 mm in length), RV (1–2 mg), and LV (1–2 mg) isolated from WT and SOD1 KO mice incubated in HEPES-PSS for 30 min. The hydrogen peroxide concentration was calculated from a standard curve and normalized by total protein measured by Bradford Assay (Bio-Rad, Hercules, CA) and reported as the difference from the fluorescence signal obtained in tissues preincubated with polyethylene glycol (PEG)-catalase (250 U/ml) for 1 h. PEG-catalase was used to demonstrate the proportion of the signal that corresponds to hydrogen peroxide because it has been shown that one-electron oxidants such as radicals derived from ONOO− are able to cause oxidation of Amplex Red to resorufin, although at a considerably lower yield compared with peroxide-mediated oxidation (105).

Luciferase activity. Luciferase activity was measured in isolated intrapulmonary arteries, RV, and LV using a Luciferase Assay System kit (Promega, Madison, WI), and light detected with a luminometer (TD20/
RESULTS

Superoxide levels are elevated, and hydrogen peroxide production is decreased in SOD1 KO mice. Figure 1, A and B, shows elevated DHE fluorescence intensity in pulmonary arteries of lung sections from SOD1 KO compared with WT mice. DHE fluorescence was reduced by incubation with PEG covalently linked to SOD (120 U/ml). Figure 1C shows that hydrogen peroxide production over a 30-min period was significantly lower in isolated pulmonary arteries from SOD1 KO vs. WT mice under normoxia. CH exposure (5 days) also significantly decreased hydrogen peroxide levels in pulmonary arteries from WT mice but did not further reduce the levels present in arteries from SOD1 KO mice (Fig. 1C). These results suggest that CH is as effective as SOD1 knockout in decreasing hydrogen peroxide production in pulmonary arteries.

No significant difference in hydrogen peroxide production was found in RV and LV between genotypes (ΔH2O2 nmol/µg protein from PEG-catalase RV: WT = 0.0006 ± 0.0002 vs. KO = 0.0010 ± 0.0002, n = 6. LV: WT = 0.0015 ± 0.0004 vs. KO = 0.0005 ± 0.0001, n = 5).

SOD1 KO mice display signs of PH that are not exacerbated by CH exposure. RVSP was significantly elevated in SOD1 KO compared with WT mice under normoxia (Fig. 2A). However, SOD1 KO mice showed no significant RV hypertrophy (Fig. 2, B and C, and Table 1). No significant sex-related effect was found. After exposure to CH, only WT mice responded with a significant increase in RVSP (Fig. 2A). Both genotypes display RV hypertrophy after exposure to CH (Fig. 2, B and C, and Table 1). RV/LV+S was significantly higher in SOD1 KO exposed to CH compared with WT mice but was to the expense of decreased LV+S weight (Fig. 2, B and D, Table 1). LV/BW was not different between WT and SOD1 KO mice under normoxia (Fig. 2D and Table 1). Consistent with a lack of exacerbated CH-induced RV hypertrophy, RVSP was not different between normoxia- and CH-exposed SOD1 KO mice (Fig. 2A). Also, RVSP of CH-exposed WT was not different from normoxia-exposed SOD1 KO mice (Fig. 2A), suggesting that CH is as effective as knocking out SOD1 to increase RVSP. Both genotypes of mice display the same degree of polycythemia (Fig. 2F). No differences in HR were observed between groups (Fig. 2C).

Additionally, we found no differences in MAP (WT = 98 ± 1 vs. SOD1 KO = 98 ± 1 mmHg, n = 3) between genotypes under normoxia as previously reported (13). Total ventricle weights normalized by BW were not different between genotypes under normoxia (Table 1), contrary to what was previously reported (13). These results suggest that the elevated RVSP in the SOD1 KO mice is not a consequence of LV dysfunction.

To elucidate the reason for the lack of significant RV hypertrophy in ~11- wk-old SOD1 KO mice under normoxia despite elevated RVSP, we determined RV/LV+S in 5-mo-old WT and SOD1 KO mice. The rationale for this study was that PH might have not been present for a sufficient duration to induce RV hypertrophy at 11 wk of age. However, we found no significant difference in RV/LV+S between WT (34.2 ± 1.2%, n = 5) and SOD1 KO mice (35.4 ± 0.7%, n = 7) at 5
mo of age. We also found no differences in BW, RV/BW, LV/BW, or total ventricular weight/BW between genotypes (Table 1).

**NFATc3 is activated in PASMC of SOD1 KO mice.** We have previously demonstrated that CH induces NFATc3 activation in mouse pulmonary arteries (28). To determine whether NFAT is activated in SOD1 KO compared with WT mice, luciferase activity was measured in isolated intrapulmonary arteries. Luciferase activity was significantly increased in pulmonary arteries from SOD1 KO under normoxic conditions (Fig. 3A) compared with WT mice. In this study, CH (21 days) again induced NFAT activation in pulmonary arteries from WT mice (Fig. 3A). However, consistent with the similar RVSP between normoxic and CH SOD1 KO mice (Fig. 2A), CH did not cause a further increase in NFAT activity in pulmonary arteries from SOD1 KO mice (Fig. 3A).

It has been previously demonstrated that NFAT plays a major role in pathological cardiac hypertrophy (68, 100–102). Therefore, NFAT activity was measured in RV of WT and SOD1 KO ~11-wk-old mice exposed to normoxia or CH. Contrary to our findings in pulmonary arteries, RV NFAT activity was not different between genotypes under normoxia but significantly increased upon exposure to 21 days of CH (Fig. 3B). This is consistent with the significant increase in RV/BW in response to CH in SOD1 KO mice (Fig. 2C). These findings suggest that increased RV afterload might not be sufficient to activate NFAT-mediated RV hypertrophy under normoxia in SOD1 KO mice.

NFAT activity was also measured in LV of normoxic WT and SOD1 KO mice, and no significant difference in luciferase activity was found between genotypes (NFAT activity: WT = 1.96 ± 0.21 vs. KO = 2.57 ± 0.59 relative light unit/µg protein, n = 4).

One of the biomarkers of PH is the elevated expression of ET-1 (2, 86). We have demonstrated that CH-induced NFATc3 activation in pulmonary arteries requires ET-1 upregulation and activation of endothelin A receptors (27). In addition, it has been shown that superoxide increases ET-1 levels in arteries and plasma (50, 56, 95). Therefore, we measured lung, RV, and pulmonary arterial pre-pro ET-1 transcripts and ET-1 plasma levels in normoxic WT and SOD1 KO mice. Contrary to our expectations, pre-pro ET mRNA levels (Fig. 3C) and ET-1 plasma (Fig. 3D) were not significantly different between genotypes.

As mentioned above, the NFATc3 isoform has been implicated in the development of chronic hypoxic pulmonary hypertension in mice (6, 26–28). The contribution of other NFAT isoforms was ruled out in that study by crossing NFAT-luciferase reporter mice with NFATc3 KO mice and demonstrating no significant increase in luciferase activity in pulmonary arteries after these mice were exposed to CH (28). However, NFATc2 has been implicated in PAH. For example, it has been shown that NFATc2 nuclear localization is increased in PASMC from patients with PAH and from rats with monocrotaline-induced PH (9). In that study, NFATc3 nuclear...
localization was also found to be increased but in a lower proportion of cells. Therefore, to determine which isoform is activated in PASMC of SOD1 KO mice, NFATc2 and NFATc3 nuclear localization was assessed by immunofluorescence confocal microscopy. We found that NFATc3 but not NFATc2 nuclear localization is increased in PASMC of SOD1 KO mice (Fig. 4, A and B). Because NFATc2 is regulated not only by its subcellular localization but also by the transcription factor signal transducers and activators of transcription protein (STAT3), NFATc2 and NFATc3 transcript levels were determined in isolated intrapulmonary arteries. We found no differences in NFATc2 mRNA levels. However, a small but significant increase in NFATc3 mRNA in arteries from SOD1 KO was found compared with WT mice (2^−ΔΔCT; NFATc2 = 0.99 ± 0.07 WT vs. 0.98 ± 0.03 KO, n = 7; NFATc3 = 1.14 ± 0.09 WT vs. 1.51 ± 0.09 KO, P < 0.01, n = 7). We also determined NFATc1, c2, c3, c4, and NFAT5 transcript levels in lung from WT and SOD1 KO mice. We found no significant differences in any of the isoforms between genotypes (2^−ΔΔCT). NFATc1 = 0.85 ± 0.04 WT vs. 1.09 ± 0.19 KO, n = 5; NFATc2 = 0.75 ± 0.07 WT vs. 1.10 ± 0.23 KO, n = 5; NFATc3 = 0.91 ± 0.04 WT vs. 0.85 ± 0.10 KO, n = 5; NFATc4 = 0.89 ± 0.08 WT vs. 0.88 ± 0.19 KO, n = 5; and NFAT5 = 0.89 ± 0.06 WT vs. 1.14 ± 0.27 KO, n = 5).

**NFAT is required for the development of PH in SOD1 KO mice.** Because NFATc3 activity is increased in PASMC of SOD1 KO mice, we determined whether administration of the selective NFAT inhibitor A-285222 (33, 94) prevents the development of PH in these mice. Indeed, NFAT activation was prevented by A-285222 administration (Fig. 5A). More importantly, administration of A-285222 significantly prevented the increase in RVSP in SOD1 KO mice (Fig. 5B).

**Table 1. Heart and body weights in normoxic and CH WT and young and older SOD1 KO mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Exposure</th>
<th>BW, g</th>
<th>RV, g</th>
<th>LV+S, g</th>
<th>TV/BW, g/kg</th>
<th>n (mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1 WT</td>
<td>Normoxia</td>
<td>28.4 ± 2.0</td>
<td>0.026 ± 0.001</td>
<td>0.092 ± 0.004</td>
<td>3.29 ± 0.08</td>
<td>13</td>
</tr>
<tr>
<td>SOD1 KO</td>
<td>CH</td>
<td>26.7 ± 1.3</td>
<td>0.034 ± 0.003*</td>
<td>0.094 ± 0.005</td>
<td>4.85 ± 0.23*</td>
<td>6</td>
</tr>
<tr>
<td>SOD1 KO</td>
<td>Normoxia</td>
<td>26.6 ± 2.0</td>
<td>0.026 ± 0.001</td>
<td>0.082 ± 0.004</td>
<td>3.09 ± 0.09</td>
<td>14</td>
</tr>
<tr>
<td>SOD1 KO</td>
<td>CH</td>
<td>26.7 ± 1.8</td>
<td>0.033 ± 0.004*</td>
<td>0.081 ± 0.007*</td>
<td>4.27 ± 0.18†</td>
<td>6</td>
</tr>
<tr>
<td>SOD1 WT 5 mo</td>
<td>Normoxia</td>
<td>30.6 ± 2.9</td>
<td>0.92 ± 0.05</td>
<td>2.67 ± 0.08</td>
<td>3.59 ± 0.13</td>
<td>5</td>
</tr>
<tr>
<td>SOD1 KO 5 mo</td>
<td>Normoxia</td>
<td>28.1 ± 0.8</td>
<td>0.92 ± 0.01</td>
<td>2.61 ± 0.04</td>
<td>3.54 ± 0.05</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are means ± SE. In the bottom 2 rows, right ventricle (RV) and left ventricle + septum weight (LV+S) values are per body weight (BW) and are in g/kg. CH, chronic hypoxia; WT, wild-type; SOD1, superoxide dismutase 1; KO, knockout; TV, total ventricular weight. *P < 0.05 vs. normoxia. †P < 0.05 vs. WT.

Fig. 2. SOD1 KO mice have signs of pulmonary hypertension (PH), which are not exacerbated by CH. A: right ventricular systolic pressure (RVSP) (n = 6–7). B: % RV/left ventricle and septum (LV+S) weight (n = 13–14 for normoxia, n = 6 for CH). C: RV/body weight (BW) (n = 13–14 for normoxia, n = 6 for CH). D: LV+S/BW (n = 13–14 for normoxia, n = 6 for CH). E: heart rate (HR) (n = 6–7). F: hematocrit (HCT) % (n = 6–7) of WT and SOD1 KO mice exposed to normoxia and 21 days of CH. *P < 0.05 vs. normoxia, #P < 0.05 vs. WT.
Additional hallmarks of PH are pulmonary arterial remodeling and increased vasoreactivity. Medial area and arterial wall thickness were determined in both SOD1 KO and WT mice by two different approaches (see MATERIALS AND METHODS). SOD1 KO mice displayed elevated pulmonary arterial wall thickness (Fig. 5C; 120–150 μm outer diameter) and media area (Fig. 5, D and E; <50 μm outer diameter) compared with WT mice. The arterial wall Ca$^{2+}$ (Fig. 6A) and associated constric- tor response to ET-1 (10$^{-7}$ M) were significantly greater in small pulmonary arteries from SOD1 KO vs. WT mice at 35 mmHg intraluminal pressure. Administration of A-285222 significantly prevented the increased arterial wall thickness, Ca$^{2+}$, and associated constric- tor response to ET-1 in SOD1 KO mice (Fig. 6, A and B). A-285222 had no effect on RVSP and NFAT activity in WT mice (Fig. 5, A and B); therefore, effects of this drug were not subsequently tested for the other parameters in WT mice.

Normalization of superoxide/hydrogen peroxide imbalance with an SOD mimetic prevents NFATc3 activation and PH in SOD1 KO mice. Administration of the SOD mimetic, tempol (19, 30, 35, 79, 95), to WT and SOD1 KO mice for 3 wk prevented the increased NFAT activity (Fig. 7A), NFATc3 nuclear localization (Fig. 7B), RVSP (Fig. 7C), and the elevated superoxide (Fig. 7D) in SOD1 KO mice.

DISCUSSION

Our study generated major new findings related to the role of SOD1 and NFAT activity in the genesis of PH. The major new finding of this study is that PH developed in ~11-wk-old SOD1 KO mice under normoxia. This response was not affected by exposure to CH. Second, SOD deficiency and CH caused a decrease in pulmonary arterial hydrogen peroxide levels. Third, SOD deficiency caused NFAT activation in pulmonary arteries and NFATc3 but not NFATc2 nuclear accumulation in PASMC, responses that were prevented by administration of the SOD mimetic, tempol. Finally, both NFAT inhibition and tempol prevented indices of PH present in SOD1 KO mice, demonstrating that NFAT contributes to the genesis of PH in this mouse model, which is associated with an imbalance in superoxide/hydrogen peroxide levels.

A reduction in SOD activity has been implicated in patients with PAH and animal models of PH (10, 15, 18, 23, 29–31, 35, 47, 57, 72, 73, 96). Mice deficient in SOD3 and rats that carry a loss-of-function mutation in SOD3 display exacerbated CH-induced PH (103). Furthermore, fawn-hooded rats, which exhibit decreased expression and activity of mitochondrial SOD2, develop spontaneous PAH (3, 88). SOD1 activity and expression are also diminished in pulmonary arteries of piglets exposed to CH (31). This decrease is accompanied by increased pulmonary arterial superoxide, increased NOX1, and decreased hydrogen peroxide. The authors concluded that ROS derived from NOX1, as well as from decreased SOD1, contribute to aberrant pulmonary resistance artery responses in newborn piglets with CH-induced pulmonary hypertension. In addition, SOD1 expression and activity are decreased in rats with monocrotaline-induced PH, a response associated with...
increased markers of oxidative stress and decreased antioxidant capacity, despite increased SOD2 expression (103). However, whether PH develops in response to SOD1 deficiency has not previously been studied. Our present data support the existence of PH in SOD1 KO mice, as demonstrated by elevated RVSP, pulmonary arterial remodeling, and increased vasoreactivity to ET-1 compared with WT mice.

It has been previously demonstrated that SOD1 KO mice have elevated superoxide levels determined by DHE staining in cerebral arterial wall (4), carotid arteries (32), and renal afferent arterioles (13). Superoxide is also increased in the aorta of these mice, as determined by the lucigenin assay (32). Consistent with these reported elevations in vascular superoxide, we found higher DHE intensity staining in the pulmonary arterial wall of SOD1 KO compared with WT mice. Despite the elevated superoxide levels and decreased SOD activity reported in these mice, there are no studies addressing whether hydrogen peroxide levels and/or production are decreased in this mouse model. In this study, we found that pulmonary arteries from SOD1 KO mice produce significantly less hydrogen peroxide than arteries from WT mice. We also found that CH is as effective as knocking out SOD1 in reducing hydrogen peroxide production in pulmonary arteries. This observation is consistent with the reported reduction in pulmonary arterial hydrogen peroxide levels in fawn-hooded rats, in which expression and activity of mitochondrial SOD2 are decreased (3). It is also consistent with the decreased pulmonary arterial hydrogen peroxide levels found in pulmonary arteries from CH-exposed piglets (31) in which SOD1 expression is decreased.

In this study, we also report the novel finding that increased superoxide and decreased hydrogen peroxide is associated with
NFAT activation in PASMC of SOD1 KO mice. We (6, 26–28, 87) and others (8, 9, 22, 76, 92, 93) have previously demonstrated a role for NFAT in different PH animal models and in patients with PAH. Accordingly, the administration of a selective NFAT inhibitor (A-285222) to SOD1 KO mice completely prevented the increase in RVSP. In addition, this inhibitor significantly decreased the elevated pulmonary arterial wall thickness, NFAT activity, and vasoreactivity to ET-1 present in vehicle-treated SOD1 KO mice. A-285222 inhibits NFAT without affecting calcineurin activity and has no out-of-target side effects in either primates (7, 94) or rodents (70). These findings demonstrate that NFAT activation drives the development of PH in SOD1 KO mice. NFATc3 nuclear localization but not NFATc2 is increased in PASMC of SOD1 KO mice, strongly suggesting that the NFATc3 isoform is involved in the development of PH in these mice.

A causal association between the increased superoxide/hydrogen peroxide ratio and NFAT activation that we found in SOD1 KO mice is strongly supported by the fact that the SOD mimetic (19, 30, 35, 95), tempol, prevented the elevated NFAT activity, PASMC NFATc3 nuclear localization, and RVSP present in SOD1 KO mice. This association is also in agreement with a reported role for superoxide as an activator and hydrogen peroxide as an inhibitor of NFAT in epidermal cells (53) and also with reports showing that hydrogen peroxide inhibits both NFAT DNA binding (5, 40, 44, 75) and calcineurin activity (14, 36, 58, 60). NFAT activation requires Ca$^{2+}$/H$^{+}$-dependent calcineurin-mediated dephosphorylation (reviewed in Ref. 81). Hydrogen peroxide-induced calcineurin inhibition involves multiple mechanisms. It has been demonstrated that hydrogen peroxide triggers proteolytic cleavage and inactiva-
tion of calcineurin in T cells (58). Also in T cells, hydrogen peroxide can directly oxidize the calmodulin-binding domain of calcineurin, thereby inactivating the enzyme (14). Finally, in neurons, Down Syndrome Candidate Region 1 (DSCR1, Adapt78, or RCAN1) protein inactivates calcineurin in response to elevations in hydrogen peroxide or large and acute increases in intracellular Ca\(^{2+}\) (36, 60). DSCR1 also inhibits the calcineurin/NFAT pathway in systemic vascular smooth muscle cells (59); however, its expression in PASMC has not yet been reported. In contrast, others have shown that NFAT nuclear import is enhanced by hydrogen peroxide-induced increases in intracellular Ca\(^{2+}\) in a leukemia cell line, neuroblastoma cells, and cardiac myocytes (41, 54, 97). Beside Ca\(^{2+}\) and calcineurin, additional mechanisms are implicated in the regulation of NFAT activity. We have recently demonstrated that RhoA/Rho kinase-dependent actin cytoskeleton remodeling is required for ET-1-mediated NFATc3 activation in PASMC from chronically hypoxic PH mice (27). Both ET-1 (12, 17) and CH (10, 82) decrease SOD activity, and ROS regulate RhoA/Rho kinase (11, 49) activity and actin cytoskeleton polymerization (66). In the present study, we found that plasma ET-1 and lung RV and pulmonary arterial pre-pro ET-1 transcript levels were not different between genotypes, suggesting that ET-1 might not be involved in the elevated NFAT activity and RVSP observed in SOD1 KO mice under normoxia. However, further studies are needed to determine whether the RhoA/Rho kinase/actin polymerization pathway participates in NFAT activation under conditions in which superoxide is increased and hydrogen peroxide is decreased.

On the other hand, Bonnet and colleagues (9, 21, 76) have demonstrated a distinct activation pathway for NFATc2 in PASMC from patients with PAH and animal models. Increased circulating levels of growth factors, agonists, and cytokines trigger activation of the transcription factor STAT3, resulting in enhanced NFATc2 transcription and increased Pim1 activation. Once activated, Pim1 triggers NFATc2 activation (nuclear translocation) (9, 21, 76). In our study, we did not find differences in lung and pulmonary arterial NFATc2 expression between WT and SOD1 KO, suggesting that this pathway might not be involved in PH resulting from SOD1 deficiency. However, this pathway could be implicated in the small but significant increase in pulmonary arterial NFATc3 transcripts found in SOD1 KO mice, but its contribution remains to be determined.

Although we measured an increase in RVSP, a very close approximation of pulmonary artery systolic pressure, in 11-wk-old SOD1 KO mice, we did not observe significant RV hypertrophy at this age. RV hypertrophy was also absent in 5-mo-old SOD1 KO mice, suggesting that the duration of elevated RVSP might not be a contributing factor for this phenotype. We did not study older mice because it has been reported that SOD1 KO mice develop motor axonopathy at about 6 mo of age (90). Interestingly, this is not the only animal model in which PH develops without a concomitant increase in RV weight. Nitric oxide synthase 3-deficient (NOS3 KO) mice (91) and young transgenic Ren2 rats (30) also display increased RVSP without RV hypertrophy. The reason for the dissociation between elevated RVSP and RV hypertrophy was not determined in the study of NOS3 KO mice (91). In the case of Ren2 rats, aging might be a contributing factor because RV hypertrophy is not evident in young rats but develops in 13–14-wk-old rats (78). Nonetheless, it is intriguing that increased RVSP and pulmonary vascular remodeling occur in the absence of RV hypertrophy in Ren2 rats, NOS2 KO, and SOD1 KO mice. This is in contrast with models of CH- and monocrotaline-induced PH, in which RV hypertrophy parallels the rise in pulmonary arterial pressure. Interestingly, in these two models, ET-1 levels are elevated in pulmonary arteries and/or RV (27, 48, 80), whereas ET-1 levels are not different between WT and SOD1 KO mice under normoxia. Therefore, our study suggests that increased RV afterload might not be sufficient to induce RV hypertrophy, and activation of the ET-1 system might also be required for
the hypertrophic response. This possibility is supported by the increased NFAT activity in the RV in response to CH in both genotypes but absence of NFAT activation under normoxia. It is also consistent with the important role that NFAT plays in pathological cardiac hypertrophy (68, 100–102). This possibility is further supported by the fact that LV hypertrophy is ET-1 dependent in both older (12–16 wk old) and younger Ren2 rats (6–9 wk old) on a high-salt diet but not in those younger rats on a normal-salt diet (27, 34, 85, 89). An alternative explanation is that a decrease in hydrogen peroxide might be required for NFAT activation in the RV. Because RV hydrogen peroxide levels are not different between genotypes then NFAT activation could not occur in response to increased RV afterload. The reason for a lack of decreased hydrogen peroxide in the RV of SOD1 KO mice remains to be determined. Further studies are also needed to identify the precise mechanism of the dissociation between elevated pulmonary pressure and RV hypertrophy in young Ren2 rats, NOS3 KO, and SOD1 KO mice.

In conclusion, our study demonstrates that NFATc3 is required for the development of PH in SOD1 KO mice, likely due to an elevation in pulmonary arterial superoxide and a reduction in hydrogen peroxide. The NFATc3-regulated genes that cause PH in this and other animal models in which ROS levels are altered remain to be determined.

ACKNOWLEDGMENTS

We thank Mary Walsh and Dr. Mary K. Walker (School of Pharmacy, University of New Mexico) for assisting on systemic blood pressure measurements by telemetry. Images in this paper were generated in the University of New Mexico & Cancer Center Fluorescence Microscopy Shared Resource, funded as detailed on http://hsc.unm.edu/crtc/microscopy/acknowledgement.shtml.

GRANTS

This work was supported by NIH Grants R01-HL-088151 (and a supplement from the American Recovery and Reinvestment Act of 2009 to R01-HL-088151), R01-HL-088192, T32-HL-007736, and a CTSC Pilot Award from the University of New Mexico Health Sciences Center.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


