Chronic hypoxia limits H2O2-induced inhibition of ASIC1-dependent store-operated calcium entry in pulmonary arterial smooth muscle

Danielle R. Plomaritas, Lindsay M. Herbert, Tracylyn R. Yellowhair, Thomas C. Resta, Laura V. Gonzalez Bosc, Benjimen R. Walker, and Nikki L. Jernigan

Vascular Physiology Group, Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

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Plomaritas DR, Herbert LM, Yellowhair TR, Resta TC, Gonzalez Bosc LV, Walker BR, Jernigan NL. Chronic hypoxia limits H2O2-induced inhibition of ASIC1-dependent store-operated calcium entry in pulmonary arterial smooth muscle. Am J Physiol Lung Cell Mol Physiol 307: L419–L430, 2014. First published July 3, 2014; doi:10.1152/ajplung.00095.2014.—Our laboratory shows that acid-sensing ion channel 1 (ASIC1) contributes to the development of hypoxic pulmonary hypertension by augmenting store-operated Ca2+ entry (SOCE) that is associated with enhanced agonist-induced vasoconstriction and arterial remodeling. However, this enhanced Ca2+ influx following chronic hypoxia (CH) is not dependent on an increased ASIC1 protein expression in pulmonary arterial smooth muscle cells (PASMC). It is well documented that hypoxic pulmonary hypertension is associated with changes in redox potential and reactive oxygen species homeostasis. ASIC1 is a redox-sensitive channel showing increased activity in response to reducing agents, representing an alternative mechanism of regulation. We hypothesize that the enhanced SOCE following CH results from removal of an inhibitory effect of hydrogen peroxide (H2O2) on ASIC1. We found that CH increased PASMC superoxide (O2·−) and decreased rat pulmonary arterial H2O2 levels. This decrease in H2O2 is a result of decreased Cu/Zn superoxide dismutase expression activity and as well as increased glutathione peroxidase (GPx) expression and activity following CH. Whereas H2O2 inhibited ASIC1-dependent SOCE in PASMC from control and CH animals, addition of catalase augmented ASIC1-mediated SOCE in PASMC from control rats but had no further effect in PASMC from CH rats. These data suggest that, under control conditions, H2O2 inhibits ASIC1-dependent SOCE. Furthermore, H2O2 levels are decreased following CH as a result of diminished dismutation of O2− and increased H2O2 catalysis through GPx-1, leading to augmented ASIC1-dependent SOCE.

superoxide dismutase; di-(4-carboxybenzyl) hyponitrite-1; glutathione peroxidase; catalase; pulmonary hypertension; degenerin/epithelial sodium channel

THE NONSELECTIVE CATION CHANNEL, acid-sensing ion channel 1a (ASIC1α), belongs to the degenerin/epithelial sodium channel family and is the major ASIC subtype with Ca2+ permeability (53, 59). Our laboratory has recently demonstrated that ASIC1 contributes to the development of hypoxic pulmonary hypertension (32). Following exposure to chronic hypoxia (CH), ASIC1-mediated store-operated Ca2+ entry (SOCE) is augmented in pulmonary arterial smooth muscle cells (PASMC), resulting in enhanced agonist-induced vasoconstriction, vascular smooth muscle remodeling, and right ventricular hypertrophy (23, 32). Despite the requirement of ASIC1 for enhanced Ca2+ influx, these responses are not dependent on an increased ASIC1 protein expression (32). Therefore, the goal of the present study was to examine alternative regulatory mechanisms of ASIC1 function.

Cellular redox status affects the function of various proteins, including ASIC1a. ASIC subunits are cysteine rich, and the cellular redox status can modify both extracellular and intracellular cysteine residues (11, 60). Reducing agents potentiate ASIC1 activity, acid-induced membrane depolarization, and Ca2+ influx. More specifically, reducing agents increase ASIC1 current amplitude and pH sensitivity while decreasing channel inactivation (2, 10, 11, 60). In addition, oxidizing agents like hydrogen peroxide (H2O2) may introduce inter-subunit disulfide bonds, thereby decreasing the amount of ASIC1a present on the cell surface and reducing acid-evoked currents (60). Together, these studies suggest that redox regulation of ASIC1 is an important factor in determining the overall physiological function of these channels. Furthermore, because cellular redox status can change dramatically under pathological states, this represents a potential mechanism by which ASIC1 contributes to the progression of many diseases (5, 12, 57).

It is well documented that hypoxic pulmonary hypertension is associated with alterations in the cellular redox environment. However, whether the generation of reactive oxygen species (ROS) is enhanced or diminished during hypoxia is still debated (34, 42, 54). Furthermore, the relative importance of changes in ROS levels vs. redox status is unclear. Numerous studies show that ROS, in particular superoxide (O2−), and subsequent oxidative stress contribute to hypoxic pulmonary hypertension (34). Indeed, our laboratory has consistently shown that pulmonary arterial O2− levels increase following exposure to CH (7, 24, 33). In contrast, CH has been shown to decrease H2O2 levels in several different species (6, 13, 40). Various models of pulmonary hypertension are associated with a dysregulation of superoxide dismutase (SOD), and possibly other antioxidant pathways, that could lead to lowering of H2O2 levels (4, 6, 13, 15, 40). However, neither the mechanisms by which CH mediates this decrease in H2O2 nor the importance of this response to regulation of pulmonary arterial smooth muscle calcium homeostasis are clearly defined. Consequently, the present study examined effects of CH on both O2− dismutation and H2O2 degradation pathways in pulmonary arterial smooth muscle. In addition, we evaluated the importance of H2O2 in regulation of ASIC1, testing the hypothesis that enhanced SOCE following CH results from removal of an inhibitory effect of H2O2 on ASIC1.
MATERIALS AND METHODS

Animals and Chronic Hypoxic Exposure

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM). Male Wistar rats (~12 wk old, Harlan Industries) designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 mmHg for 4 wk. Age-matched control rats were housed at ambient barometric pressure (~630 mmHg in Albuquerque, NM). We have previously shown that exposure of rats and mice to CH causes pulmonary hypertension, along with the associated right ventricular hypertrophy, arterial remodeling, and polycythemia (32, 44). Male and female SOD1<sup>−/−</sup> and SOD1<sup>+/+</sup> mice were obtained from Jackson Laboratory (Sod1<sup>tm1Leb/J</sup>, no.002972) and bred on a C56B6/J background. Previous reports from our laboratory indicate that SOD1<sup>−/−</sup> mice develop spontaneous pulmonary hypertension (40).

Isolation of Pulmonary Arteries and Generation of PASMC Culture

Rats were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were exposed by midline thoracotomy. The left lung was removed and immediately placed in physiological saline solution (PSS) [pH adjusted to 7.4 with NaOH containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO<sub>4</sub>, 4 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 1.18 KH<sub>2</sub>PO<sub>4</sub>, and 6 glucose]. Intrapulmonary arteries (~2nd-5th order) were dissected from surrounding lung parenchyma and either snap-frozen in liquid N<sub>2</sub> or utilized to generate short-term PASMC cultures. To isolate PASMCs, intrapulmonary arteries were enzymatically digested in reduced Ca<sup>2+</sup> HBSS containing papain (9.5 U/ml), type-I collagenase (1,750 U/ml), and dithiothreitol (1 mM) at 37°C for 30 min. The cell suspension was placed on 25-mm glass coverslips and cultured in Ham’s F-12 media supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin for 3~4 days in a humidified atmosphere of 5% CO<sub>2</sub>-95% air at 37°C.

Assessment of PASMC Superoxide Levels

To determine the effect of CH on O<sub>2</sub>·<sup>−</sup> levels, PASMCs from control and CH rats were incubated with dihydroethidium (DHE; 5 μM in 0.05% pluronic acid) and TO-PRO-3 (1:2,000, Molecular Probes) at 37°C for 15 min. Cells were subsequently fixed with 2% paraformaldehyde for 10 min at room temperature. To verify the specificity of DHE in this preparation to detect O<sub>2</sub>·<sup>−</sup>, experiments were repeated with preincubation of either polyethylene glycol (PEG)-SOD (50 U/ml), the SOD mimetic tiron (10 mM), or the catalase inhibitor 3-amino-1,2,4-triazole (AT; 5 mM) and glutathione peroxidase (GPx) inhibitor mercaptosuccinic acid (3 mM).

Pulmonary arterial antioxidant capacity. The Amplex Red assay was used to examine the antioxidant capacity of tissue as previously described (50). Pulmonary arteries from control and CH rats were incubated with or without H<sub>2</sub>O<sub>2</sub> (1 μM) for 1 h at 37°C. The rate of H<sub>2</sub>O<sub>2</sub> catalyzed (R<sub>cat</sub>) was determined by R<sub>cat</sub> = [H<sub>2</sub>O<sub>2</sub> – (Sample<sub>H2O2</sub> – Sample<sub>untreated</sub>)/1 h], where H<sub>2</sub>O<sub>2</sub> is the fluorescence value of 1 μM H<sub>2</sub>O<sub>2</sub> with no tissue, Sample<sub>H2O2</sub> is the tissue sample incubated with 1 μM H<sub>2</sub>O<sub>2</sub>, and Sample<sub>untreated</sub> is an untreated tissue sample. Sample<sub>untreated</sub> was negligible, as it was not detected above background fluorescence (see Fig. 3A, inset).

Western Blotting

Snap-frozen intrapulmonary arteries were homogenized in 10 mM Tris-HCl containing 255 mM sucrose, 2 mM EDTA, 12 μM leupeptin, 4 μM pepstatin A, 1 μM aprotinin (Sigma) and centrifuged at 10,000 g at 4°C to remove insoluble debris. Supernatant was collected, and sample protein concentrations were determined by the Bradford method (Bio-Rad) or spectrophotometer (Nano Drop 2000, Thermo Scientific). Equal protein concentration of pulmonary artery lysates were separated by SDS-PAGE (Tris-HCl gels, Bio-Rad) and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h at room temperature with 5% milk and 0.05% Tween 20 and incubated with rabbit anti-SOD1 (1:5,000), rabbit anti-SOD2 (1:5,000), and rabbit anti-SOD3 (1:500, Abcam) primary antibodies. After incubation, blots were incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase (1:3,000, Bio-Rad). For immunochromelabeling, bands were detected by exposure of the blots to chemiluminescence-sensitive film (Bio-Express). Bands were quantified by densitometric analysis using ImageJ (NIH). Blots were normalized to total protein determined by dividing the target protein by the intensity of the corresponding lane in the Coomassie-stained blots because exposure to CH significantly elevates both mRNA and protein expression of the traditional loading controls, β-actin and GAPDH, in pulmonary arterial homogenates (unpublished observations).

SOD Expression, Activity, and Modulation of SOCE

Protein expression. To determine the effect of CH on SOD expression, Western blot analysis was performed in pulmonary artery lysates from control and CH rats. Blots were incubated with rabbit anti-SOD1 (1:5,000), rabbit anti-SOD2 (1:5,000), and rabbit anti-SOD3 (1:500, Abcam) primary antibodies.

Activity. The effect of CH on SOD activity was determined by an SOD Assay kit (Cayman Chemical). Intrapulmonary arteries were homogenized in 5 μl buffer (20 mM HEPES pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) per milligram of tissue and centrifuged at 1,500 g for 5 min at 4°C. Supernatant was collected, diluted 1:2.5, and transferred to a 96-well plate, where all samples were incubated in the presence or absence of NaCN (1.8 mM) to inhibit Cu/Zn SOD (SOD1 and SOD3) for 20 min at room temperature. Absorbance was detected at 450 nm using an absorbance microplate reader (ELx800, BioTek Instruments). Cu/Zn SOD activity was calculated by subtracting absorbance from the NaCN-treated samples (Mn SOD activity) from total SOD activity.

Modulation of SOCE. To determine the influence of SOD1 expression and activity on ASIC1-dependent SOCE, small intrapulmonary arteries from SOD1<sup>−/−</sup> and SOD1<sup>+/+</sup> mice were cannulated and pressurized for measurement of [Ca<sup>2+</sup>], as described previously (32). Briefly, mice were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were exposed by midline thoracotomy. The left lung was removed and immediately placed in physiological saline solution (PSS) [pH adjusted to 7.4 with NaOH containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO<sub>4</sub>, 4 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 1.18 KH<sub>2</sub>PO<sub>4</sub>, and 6 glucose]. Intrapulmonary arteries (~2nd-5th order) were dissected from surrounding lung parenchyma and either snap-frozen in liquid N<sub>2</sub> or utilized to generate short-term PASMC cultures. To isolate PASMCs, intrapulmonary arteries were enzymatically digested in reduced Ca<sup>2+</sup> HBSS containing papain (9.5 U/ml), type-I collagenase (1,750 U/ml), and dithiothreitol (1 mM) at 37°C for 30 min. The cell suspension was placed on 25-mm glass coverslips and cultured in Ham’s F-12 media supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin for 3~4 days in a humidified atmosphere of 5% CO<sub>2</sub>-95% air at 37°C.
ip. The left lung was removed, and small intrapulmonary arteries (4th-5th order) were dissected free and transferred to a vessel chamber (Living Systems) and secured to tethered glass pipettes with a single strand of silk ligature. After cannulation, the artery was pressurized with a servo-controlled peristaltic pump (Living Systems) to 12 mmHg. Any artery that failed to maintain pressure upon switching off of the servo-controller was discarded. The vessel chamber was superfused with HEPES-based PSS (5 ml/min at 37°C). Arteries were incubated abuminally with fura 2-AM (2 μM and 0.05% pluronic acid in PSS, Molecular Probes) for 45 min at room temperature. Fura 2-loaded arteries were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual-excitation light source (IonOptix), and the respective 510-nm emissions were detected with a photomultiplier tube. Following baseline fura 2 measurements, arteries were superfused (5 ml/min at 37°C) with Ca2+-free PSS containing 3 mM EGTA to chelate extracellular Ca2+, 50 μM diltiazem to prevent Ca2+ entry through L-type voltage-gated Ca2+ channels, and 10 μM cyclopiazonic acid (CPA) to deplete intracellular Ca2+ stores and prevent Ca2+ reuptake through the sarcoplasmic reticulum Ca2+-ATPase. SOCE was determined by measuring the changes in [Ca2+]i (area under curve) upon repbullation of PSS containing 1.8 mM CaCl2 in the continued presence of diltiazem and CPA. Parallel experiments were performed in the presence of the specific ASIC1 inhibitor, psalmotoxin 1 (PcTX1; 20 nM).

ASIC1 protein expression. To determine the effect of SOD1 on ASIC1 expression, Western blot analysis was performed in pulmonary artery lysates from control and CH rats. Blots were incubated abluminally with rabbit anti-GPx-1 (1:5,000, Abcam).

Catalase Expression and Activity

Protein expression. To determine the effect of CH on catalase expression, Western blot analysis was performed as described above in pulmonary artery lysates from control and CH rats. Blots were incubated with rabbit anti-catalase (1:2,000, Pierce Thermo Scientific) primary antibody.

Activity. The effect of CH on catalase activity was determined by a catalase assay kit (Cayman Chemical) according to the manufacturer’s directions. Intrapulmonary arteries were homogenized in 5 μl buffer (50 mM potassium phosphate pH 7.0 containing 1 mM EDTA) per milligram of tissue and centrifuged at 10,000 g for 15 min at 4°C. Supernatant was collected, diluted 1:5, and transferred to a 96-well plate. Absorbance was detected at 540 nm using an absorbance microplate reader (SpectraMax Plus384, Molecular Devices).

GPx Expression and Activity

Protein expression. To determine the effect of CH on GPx expression, Western blot analysis was performed as described above in pulmonary artery lysates from control and CH rats. Blots were incubated with rabbit anti-GPx-1 (1:5,000, Abcam).

Activity. The effect of CH on GPx activity was determined by an assay kit (Cayman Chemical). Intrapulmonary arteries were homogenized in 5 μl buffer (50 mM potassium phosphate pH 7.0 containing 1 mM EDTA) per milligram of tissue and centrifuged at 10,000 g for 15 min at 4°C. Supernatant was collected, diluted 1:1 and 1:5, and transferred to a 96-well plate. Absorbance was detected at 340 nm using an absorbance microplate reader (SpectraMax Plus384, Molecular Devices).

H2O2 Regulation of ASIC1-dependent SOCE

To assess the role of H2O2 on ASIC1-dependent SOCE, a similar protocol to that described above was used to measure SOCE in PASMC from control and CH rats following fura 2 loading (2 μM and 0.05% pluronic acid for 30 min at 32°C). PASMC were treated with PEG-catalase (250 U/ml); H2O2 (25 μM); GPx mimetic, ebulesen (30 μM); or GPx inhibitor, mercaptosuccinic acid (3 mM) throughout the experiment. The contribution of ASIC1 to SOCE was determined in paired experiments by subsequently repeating the SOCE response following 20-min incubation with PcTX1 (20 nM). Data are expressed as percentage of control vehicle taken as an average of the control vehicle responses.

Calculations and Statistics

All data are expressed as means ± SE. Values of n refer to number of animals in each group unless otherwise stated. A t-test or two-way ANOVA was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared with the Student-Newman-Keuls test. A Mann-Whitney rank sum t-test or Kruskal-Wallis one-way ANOVA was used to make comparisons for data converted to percentage. A probability of P < 0.05 was accepted as significant for all comparisons.

RESULTS

CH Increases O2− and Decreases H2O2 Levels

Exposure to CH resulted in a significant increase in right ventricular hypertrophy expressed as right ventricular plus septum weight as an index of pulmonary hypertension (control: 0.28 ± 0.01 and CH: 0.53 ± 0.02; P < 0.001). Consistent with previous studies from our laboratory in isolated pressurized vessels (7, 24, 33), DHE fluorescence was increased in PASMC from CH rats compared with controls (Fig. 1). Dismutation of O2− by PEG-SOD reduced fluorescence in both groups, DHE fluorescence was not altered by PEG-catalase in PASMC from control animals, suggesting that DHE selectively detects O2− over H2O2. However, the augmented DHE fluorescence in PASMC from CH rats was diminished by preincubation with PEG-catalase.

In initial measurements of H2O2 in untreated pulmonary artery segments, detection of Amplex Red fluorescence was below the linear range of the standard curve (Fig. 3, A, inset, and B). Therefore, O2− and subsequent H2O2 production was stimulated with SOTS-1 (0.01, 0.1, 1.0 mM). SOTS-1 resulted in a concentration-dependent increase in H2O2 fluorescence that was abolished by coincubation with PEG-SOD but not PEG-catalase (P = 0.073; Fig. 2). In addition to increasing cellular O2− levels, SOTS-1 caused a concentration-dependent increase in H2O2 as measured by Amplex Red fluorescence (Fig. 3). SOTS-1 at 1 mM caused profound PASMC contraction and saturated the Amplex Red fluorescence signal. Therefore, experiments in Fig. 3B were performed using the lowest concentration (0.01 mM SOTS-1) that resulted in a detectable increase in H2O2 production. In contrast to effect of CH on O2− levels, CH caused a decrease in pulmonary arterial SOTS-1-induced H2O2 production (Fig. 3). PEG-catalase significantly reduced H2O2 levels in both groups.

CH Decreases SOD Expression and Activity

One possibility for the reciprocal levels of O2− and H2O2 following CH is dysregulation of SOD, which has been
shown in several models of pulmonary hypertension (4, 13, 15, 26, 35, 40). Consistent with this possibility, CH decreased Cu/Zn SOD1 (cytosolic) and Cu/Zn SOD3 (extracellular) protein expression (Fig. 4, A and B). This correlated with diminished total SOD and Cu/Zn SOD (SOD1 and SOD3) activity (Fig. 4C). There was no detectable
difference in Mn SOD2 (mitochondrial) protein expression or activity between groups.

**ASIC1-dependent SOCE is Increased in SOD1<sup>−/−</sup> Mice**

Our laboratory has previously demonstrated that SOD1<sup>−/−</sup> mice develop spontaneous pulmonary hypertension (40). To determine whether the expression and activity of SOD1 influences ASIC1 activity, we examined SOCE in isolated-pressurized pulmonary arteries (average inner diameter: 138 ± 17 µm). SOCE was greater in pulmonary arteries from SOD1<sup>−/−</sup> compared with SOD1<sup>+/+</sup> mice (Fig. 5). The specific ASIC1α inhibitor, PCtx1, attenuated SOCE in both groups, and SOCE was not different between groups in the presence of PCtx1. Expression of ASIC1 was similar in pulmonary arteries from SOD1<sup>+/+</sup> and SOD1<sup>−/−</sup> mice (Fig. 5, B and C). Expression of SOD1 was only present in pulmonary arteries from SOD1<sup>+/+</sup> mice, confirming knockout of this protein (Fig. 5B). These data suggest that a decrease in SOD1 expression and activity is sufficient to increase ASIC1-mediated SOCE, and this is further independent of a change in ASIC1 protein expression, as previously reported (32).

**CH Increases Pulmonary Arterial Antioxidant Capacity**

If SOD is the rate-limiting step in the formation of H<sub>2</sub>O<sub>2</sub>, then addition of PEG-SOD should increase H<sub>2</sub>O<sub>2</sub> levels to similar levels in pulmonary arteries from control and CH rats. PEG-SOD and the SOD mimetic, tiron, increased Amplex Red fluorescence in both groups above that observed in untreated tissue (Fig. 3, A, inset and Fig. 6A), and, therefore, SOTS-1 was not utilized in these experiments. However, H<sub>2</sub>O<sub>2</sub> levels were still less in pulmonary arteries from CH rats (Fig. 6B), suggesting that antioxidant mechanisms other than SOD contribute to the overall H<sub>2</sub>O<sub>2</sub> levels. Consistent with this possibility, preventing the catalysis of H<sub>2</sub>O<sub>2</sub> with blockers of catalase (AT) and GPx (mercaptosuccinic acid) increased H<sub>2</sub>O<sub>2</sub> levels similarly in pulmonary arteries from control and CH rats (Fig. 6B).

To specifically examine differences in the rate of H<sub>2</sub>O<sub>2</sub> catalysis, we measured the rate of H<sub>2</sub>O<sub>2</sub> decomposition following administration of 1 µM H<sub>2</sub>O<sub>2</sub> to the arteries from each group (Fig. 6C). After 1 h, pulmonary arteries from CH rats had degraded significantly more H<sub>2</sub>O<sub>2</sub> (637 ± 34 nM) compared with control tissue (373 ± 26 nM). Together, these data suggest that CH increases the rate of H<sub>2</sub>O<sub>2</sub> catalysis in pulmonary arteries.

**CH Increases GPx Expression and Activity**

Next we determined the effect of CH on the expression and activity of the two main enzymes responsible for H<sub>2</sub>O<sub>2</sub> degradation in the vasculature, catalase, and GPx-1. Although there was a tendency for catalase expression and activity to decrease following CH, there was no a statistically significant difference between control and CH groups (Fig. 7). Conversely, GPx-1 expression and activity were augmented in pulmonary arteries from CH rats (Fig. 7).

**H<sub>2</sub>O<sub>2</sub> Inhibits ASIC1-dependent SOCE**

We examined the PCtx1-sensitive component of SOCE to determine the influence of H<sub>2</sub>O<sub>2</sub> on ASIC1. In time-control experiments, we found that SOCE was similar between the first and second response (Fig. 8B). Therefore, data are expressed as the PCtx1-sensitive component of SOCE, which is derived from subtracting the SOCE response in the presence of PCtx1 from that of vehicle (Fig. 8C). Consistent with previous data from our laboratory (23, 32), we observed an increase in ASIC1-mediated SOCE in PASMC from CH rats (Fig. 9). Decreasing levels of H<sub>2</sub>O<sub>2</sub> with PEG-catalase (Fig. 9A) or the GPx mimetic, ebselen (Fig. 9B), increased SOCE in PASMC from control rats but had no further effect to increase SOCE in PASMC from CH rats. Addition of H<sub>2</sub>O<sub>2</sub> largely attenuated SOCE in PASMC from both control and CH rats (Fig. 9A). Likewise, increasing H<sub>2</sub>O<sub>2</sub> levels by blocking GPx with mercaptosuccinic acid diminished ASIC1-dependent SOCE (Fig. 9B). Together these data suggest that H<sub>2</sub>O<sub>2</sub> negatively regulates ASIC1 activity.

**DISCUSSION**

Previous studies from our laboratory show that the contribution of ASIC1 to the development of hypoxic pulmonary hypertension is independent of an increase in ASIC1 protein expression (32). Changes in ROS occur following CH that could potentially alter the activity of ASIC1 because ASIC1 is redox sensitive. The goal of the current study was to better understand the changes in ROS that occur following CH and how these changes influence ASIC1-dependent SOCE.
major findings from this study are that 1) CH increased PASMC O$_2^{-}$ and decreased pulmonary arterial H$_2$O$_2$ production; 2) CH attenuated Cu/Zn SOD expression and activity while increasing GPx expression and activity; 3) H$_2$O$_2$ inhibited ASIC1-dependent SOCE in PASMC from both control and CH animals; and 4) catalase and the GPx mimic, ebselen, augmented ASIC1-mediated SOCE in PASMC from control rats but had no further effect in PASMC from CH rats. These data suggest that, under control conditions, H$_2$O$_2$ inhibits ASIC1-dependent SOCE. Furthermore, H$_2$O$_2$ levels are decreased following CH as a result of diminished O$_2^{-}$ dismutase and increased H$_2$O$_2$ catalysis through GPx, leading to the augmented ASIC1-dependent SOCE (Fig. 10).

Endogenous O$_2^{-}$ and H$_2$O$_2$ are physiologically important second messengers that regulate a variety of downstream signaling pathways. However, an imbalance in ROS homeostasis has been implicated in the progression of various disease states, including pulmonary hypertension. Several lines of evidence, including studies from our laboratory, suggest that CH increases O$_2^{-}$ generation from multiple sources, including mitochondrial electron transport chain, xanthine oxidase, cytochrome P-450, nitric oxide synthase, and NADPH oxidase (34, 38, 43). Our laboratory further demonstrates an important contribution of O$_2^{-}$ to induce RhoA activation, myofilament Ca$^{2+}$ sensitization, and enhanced vasoconstriction in hypertensive pulmonary arteries (7, 8, 24, 33). Consistent with our previous studies, which examined O$_2^{-}$ generation in whole
artery preparations (7, 24, 33), in the current study, we found that O$_2^-$ is specifically elevated in PASMC from CH rats. Pretreatment of PASMCs with PEG-SOD decreased O$_2^-$ in both groups and prevented the CH-induced increase in O$_2^-$. DHE is expected to selectively detect O$_2^-$ over H$_2$O$_2$, and this is demonstrated in PASMC from control animals. Interestingly, we found that PEG-catalase diminished O$_2^-$ in PASMC from CH animals. We also observed a tendency for PEG-catalase to decrease SOTS-1-induced O$_2^-$ (i.e., DHE fluorescence); however, this did not reach statistical significance (Fig. 2; $P = 0.073$). Although the reason(s) for this effect of PEG-catalase is unclear, it is possible that catalase is removing a H$_2$O$_2$-mediated inhibition of SOD (20, 21, 29, 56). Exogenous H$_2$O$_2$ has been shown to inactivate Cu/ZnSOD through oxidation of copper at the enzyme active site (20, 21). In theory, PEG-catalase would increase SOD activity. Under conditions such as CH and SOTS-1 where there is more O$_2^-$ substrate for SOD, the effect of PEG-catalase to enhance SOD activity resulting in increased O$_2^-$ dismutation may be more apparent compared with control conditions. Although we did not spe-

CH increases pulmonary arterial antioxidant capacity. A: Amplex Red RFU from control intrapulmonary arteries following treatment with PEG-SOD (50 U/ml), the SOD mimetic, tiron (10 mM), or inhibition of catalase and glutathione peroxidase (GPx) with 3-amino-1,2,4-triazole (AT; 5 mM) and mercaptosuccinic acid (MSA; 3 mM) are within the linear range of the background-subtracted standard curve. B: summary data of H$_2$O$_2$ levels in intrapulmonary arteries from control and CH rats following above treatments. C: rate of H$_2$O$_2$ catalysis (1 µM) by control and CH intrapulmonary arteries measured by Amplex Red fluorescence. Values are means ± SE; $n = 6–8$ animals per group; *$P < 0.05$ vs. untreated pulmonary arteries from control rats.

GPx expression and activity are increased following CH. Representative Western blots (A) and summary data showing protein expression (B) and enzyme activity (C) of catalase and GPx in isolated pulmonary arteries from control and CH rats. Protein expression was normalized to total protein using the corresponding Coomassie-stained blot. Values are means ± SE; $n = 6$ animals per group; *$P < 0.05$ vs. control.
specifically measure the effect of PEG-catalase on SOD activity, important studies from Wedgwood et al. (56) have recently reported this in the fetal pulmonary circulation. Relative to fetal control lambs, DHE fluorescence was increased, and SOD3 activity was decreased in a lamb model of persistent pulmonary hypertension of the newborn (PPHN) (56). This was further exacerbated in fetal PPHN lambs ventilated with 100% O2. Catalase treatment decreased DHE fluorescence similar to levels observed in fetal control lambs and greatly augmented SOD3 activity in ventilated PPHN lambs (56). In addition, Wedgwood et al. (56) show that exogenous H2O2 increases and PEG-catalase decreases SOD3 mRNA expression. This correlation between H2O2 and SOD expression is consistent with our current findings, where CH resulted in a decrease in H2O2 levels and SOD1 and SOD3 expression.

In contrast to effects of CH to increase O2·− levels, we found that CH exposure resulted in a paradoxical decrease in H2O2 levels. Although O2·− is generally associated with contraction of pulmonary arteries, both contraction and relaxation have been observed in response to H2O2 (reviewed in Ref. 25). This varied response to H2O2 in the pulmonary circulation appears to be dependent on the level of vascular tone present and the concentration of H2O2 applied. At physiological concentrations, H2O2 is a vasodilatory and antiproliferative signaling molecule (9, 30). In response to acute hypoxia, basal production of H2O2 in the rat pulmonary circulation is diminished (3, 30). This prompted the general notion that, under normoxic conditions, H2O2 inhibits smooth muscle contraction, whereas under hypoxia H2O2 levels fall, resulting in hypoxic pulmonary vasoconstriction. A decrease in H2O2 levels has also been reported following long-term exposure to CH (6, 13, 40, 41), as well as in other experimental models of spontaneous pulmonary hypertension and in humans with pulmonary arterial hypertension (4, 6, 40). This decrease in H2O2 is thought to contribute to proproliferative and antiapoptotic effects that are, in part, mediated by hypoxia-inducible factor 1α (HIF-1α) and

![Graph](http://ajplung.physiology.org/)
Superoxide is highly reactive and rapidly dismutates to \( \text{H}_2\text{O}_2 \); therefore, an additional means of regulating \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) levels is through SOD. Indeed, a number of studies have implicated the downregulation of various SOD isoforms in the development of pulmonary hypertension. Although SOD can be regulated by a variety of transcription factors, HIF-1\( \alpha \) and HIF-2\( \alpha \) directly bind the hypoxia-responsive element of SOD2 and SOD1 promoter, resulting in negative regulation of gene expression. A loss of SOD1, SOD2, and SOD3 leads to the hypoxic activation of HIF-1\( \alpha \), suggesting that stabilization of HIF-1\( \alpha \) is redox sensitive and closely linked to SOD function and the oxidant/antioxidant equilibrium.

Our laboratory has recently shown that SOD1\(^{-/-}\) mice develop spontaneous pulmonary hypertension, and this response is associated with increases in pulmonary arterial \( \text{O}_2^- \) and decreases in \( \text{H}_2\text{O}_2 \) levels. In the current study, we demonstrate that the loss of SOD1 and subsequent imbalance in \( \text{O}_2^-/\text{H}_2\text{O}_2 \) mimics the effects of hypoxia and leads to enhanced ASIC1-dependent SOCE. Similar to our findings following CH (32), this occurs independently of a significant change in ASIC1 protein expression in pulmonary arteries from SOD\(^{-/-}\) compared with SOD\(^{+/+}\) mice. Together, these data strongly support the concept that the downregulation of SOD1 and SOD3, and subsequent decrease in \( \text{H}_2\text{O}_2 \), following CH contributes to ASIC1 activation. Additionally, we found that the addition of PEG-SOD or tiron improved, but did not normalize, \( \text{H}_2\text{O}_2 \) levels in pulmonary arteries from CH rats to those of controls. This suggested that CH may additionally augment the enzymatic decomposition of \( \text{H}_2\text{O}_2 \) by catalase and/or GPx. Consistent with this possibility, we found that expression and activity of GPx-1 was augmented in pulmonary arteries from CH rats. Other studies also indicate that CH exposure in rats results in increased lung GPx-1 (17, 31, 58).

Because CH alters the state of various redox couples, it is difficult to determine the physiological impact of increased GPx-1 expression and activity on the overall cellular redox environment. This is further complicated by the fact that redox responses can differ markedly among subcellular compart-
ments (55). Nonetheless, changes to the redox potential represent an important mechanism of ion channel regulation, including regulation of ASIC1a.

Although H$_2$O$_2$ has been shown to increase [Ca$^{2+}$], and constriction of pulmonary arteries, these responses were unaffected by removal of extracellular Ca$^{2+}$ or by nonselective channel inhibitors, arguing against involvement of store- and receptor-operated Ca$^{2+}$ channels (28, 39). Rather than activation, H$_2$O$_2$ seems to cause a profound inhibition of SOCE in vascular smooth muscle and endothelial cells (14, 16, 37, 47), an effect widely observed in a variety of nonvascular cells (49, 51). This is consistent with our current findings that H$_2$O$_2$ inhibited ASIC1-dependent SOCE in PASMC from both control and CH rats. Consistently, removing H$_2$O$_2$ through the addition of PEG-catalase or the GPx mimetic, ebselen, increased SOCE in PASMC from control animals. In contrast, these inhibitors were without effect on SOCE following CH, suggesting that inhibition of SOCE by endogenous H$_2$O$_2$ is absent in PASMC from CH animals. However, whether this is due to a direct effect of decreased H$_2$O$_2$ levels on ASIC1 or an indirect effect mediated by a shift in the cytosolic redox environment is currently unknown. Furthermore, whether CH induces an oxidized or reduced shift in the redox potential is unclear, as both have been reported (41, 55).

Reducing agents such as dithiothreitol and glutathione potentiate ASIC1 current, increase pH sensitivity, and decrease channel inactivation (2, 10, 11, 60). In cortical neurons, this leads to enhanced acid-induced membrane depolarization and Ca$^{2+}$ influx (11). Site-directed mutagenesis identified involvement of extracellular cysteine and lysine residues in the modulation of ASICs by oxidizing and reducing agents, respectively (11). Other studies show that intracellular COOH-termina
cysteine residues are important in the intersubunit disulfide bond formation induced by H$_2$O$_2$. The formation of intersubunit disulfide bonds inhibits acid-evoked currents and reduces the amount of ASIC1a present on the cell surface (60). It is therefore likely that redox reagents affect both trafficking of ASIC1 to the membrane as well as altering the kinetics of channel activation.

Oxidative stress reflects an imbalance in the production of free radicals and antioxidant defenses. In lungs, the main enzymatic antioxidants are SOD, catalase, GPx, and thiore
doxin (38). In the current study, we show that CH decreases pulmonary arterial SOD1 and SOD3 and increases GPx-1 expression and activity. The resulting decrease in H$_2$O$_2$ production and increase in degradation lead to an overall reduction in pulmonary arterial H$_2$O$_2$ levels following CH. H$_2$O$_2$, either through direct mechanisms or indirectly through redox modification, inhibits ASIC1-dependent SOCE. Loss of endogenous H$_2$O$_2$ following CH leads to enhanced ASIC1-mediated SOCE (Fig. 10). Further studies are necessary to specifically address key questions regarding how the redox changes during CH correlate to ASIC1 activity and the mechanisms involved in this response.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


10. Cho J, Askwith C. Potentiation of acid-sensing ion channels by salicyl


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