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**MECHANISMS RESPONSIBLE FOR DECREASED RAT
PULMONARY ARTERIAL H₂O₂ LEVELS IN A MODEL OF
CHRONIC HYPOXIA-INDUCED PULMONARY HYPERTENSION**

by

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**B.S. BIOLOGY, LIBERTY UNIVERSITY, 2008
M.ED. SCIENCE EDUCATION, LYNCHBURG COLLEGE, 2009**

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Master of Science
Biomedical Sciences**

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Albuquerque, New Mexico

July 2014

DEDICATION

TO GRAMPIE

-You built a legacy for me in education first and foremost by example and then by helping me with school projects starting in elementary school all the way through my master's degree in science education by helping create entomology student based projects.

-You built a legacy for me in science through your work researching entomology and forestry in Wisconsin and tobacco in Virginia.

-You built a legacy for me in family by living in Virginia during the school year to mentor me and my brothers; by taking us fishing in Door County, Wisconsin; by making every moment a teachable moment about life and caring for others.

I started on this journey in a small part to say at the end, I'm Dr. Giese now too. I know you are still proud and thank you for paving the way.

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ABSTRACT

Our laboratory has demonstrated an important role for acid-sensing ion channel 1 (ASIC1) in pulmonary hypertension through augmented Ca²⁺ influx in pulmonary arterial smooth muscle cells (PASMC) following chronic hypoxia (CH). However, this enhanced calcium (Ca²⁺) influx is not dependent on an increase in ASIC1 protein expression. This suggests other regulatory mechanisms influencing ASIC1 activity are altered following CH. ASIC1 is a redox sensitive ion channel and recent studies from our laboratory have shown that the oxidant hydrogen peroxide (H₂O₂) inhibits ASIC1-dependent Ca²⁺ influx in PASMC. Reactive oxygen species are known to be altered in pulmonary hypertension, however the direction of these changes remains largely controversial. We hypothesized that H₂O₂ levels are decreased following CH due to diminished

production and enhanced degradation. To test this hypothesis we assessed H_2O_2 levels by Amplex Red fluorescence and found a decrease in H_2O_2 in pulmonary arteries from CH rats (4 wks @ 380 Torr) compared to control. To determine the effect of CH on H_2O_2 production, we examined the expression and activity of superoxide dismutase (SOD) 1, 2, and 3. The expression of SOD1 and SOD3 was decreased; and total and Cu/ZnSOD (SOD1 and SOD3) activities were reduced in pulmonary arteries from CH rats compared to those of controls. To determine the effect of CH on H_2O_2 decomposition, we examined the rate of catalysis of H_2O_2 and the expression and activity of the enzymes responsible for the catalysis of H_2O_2 : catalase and glutathione peroxidase. We found the rate of H_2O_2 degradation was greater in pulmonary arteries from CH rats compared to control. While there was no difference in catalase expression or activity between groups, glutathione peroxidase expression and activity was augmented following CH. Together these data suggest the decrease in pulmonary arterial H_2O_2 levels in CH-induced pulmonary hypertension is a result of 1) decreased production due to diminished SOD1 and SOD3 expression and activity; and 2) increased catalysis via glutathione peroxidase. The decreased H_2O_2 levels correlate with enhanced ASIC1-dependent Ca^{2+} influx in PASMC following CH. Further studies are needed to determine the mechanism by which H_2O_2 regulates ASIC1 Ca^{2+} influx.

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Chapter 1 – Introduction

Pulmonary Hypertension

Pulmonary hypertension (PH) is defined by mean pulmonary arterial pressure greater than 25 mmHg (162). This disease has multiple etiologies that allow for various classifications as defined by the World Health Organization. Group III includes PH caused by chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), and/or hypoxia (162). Chronic lower respiratory diseases, including COPD, are the third leading cause of death in the United States as of 2011, with an increase in the death rate from the previous year (71). The mainstream treatments for PH include prostacyclin analogs, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors (39). However, these treatments are ineffective in Group III PH and in some cases worsen the disease (39, 96). Therefore, treatment is limited for patients for Group III PH.

Chronic hypoxia (CH)-induced pulmonary hypertension is associated with structural and functional changes in the pulmonary vasculature including vasoconstriction, vascular remodeling, and polycythemia (Figure 1)(41). The initial physiological response to alveolar hypoxia occurs within minutes initiating vasoconstriction which diverts pulmonary blood to areas of higher oxygen (171). If hypoxia is sustained, hypoxic pulmonary vasoconstriction (HPV) will persist though at a reduced level (171). In addition to HPV, several other forms of vasoconstriction occur in CH-induced pulmonary hypertension including depolarization-induced (16, 112, 120), agonist-induced (8), and pressure-induced

vasoconstriction (17) as well as Rho kinase-induced calcium (Ca^{2+}) sensitization (81). In both acute and sustained hypoxia, an increase in pulmonary artery smooth muscle cell (PASMC) Ca^{2+} occurs. Over time, the increase in Ca^{2+} facilitates vascular remodeling by stimulating cell proliferation in the medial layer. The increased thickness of the medial layer, in addition to vasoconstriction already present, reduces the pulmonary artery diameter and increases resistance (100).

Hypoxia also mediates the stabilization of hypoxia inducible factor-1 α (HIF-1 α) which induces erythropoiesis and causes polycythemia (63, 161). Polycythemia increases blood viscosity and in addition to the previously mentioned reduction in arterial diameter, increases resistance (Poiseuille's law, $R = 8\eta l / \pi r^4$) (R = resistance; η = blood viscosity; l = vessel length; r = vessel radius) and therefore increases in pulmonary arterial pressure (14, 100). In an effort to compensate, the right ventricle undergoes hypertrophy as it works against the increased afterload (12). This increase in right ventricle size is used as an index of PH. For rats exposed to 4 weeks of CH, there is a significant increase in Fulton's index (right ventricular weight / left ventricular weight plus septum) as well as hematocrit levels (Figure 2a,b).

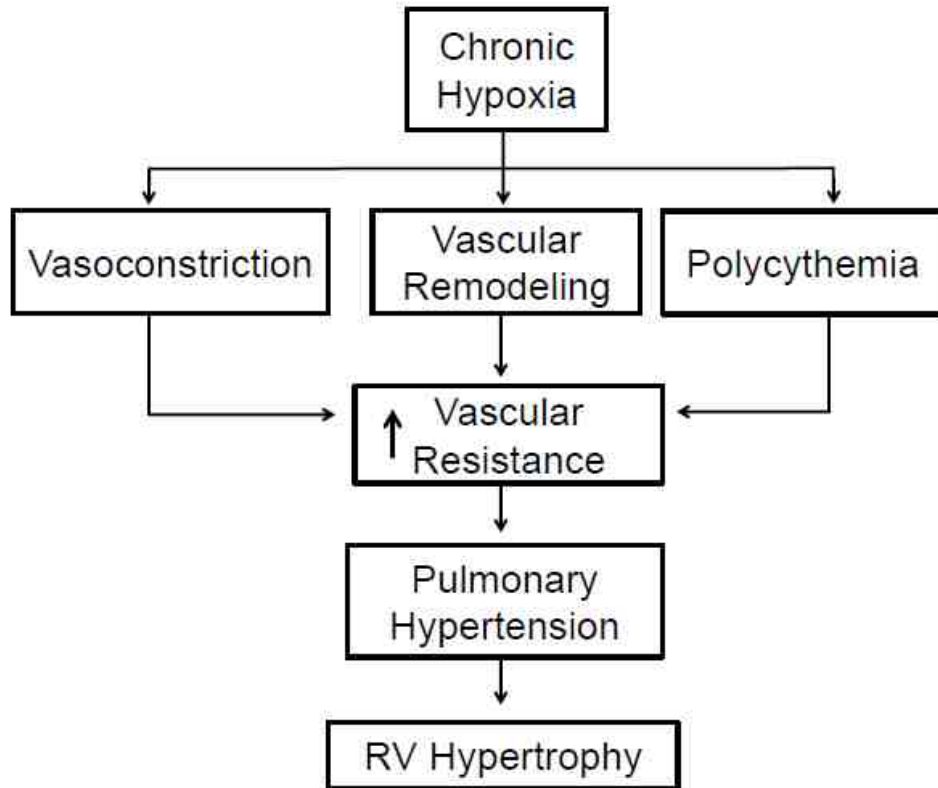


Figure 1: Model of chronic hypoxia (CH)-induced pulmonary hypertension. Structural and functional changes that occur in a model of CH-induced pulmonary hypertension (380 mmHg, 4 weeks). CH induces changes in vasoconstriction, vascular remodeling, and polycythemia. These lead to an increase in vascular resistance and the development of pulmonary hypertension and right ventricular hypertrophy.

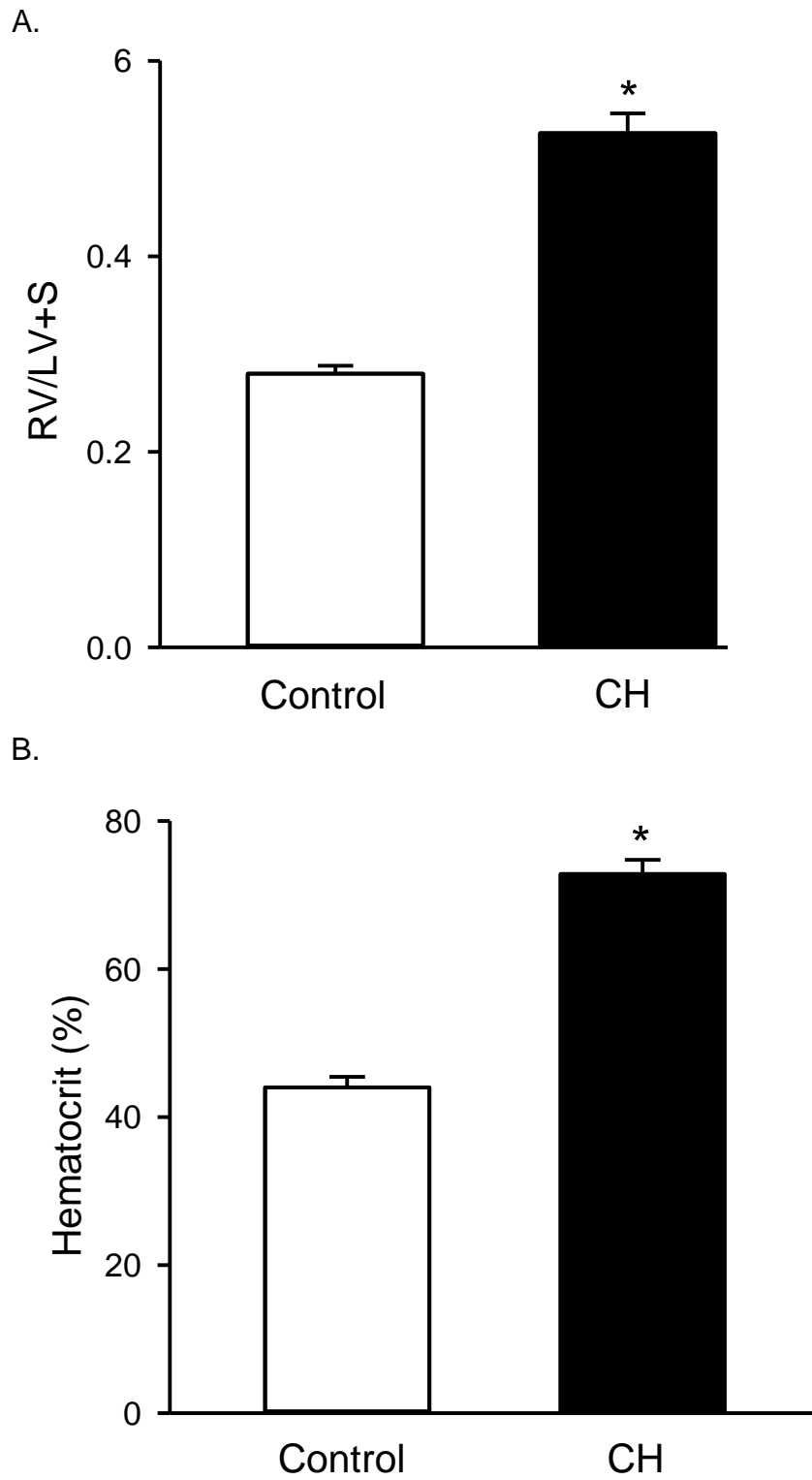


Figure 2: Right ventricular hypertrophy and polycythemia present after 4 weeks chronic hypoxia (CH). A) Fulton's index of right ventricle (RV) to left ventricle plus septum weight (LV+S) and B) hematocrit (in %) in rats exposed to control or CH conditions. Values are means \pm SE by *t*-test; n = 6/group; **p*<0.05 vs. control.

Hypoxic Pulmonary Vasoconstriction

As mentioned previously HPV matches ventilation and perfusion as a result of vasoconstriction due to acute (Phase 1) and sustained hypoxia (Phase 2)(171). The increase in pulmonary artery constriction to hypoxia is well characterized, but the underlying mechanisms behind HPV remain unresolved, although increases in PASM Ca^{2+} are implicated in both phases as well as in chronic hypoxia (100).

Identifying the oxygen sensor is one source of contention. Two underlying assumptions guide the selection of the oxygen sensor. First, this sensor should be able to interact with oxygen within a hypoxic setting, and second it must be able to signal within the PASM (155). The mitochondrion is one oxygen sensor that has been implicated in both acute and sustained HPV (155, 171). NADPH-oxidase is a second oxygen sensor and has mainly been implicated in the first phase of HPV due to acute hypoxia (171).

Chronic Hypoxia and Increased Calcium

Increases in vascular smooth muscle (VSM) intracellular Ca^{2+} accompany the large scale structural and functional changes in CH-induced pulmonary hypertension such as vasoconstriction and vascular remodeling (152, 170). There are multiple modes of entry for Ca^{2+} into PASM including L-type voltage gated calcium channels (VGCC), store operated channels (SOC), and receptor operated channels (ROC) (14)(Figure 3) and each of these in turn have their own unique regulatory pathways. L-type VGCCs are activated under depolarizing

conditions (14) and also by phosphorylation (95), but do not appear to contribute a major role to the increased Ca^{2+} observed following CH (93, 152). ROCs mediate Ca^{2+} influx upon activation by the second messenger diacylglycerol which is initiated upon G-protein coupled receptor activation (65). ROCs and SOCs include non-selective cation channels (92, 101, 125). The transient receptor potential (TRP) channel family of proteins has been implicated in receptor operated calcium entry (ROCE). Members of the canonical family of TRP, TRPC channels include TRPC3, 6, and 7 and have been implicated in ROCE (65). Following CH, ROCE is increased in rat PASMCM (93), though our laboratory has shown a decrease in pulmonary arteries (79, 154).

SOCs mediate the influx of Ca^{2+} in response to store depletion of the sarcoplasmic reticulum (SR) in smooth muscle cells and endoplasmic reticulum (ER) in other cell types in a process called store operated calcium entry (SOCE). Upon G-protein coupled receptor activation, the second messenger inositol trisphosphate binds its receptor on the SR causing release of Ca^{2+} . Stromal interaction molecule 1 (STIM1), a transmembrane protein found in the SR that binds Ca^{2+} , detects decreased Ca^{2+} levels and clusters near SOCs. The STIM1 clusters enable the protein Orai1 to colocalize with STIM1 and facilitate SOCE (42, 70, 139, 158) in which Ca^{2+} enters through SOCs to refill the SR stores (14). Several ion channels have been implicated in SOCE including TRPC1, 4, 5 and 6 (124, 141, 169, 170), as well as Orai1/2 (46, 104, 107, 165, 186).

Following CH, there is an increase in SOCE in rat PASMCM (93) and SOCE contributes to the known increase in basal Ca^{2+} following CH (170). In addition,

CH has been linked to increased STIM1 (70) and TRPC1 and 6 expression (93, 169), and following acute hypoxia STIM1 associates with TRPC1 and Orai1 in PASMC (115). In cells from patients with PH, there is higher expression of STIM2 and Orai2, and STIM2 contributes to enhanced SOCE (157).

With the use of siRNA targeting TRPC1 and 6 (93), STIM1, and Orai1 (114-116), Ca^{2+} influx is reduced, but not abolished. Experiments employing TRPC1 knockout mice reveal conflicting data, with some reports revealing a significant contribution of TRPC1 to SOCE (149) and others no contribution (38) depending on tissue type. In addition, some studies examining TRPCs in regard to SOCE utilize non-selective cation channel inhibitors (93, 170). Therefore the identity and characteristics surrounding SOCs and associated SOCE mechanisms remain in question. Additional channels exist outside of TRPC family members that are able to conduct Ca^{2+} and they include acid-sensing ion channels: homomeric 1a, homomeric 1b, and heteromeric 1a + 2b depending on the animal species (167, 184).

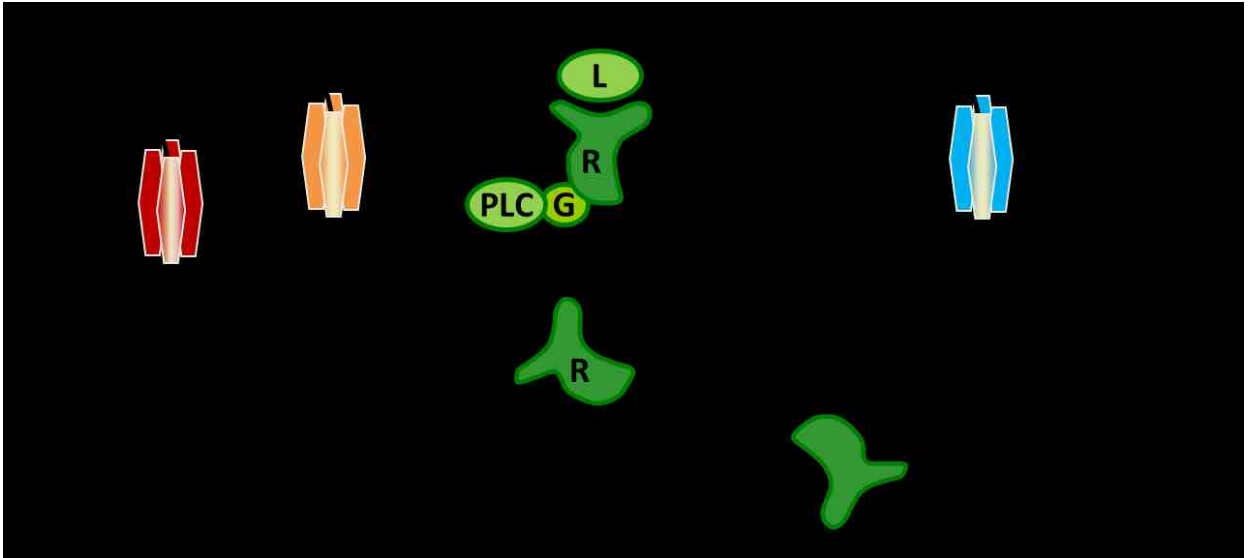


Figure 3: Calcium influx pathways in a vascular smooth muscle cell. L-type voltage gated calcium channels (VGCC) allow for the influx of calcium classically through depolarization; receptor operated channels (ROC) activate upon the binding of diacylglycerol (DAG) via G-protein coupled receptor activation; store operated channels (SOC) activate in response to depletion of calcium in the sarcoplasmic reticulum (SR).

Acid-Sensing Ion Channel 1

Acid-sensing ion channel 1 (ASIC1), part of the degenerin/epithelial Na⁺ (DEG/ENaC) channel family, is activated upon a drop in extracellular pH to conduct Ca²⁺ and Na⁺ (167). Along with other members of the DEG/ENaC family, ASICs have a large extracellular cysteine rich region (9) as well as C-terminal cysteines (185). Although they are primarily present in the nervous system, ASICs have also been observed in VSM (56, 57, 80, 118) and contribute to VSM migration (56, 57) and vasoconstriction following CH (79, 118).

Previous work from our laboratory has shown that ASIC1 is expressed in the pulmonary vasculature (80, 118) and that it conducts Ca²⁺ (80). In addition, our laboratory found that ASIC1 mediates SOCE in control PASMC (80). Following CH, ASIC1 contributes to augmented SOCE in pulmonary arteries and PASMC (79, 118). ASIC1 also contributes to agonist-induced vasoconstriction following CH (79, 118) and HPV (118). Through the use of ASIC1 knockout mice, ASIC1 directly contributes to the development of CH-induced pulmonary hypertension by measuring indices such as arterial remodeling, polycythemia, Fulton's index of right heart hypertrophy, and right ventricular systolic pressure (118).

Interestingly, our laboratory found ASIC1 to be involved in augmented SOCE following CH in rats and mice (79, 118). We assessed protein expression and found increased ASIC1 in pulmonary arteries from rats exposed to CH but not mice exposed to CH, with no changes in mRNA (79, 118). This discrepancy

in protein expression suggests other regulatory mechanisms contribute to the increase in SOCE observed independent of additional ASIC1 protein.

Other groups have shown that ASIC1 can be regulated by redox state, with oxidizing agents inhibiting and reducing agents enhancing current through the channel (32); as well as reducing agents slowing desensitization of the channel, allowing it to remain open longer (2, 31). In addition, hydrogen peroxide (H_2O_2), a known oxidizing agent, decreases current and plasma membrane localization of ASIC1 (185). The exact mechanism of this regulation is not known, but strong speculation revolves around thiol modification affecting channel kinetics and/or trafficking (31, 185).

Chronic Hypoxia and Associated Changes

HPV is known to contribute to the pathogenesis of CH-induced pulmonary hypertension in part, but not completely as studies have shown exposure of CH-exposed rats or humans with high altitude pulmonary hypertension to normoxia reduces but does not normalize pulmonary artery pressure (89, 91, 135, 161). Therefore other mechanisms contribute to CH-induced pulmonary hypertension. Numerous signaling pathways have been implicated in the disease, some of which exceed the scope of this project, however there are key regulators still under investigation (161). Hypoxia has been reported to affect superoxide (O_2^-) and H_2O_2 , however there is controversy in the literature in regards to the changes that occur to reactive oxygen species (ROS) following hypoxic exposure in phase 1 of HPV (147). Some groups have observed that hypoxia causes an increase of

ROS in PASMC (87, 99, 132, 172, 173, 181). However, other groups have observed that hypoxia causes a decrease in ROS in PASMC (102, 105, 181). Hence two major views concerning ROS in regards to hypoxia have emerged in the larger context of the phenomenon of HPV: the ROS (175) and redox hypotheses (3, 5, 179).

Oxidative Stress.

Following hypoxia, changes in ROS are known to occur. ROS can include O_2^- , hydroxyl radicals, and oxidizing agents formed from H_2O_2 among others. This leads to the first major HPV hypothesis, the ROS hypothesis which asserts an increase in ROS following hypoxia, with NADPH-oxidase as the oxygen sensor (177). This increase in ROS allows for the release of Ca^{2+} from the SR, triggering SOCE, and a subsequent initiation of contraction events (177). Recent evidence for this hypothesis implicates mitochondrial complex III Rieske iron-sulfur protein (175), which normally acts to transfer an electron from the Qo site to cytochrome c1 (62) and with hypoxia could lead to additional O_2^- formation (175).

Following CH, NADPH-oxidase expression is increased in pulmonary arteries from piglets and NADPH-oxidase contributes to ROS production (37, 49). In addition, xanthine oxidase expression and activity and uncoupled eNOS are increased in the lungs of rats exposed to CH (77), and these are known to produce O_2^- (50, 51). As a result, oxidant stress and ROS are increased following CH (77). Studies performed in PASMC under acute hypoxia show an increase in

oxidative state as assessed by glutathione (173) and the fluorescent probe roGFP with catalase blocking the increase in oxidant-based fluorescence indicating that H_2O_2 is responsible for the oxidative stress (174).

Redox.

Redox state of the cell is determined by the ratio between reducing and oxidizing agents, primarily reduced and oxidized glutathione (GSH:GSSG) and to a smaller extent NAD(P)H:NAD(P)⁺. The overall redox state is important as it has implications in regards to thiol group modification as glutathione can donate or receive electrons which can interact with cysteines on other proteins such as ion channels (156). Studies have shown that cellular redox state changes with CH shifting the whole lung to a more reduced state by increasing GSH (134, 180). With acute hypoxia in pulmonary arteries, there is an increase in NADPH (60) as well as a decrease in H_2O_2 which as an oxidant would contribute to the redox state (105). In addition, a more reduced state has been shown with the fluorescent probe roGFP in PASMC from fawn hooded rats which develop spontaneous pulmonary hypertension (4, 142).

These redox changes lead to the second major HPV hypothesis. The redox hypothesis asserts that a decrease in mitochondrial ROS leads to a more reduced redox state in the cell following hypoxia, with the mitochondria acting as the oxygen sensor (110). Specifically, activity of the electron transport chain is suppressed at complex I and III (93, 105) reducing the probability that electrons pair with molecular oxygen and generate mitochondrial ROS, which would lower

H₂O₂ levels. This allows for a buildup of NAD(P)H which in turn shifts the ratio of glutathione to GSH (177). The subsequent reduced redox state leads to an inhibition of K⁺ channels resulting in activation of L-type VGCC leading to contraction (110).

Superoxide Dismutase and Hydrogen Peroxide.

Superoxide dismutase (SOD) converts O₂⁻ to H₂O₂ and is important for maintaining appropriate ROS levels in the cell. If this system is disrupted, high ROS results leading to oxidative stress implicated in numerous diseases. The three main isoforms of SOD are found in the cytosol (SOD1), mitochondria (SOD2), and extracellular matrix (SOD3). The enzymes operate via metal cofactors, with SOD1 and SOD3 utilizing copper/zinc and SOD2 utilizing manganese. Low expression and activity of SOD have been implicated in several models of pulmonary hypertension including fawn hooded rats (4), CH-induced pulmonary hypertension (37), persistent pulmonary hypertension of the newborn model with lambs (1), spontaneous pulmonary hypertension in SOD1 knockout mice (130), and human pulmonary hypertension (4).

H₂O₂ can cause vasoconstriction or relaxation across various vascular beds (53, 54, 74, 76, 82, 122). In the pulmonary vasculature, there is evidence that H₂O₂ causes relaxation at low concentrations by activation of PKG through soluble guanylate cyclase and cGMP-dependent (20, 113) and -independent mechanisms (113). Following hypoxia, less H₂O₂ production, via mitochondrial dysfunction (105), decreases cGMP (19, 61, 109) and results in vasoconstriction

(105). With increasing concentrations, H₂O₂ causes constriction via activation of ERK MAP kinase (122), PLC (148), and cyclooxygenase-derived prostaglandins (183). Other groups report that hypoxia allows for increased intracellular Ca²⁺ in pulmonary arteries due to increased H₂O₂ from the mitochondria (172, 176), possibly by mitochondrial H₂O₂ oxidizing mitochondrial nucleotides and stimulating Ca²⁺ release from the mitochondria (138).

H₂O₂ has also been shown to activate STIM1-dependent calcium-release-activated Ca²⁺ current (I_{CRAC})(59), the current attributed to store-operated responses (69). One postulated mechanism for this is through H₂O₂ induced S-glutathionylation of STIM1, causing STIM1 clustering and initiating SOCE (13, 66). In addition, H₂O₂ could trigger I_{CRAC} via IP₃ receptor activation (59, 133). Contrastingly, H₂O₂ can attenuate SOCE in thyroid cells (163), mast cells (160), and PASMC (128).

Rationale and Specific Aims

Our laboratory has demonstrated that ASIC1 contributes to enhanced SOCE following CH in the pulmonary vasculature (79, 118), but mechanisms regulating this response remain unclear. H₂O₂ is a critical regulator of ASIC1, decreasing plasma membrane expression and current amplitude (184, 185). We have shown increased O₂⁻ following CH (81), however whether this leads to a parallel increase in H₂O₂ is unknown especially with many forms of pulmonary hypertension having decreased SOD expression and activity (1, 4, 37, 130).

Therefore we tested the hypothesis that H₂O₂ levels are decreased in pulmonary arteries following CH due to diminished production and improved degradation.

Specific Aim 1.

Determine the effect of CH on pulmonary arterial H₂O₂ levels.

Hypothesis and Approach.

We hypothesize that H₂O₂ levels are decreased following CH. This hypothesis was studied by assessing H₂O₂ levels using the reagent Amplex Red in pulmonary arteries from control rats and rats exposed to CH.

Specific Aim 2.

Determine the effect of CH on H₂O₂ production in pulmonary arteries.

Hypothesis and Approach.

We hypothesize that SOD expression and activity are decreased following CH. To examine the importance of SOD function in H₂O₂ production, protein levels were examined for SOD1, SOD2, and SOD3. In addition, we assessed enzyme activity for total SOD, MnSOD (SOD2) and Cu/ZnSOD (SOD1 and SOD3).

Specific Aim 3.

Determine the effect of CH on H₂O₂ degradation in pulmonary arteries.

Hypothesis and Approach.

We hypothesize that catalase and glutathione peroxidase expression and activity are increased following CH. To determine if CH increased H₂O₂ degradation, the two major enzymes for H₂O₂ degradation were inhibited and H₂O₂ levels were measured by Amplex Red. In addition, H₂O₂ itself was added to assess the pulmonary arteries' capability to break down the oxidant. Last, to assess the effect of CH on the two enzymes involved in H₂O₂ catalysis, protein levels and enzyme activity were assessed for catalase and glutathione peroxidase.

Chapter 2 – Methods

Animals and Chronic Hypoxic Exposure Protocol

Male Wistar rats (~12 wk old, Harlan Industries) were divided into two groups (control and CH) for each experiment. Animals designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 mmHg for 4 wk. The chamber was opened three times per week to provide animals with fresh food, water, and clean bedding. Age-matched control rats were housed at ambient barometric pressure (~630 mmHg in Albuquerque, NM). All animals were maintained on a 12:12-h light-dark cycle. 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).

Measurement of Right Ventricular Hypertrophy and Polycythemia

Rats were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart was exposed by midline thoracotomy. After isolation of the heart, the atria and major vessels were removed. Fulton's index assessing right heart hypertrophy was expressed as the ratio of right ventricle (RV) to left ventricle plus septum (LV+S) weight. Polycythemia was assessed by measuring hematocrit from blood collected in microcapillary tubes following direct cardiac puncture.

Pulmonary Artery Isolation

Rats were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were exposed by midline thoracotomy. Lungs were removed and immediately placed in physiological saline solution (PSS) [pH adjusted to 7.4 containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO₄, 4 NaHCO₃, 1.8 CaCl₂, 10 HEPES, 1.18 KH₂PO₄, 6 glucose]. Intrapulmonary arteries were dissected from surrounding lung parenchyma and snap-frozen in liquid N₂ to utilize for further experiments.

Amplex Red Assay / Hydrogen Peroxide Degradation Assay

Hydrogen peroxide levels were determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay (Life Technologies). The assay was performed according to manufacturer's directions with some modifications. The descending branch of the left pulmonary artery was isolated from control and CH-exposed rats. This branch was dissected into 2 mm segments over ice in cold HEPES-PSS. Segments were incubated in vehicle (HEPES-PSS), PEG-catalase (250 U/ml), PEG-SOD (50 U/ml), the SOD mimetic tiron (10 mM), the glutathione peroxidase (GPx) inhibitor mercaptosuccinic acid (3 mM) or a combination of the catalase inhibitor 3-amino-1,2,4-triazole (5 mM) and mercaptosuccinic acid (3 mM) for 30 minutes at 37°C. The supernatant was transferred to a 96-well plate and incubated with Amplex Red reagent for 30 minutes at 37°C. Amplex Red fluorescence was excited at 550 nm and detected at 610 nm using a fluorescence microplate reader (Tecan Infinite® M200).

Since Amplex Red fluorescence in the vehicle treated group was below standard curve values (Figure 4a,b), H₂O₂ production was stimulated in pulmonary artery segments with Di-(4-carboxybenzyl) hyponitrite (SOTS-1; 10 μM) for 1 hr at 37°C prior to addition of Amplex Red (75, 88). To verify that SOTS-1 resulted in slow release of H₂O₂, we examined H₂O₂ levels via Amplex Red in response to increasing concentrations of SOTS-1 (0.01, 0.1, 1.0 mM) (Figure 4b). Protein concentration was determined for each segment following the assay to verify equal amounts of sample were used for each group.

The antioxidant capacity and efficiency of the pulmonary arteries to degrade H₂O₂ was measured using the Amplex Red assay as described above. Pulmonary artery segments were incubated with H₂O₂ (1 μM) for 1 hr at 37°C and the supernatant was transferred to a 96 well plate and incubated with Amplex Red reagent for 30 minutes. H₂O₂ that was quenched by the tissue was determined by subtracting the fluorescence values of the samples from the values of 1 μM H₂O₂ alone (Figure 5).

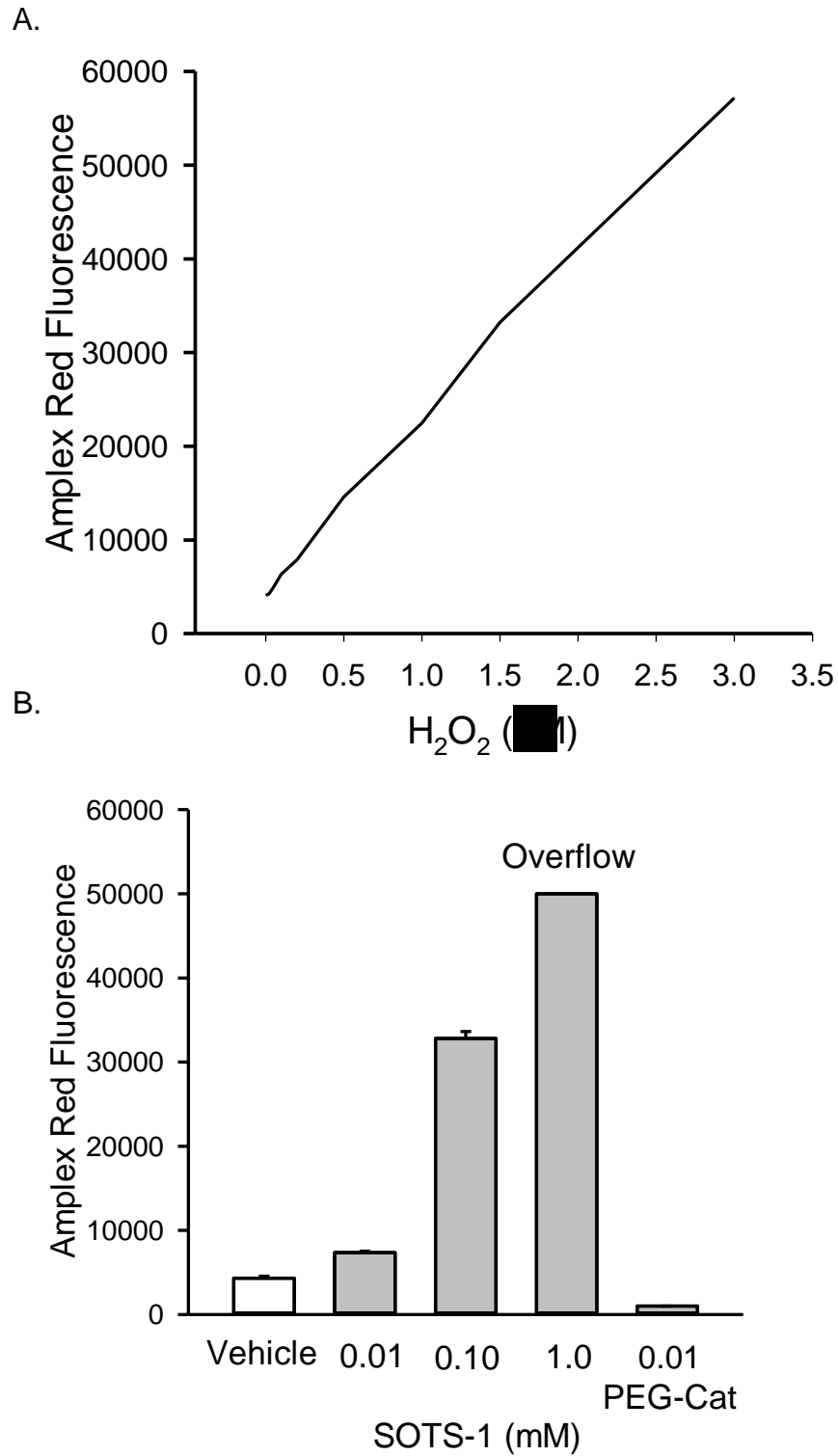


Figure 4: SOTS-1 Increases H₂O₂. A) Standard curve for Amplex Red assay measuring H₂O₂ levels from 0 to 3 μM. B) Summary data for Amplex Red fluorescence in isolated pulmonary arteries from control rats for increasing concentrations of SOTS-1 (0.01, 0.1, 1.0 mM) and SOTS-1 (0.01 mM) plus PEG-catalase (250 U/ml). Overflow indicates saturation in the reading from the microplate reader. Values are means ± SE by one way ANOVA; n = 3-4/group.

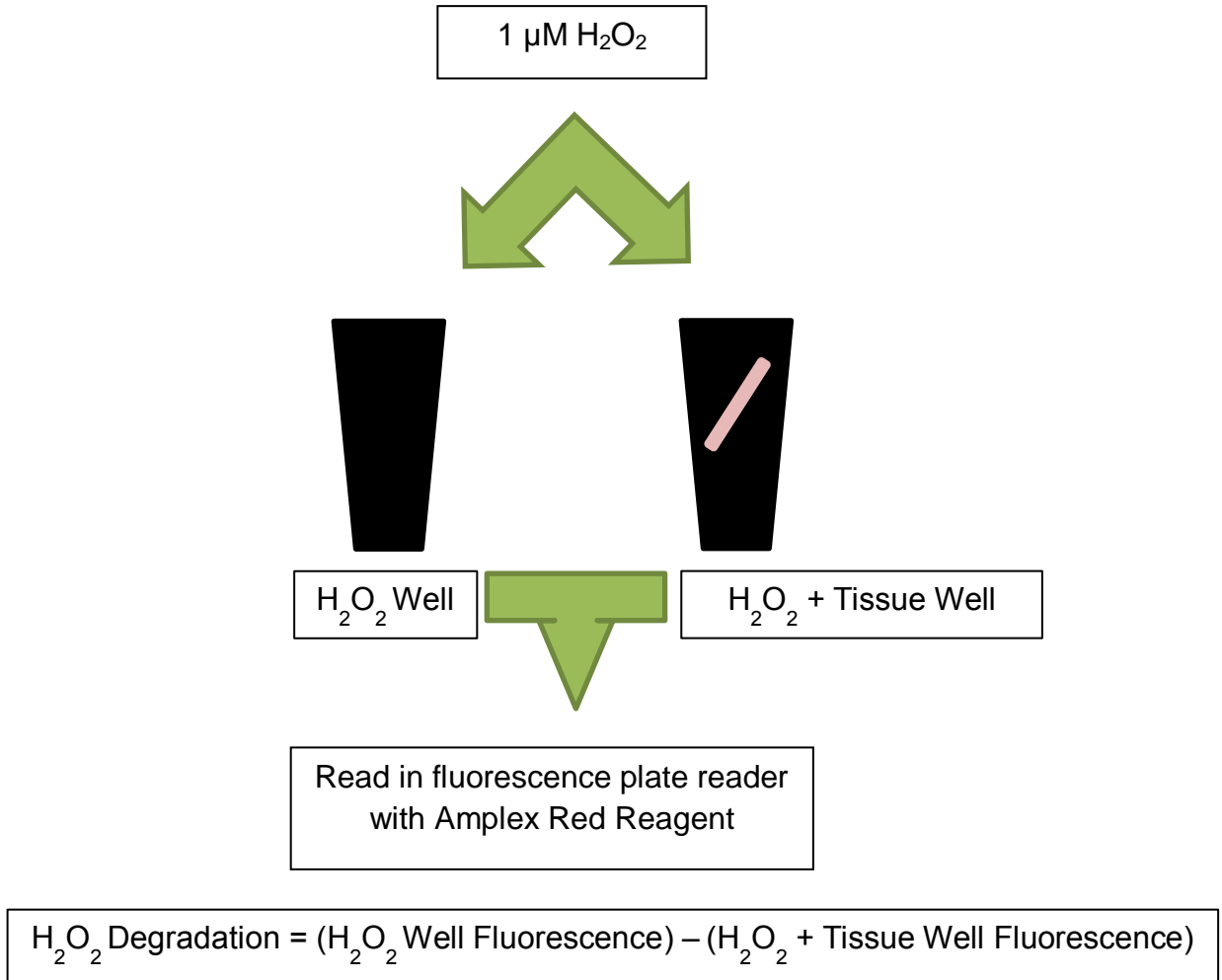


Figure 5: Method for H_2O_2 degradation. 2mm pulmonary artery segments from control rats and rats exposed to chronic hypoxia were incubated with 1 μM H_2O_2 for 1 hr at RT. Supernatant was transferred to a 96-well plate and incubated with Amplex Red reagent according to manufacturer's directions for 30 min at RT. Fluorescence was read in a microplate reader at 610 nm. The amount of H_2O_2 catalyzed by the tissue was assessed by taking the raw fluorescence value, indicative of the amount of H_2O_2 left in the tissue, and subtracting this from the 1 μM H_2O_2 standard well without tissue.

Western Blotting

Pulmonary arteries were dissected in ice-cold HEPES-PSS and snap frozen in liquid N₂. Samples 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). Pulmonary artery lysates were separated by SDS-PAGE (Tris-HCl gels, Bio-Rad) and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 hr at RT with 5% milk and 0.05% Tween 20 (Bio-Rad) in Tris-buffered saline (TBS) containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.5). Blots were incubated with rabbit anti-SOD1 (1:5,000 Abcam), rabbit anti-SOD2 (1:5,000 Abcam), rabbit anti-SOD3 (1:500; Abcam), rabbit anti-catalase (1:2,000; Pierce Thermo Scientific), or rabbit anti-GPx-1 (1:5,000; Abcam). For immunochemical labeling, blots were incubated for 1 hr at RT with goat anti-rabbit IgG-horseradish peroxidase (1:3,000; Bio-Rad). After chemiluminescence labeling (ECL, Pierce Thermo Scientific), bands were detected by exposing the blots to chemiluminescence-sensitive film (Bio-Express). Bands were normalized to Coomassie staining. β-actin and GAPDH as traditional loading controls were first tested but were significantly elevated in the CH group ($p < 0.05$) whereas there was not a statistically significant difference between control and CH with Coomassie staining. Bands were quantified by densitometric analysis using ImageJ (NIH).

SOD Activity Assay

SOD activity was determined by an SOD Assay kit (Cayman Chemical). The assay was performed according to manufacturer's directions with some modifications. Pulmonary arteries from the right descending branch were dissected in ice-cold HEPES-PSS and snap frozen in liquid N₂. Samples 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 1:25 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/ZnSOD for 20 minutes at RT. Absorbance was detected at 450 nm using an absorbance microplate reader (BioTek Instruments, Inc. ELx800). Sample protein concentrations were determined by spectrophotometer (Nano Drop 2000; Thermo Scientific).

Catalase Activity Assay

Catalase activity was determined by a Catalase Assay kit (Cayman Chemical). The assay was performed according to manufacturer's directions. Pulmonary arteries were dissected in ice-cold HEPES-PSS and snap frozen in liquid N₂. Samples 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 (Molecular Devices; SpectraMax Plus384).

Glutathione Peroxidase Activity Assay

Glutathione peroxidase activity was determined by a Glutathione Peroxidase Assay kit (Cayman Chemical). The assay was performed according to manufacturer's directions with some modifications. Pulmonary arteries were dissected in ice-cold HEPES-PSS and snap frozen in liquid N₂. Samples 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, once every minute for 6 minutes, using an absorbance microplate reader (Molecular Devices; SpectraMax Plus384).

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, one-way ANOVA, 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 was used to make comparison for data converted to percent. A probability of $P < 0.05$ was accepted as significant for all comparisons.

Chapter 3 – Results

Specific Aim 1

Determine the effect of CH on pulmonary arterial H₂O₂ levels.

Hypothesis.

We hypothesize that H₂O₂ levels are decreased following CH.

Pulmonary Arterial H₂O₂ Levels Decreased Following CH.

To assess the effect of CH on pulmonary arterial H₂O₂ levels, an Amplex Red assay was performed. Following incubation in 10 μM SOTS-1, H₂O₂ levels in pulmonary artery segments from rats exposed to CH were significantly lower than in those from control rats (Figure 6). With the addition of PEG-catalase (250 U/ml), Amplex Red fluorescence was significantly attenuated in both groups indicating the fluorescence with SOTS-1 treatment was from H₂O₂ (Figure 6).

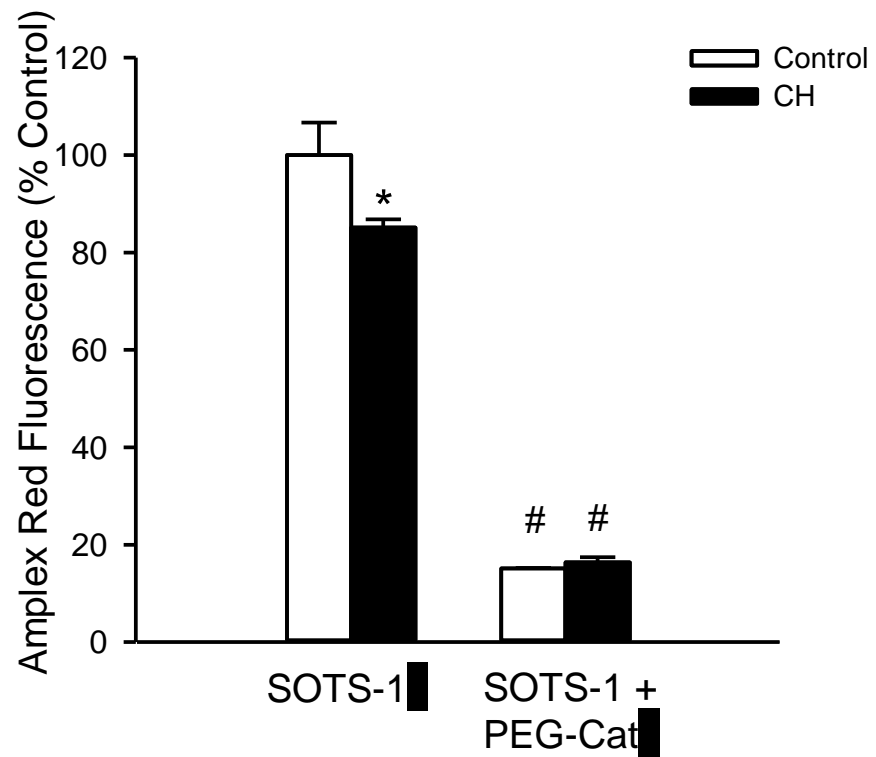


Figure 6: CH decreases pulmonary arterial H₂O₂ levels. Summary data for Amplex Red fluorescence in pulmonary arteries from control rats and rats exposed to CH. H₂O₂ was stimulated with the addition of 10 μM SOTS-1. Segments were additionally treated with PEG-catalase (250U/ml). Values are means ± SE by two-way ANOVA; n = 3/group; **p*<0.05 vs. control.

Specific Aim 2

Determine the effect of CH on H₂O₂ production in pulmonary arteries.

Hypothesis.

We hypothesize that SOD expression and activity are decreased following CH.

SOD Expression and Activity Are Decreased Following CH.

To determine if production of H₂O₂ is impaired following CH, we examined the expression and activity of SOD1, SOD2, and SOD3. In pulmonary arteries from rats exposed to CH, protein expression of SOD1 and SOD3 was significantly decreased compared to control when normalized to Coomassie staining. However, there was no significant change in SOD2 protein expression following CH (Figure 7a,b). Consistent with this result, we found that total SOD and Cu/ZnSOD (SOD1 and SOD3) activity was significantly decreased in pulmonary arteries from rats exposed to CH compared to control, with no statistically significant change in MnSOD activity (SOD2)(Figure 8).

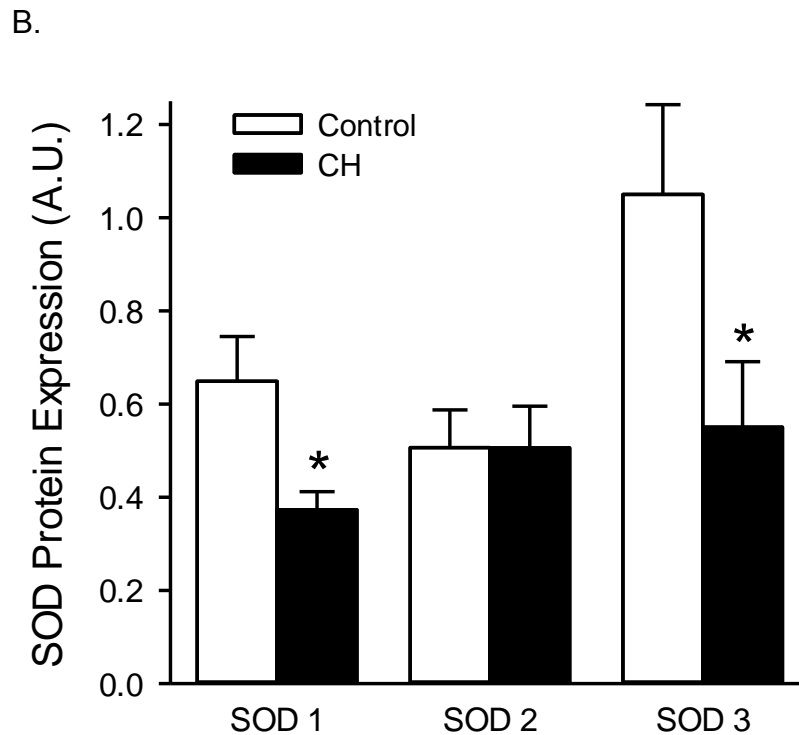
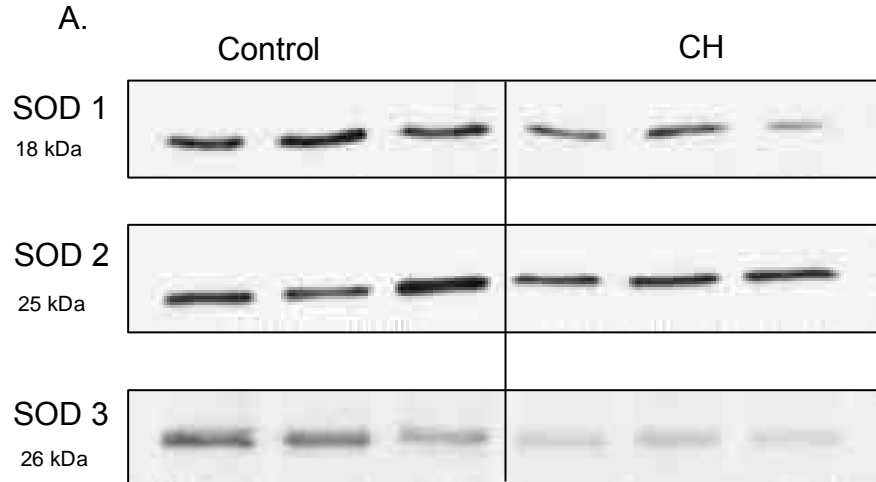


Figure 7: CH decreases pulmonary arterial SOD expression. A) Representative western blots for SOD1 (18kDa), SOD2 (25kDa), and SOD3 (26kDa) protein expression in isolated pulmonary arteries from control rats and rats exposed to CH. B) Summary data for analysis of SOD1, SOD2, SOD3 protein expression in isolated pulmonary arteries from control rats and rats exposed to CH normalized to Coomassie staining. Values are means \pm SE by *t*-test; *n* = 6-9/group; **p* \leq 0.05 vs. control.

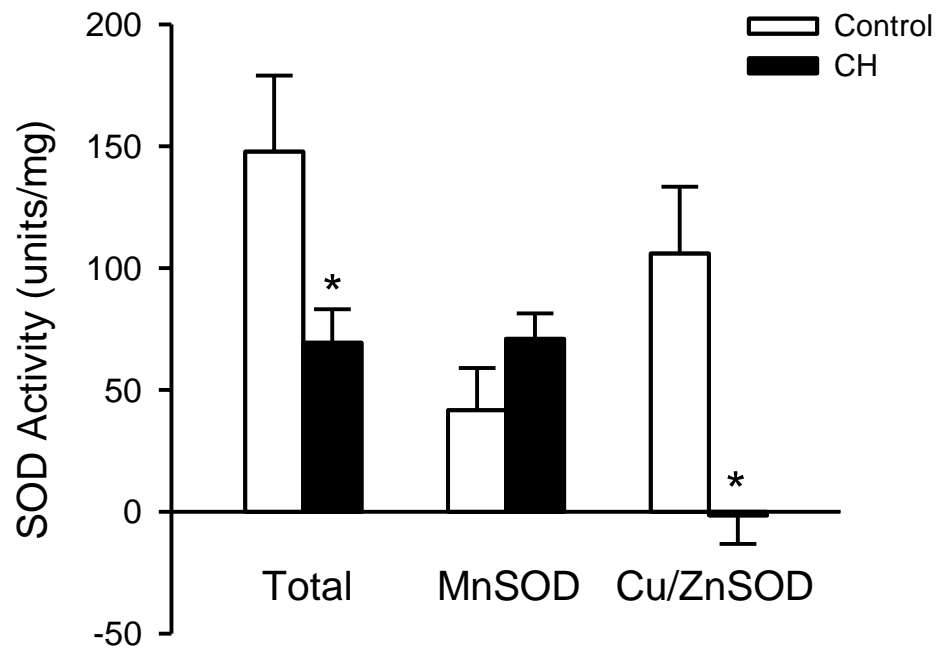


Figure 8: CH decreases pulmonary arterial SOD activity. Summary data for total, MnSOD (SOD2), and Cu/ZnSOD (SOD1 and SOD3) activity in isolated pulmonary arteries from control rats and rats exposed to CH. Values are means \pm SE by *t*-test; *n* = 6/group; **p*<0.05 vs. control.

Administering SOD Does Not Normalize Pulmonary Arterial H₂O₂ Levels Following CH.

Next we sought to determine if restoring SOD would normalize the decreased H₂O₂ levels observed following CH. The addition of PEG-SOD and the SOD mimetic tiron to pulmonary artery segments from control rats and rats exposed to CH did not normalize endogenous H₂O₂ levels as assessed by Amplex Red (Figure 9).

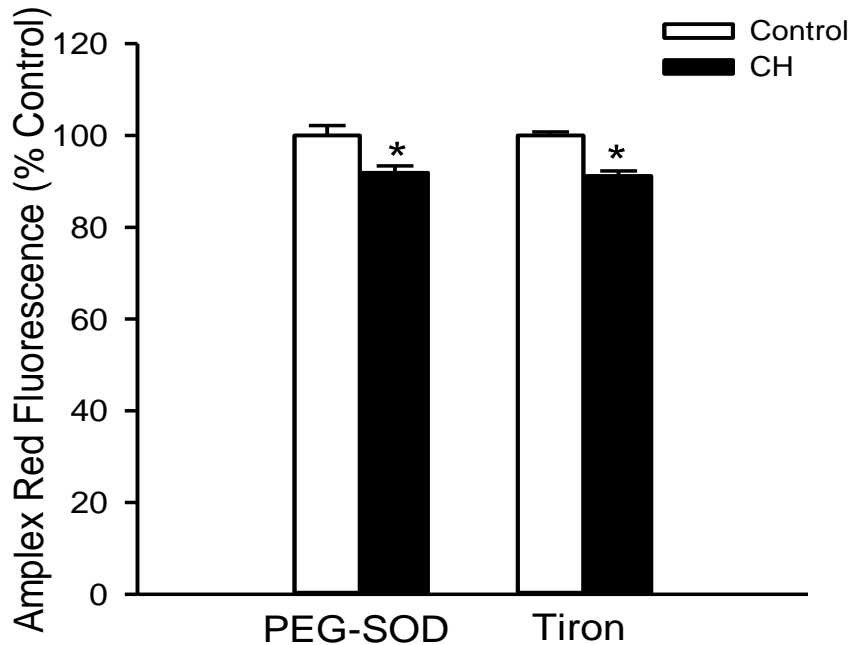


Figure 9: Administering SOD does not normalize pulmonary arterial H₂O₂ levels following CH. Summary data for H₂O₂ as affected by PEG-SOD (50 U/ml) or tiron (10 mM) in pulmonary artery segments from control rats and rats exposed to CH. Values are means \pm SE by rank sum *t*-test; n = 6/group; **p*<0.05 vs. control.

Specific Aim 3

Determine the effect of CH on H₂O₂ degradation in pulmonary arteries.

Hypothesis.

We hypothesize that catalase and glutathione peroxidase expression and activity are increased following CH.

H₂O₂ Degradation Increased Following CH.

Since administering PEG-SOD and tiron did not restore H₂O₂ levels to control levels, we focused on H₂O₂ degradation. Inhibiting catalase with 3-amino-1,2,4-triazole and glutathione peroxidase (GPx) with mercaptosuccinic acid normalized endogenous H₂O₂ levels in pulmonary artery segments between control rats and rats exposed to CH (Figure 10a). With the addition of H₂O₂ to pulmonary artery segments, tissue from rats exposed to CH degraded H₂O₂ more efficiently than control tissue (Figure 10b).

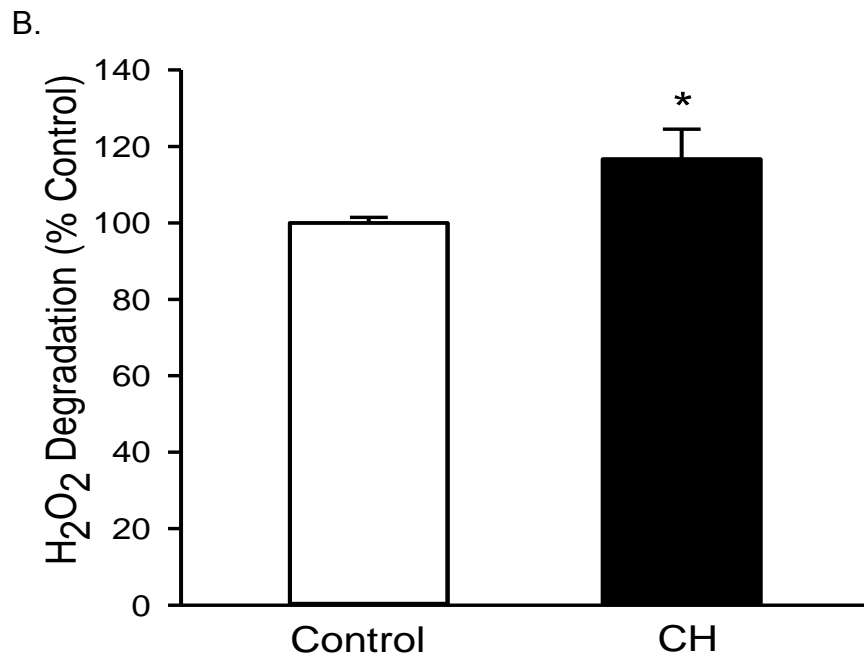
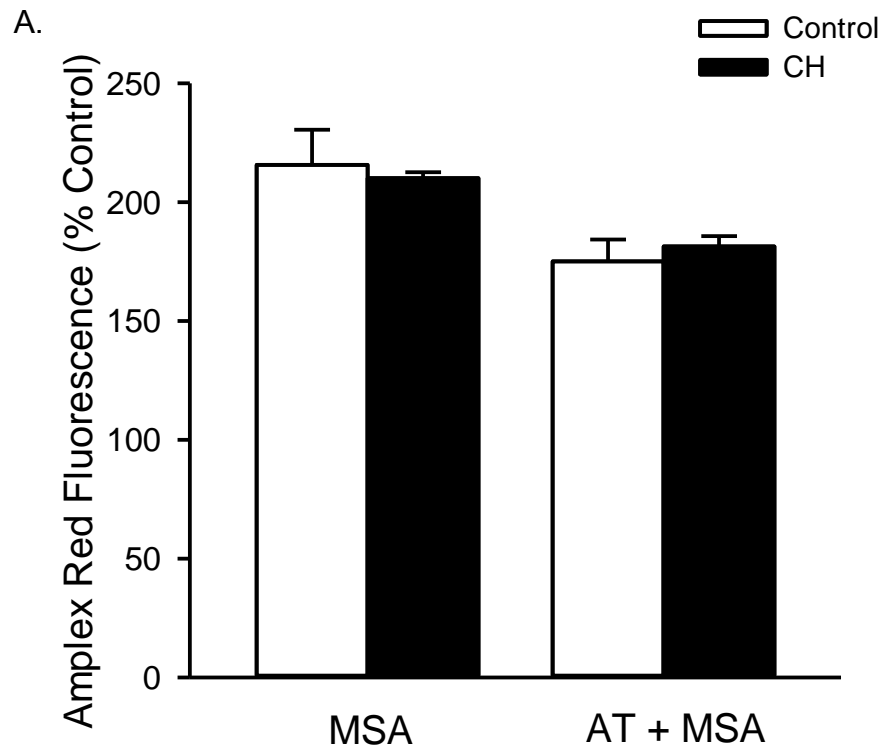


Figure 10: H₂O₂ catalysis increased in pulmonary arteries following CH. A) Summary data for endogenous H₂O₂ as affected by mercaptosuccinic acid (MSA)(3 mM) and 3-amino-1,2,4-triazole (AT)(5 mM) in pulmonary artery segments from control rats and rats exposed to CH. B) Summary data for H₂O₂ degradation as measured by Amplex Red with H₂O₂ treatment (1 μ M) in pulmonary artery segments from control rats and rats exposed to CH. Values are means \pm SE by rank sum *t*-test; n = 3-9/group; **p*<0.05 vs. control.

Glutathione Peroxidase Expression and Activity Are Increased Following CH.

Due to the increased catalysis of H_2O_2 observed (Figure 10b), we assessed expression and activity for the two main enzymes responsible for H_2O_2 degradation: catalase and glutathione peroxidase. There was a significantly higher amount of GPx-1 in pulmonary arteries from rats exposed to CH compared to control when normalized to Coomassie staining (Figure 11a). In addition, activity of GPx was higher in pulmonary arteries from rats exposed to CH (Figure 11b). There was not a statistically significant difference in catalase expression or activity between pulmonary arteries from control rats and rats exposed to CH (Figure 12a,b).

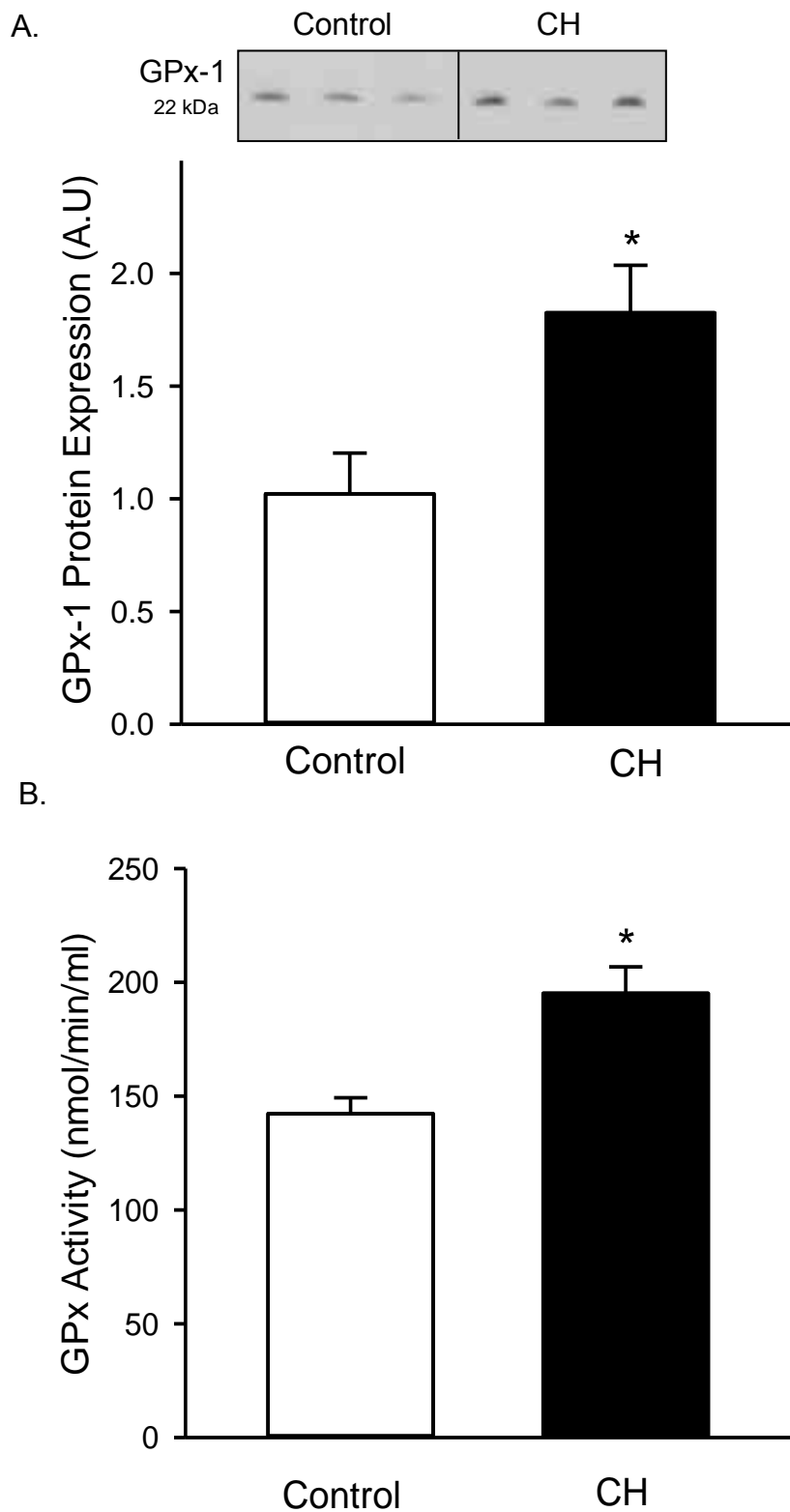


Figure 11: Glutathione peroxidase (GPx) expression and activity are increased following CH. A) Representative western blot and summary data for analysis of GPx-1 protein expression (22kDa) in isolated pulmonary arteries from control rats and rats exposed to CH normalized to Coomassie staining and B) GPx activity in pulmonary arteries from control rats and rats exposed to CH. Values are means \pm SE by *t*-test *n* = 6/group; **p*<0.05 vs. control.

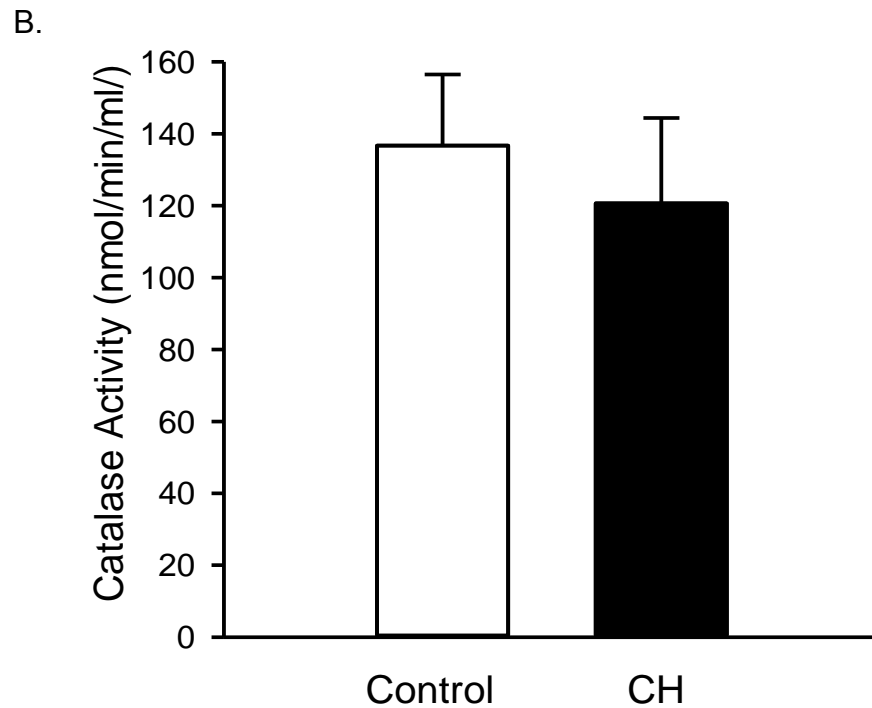
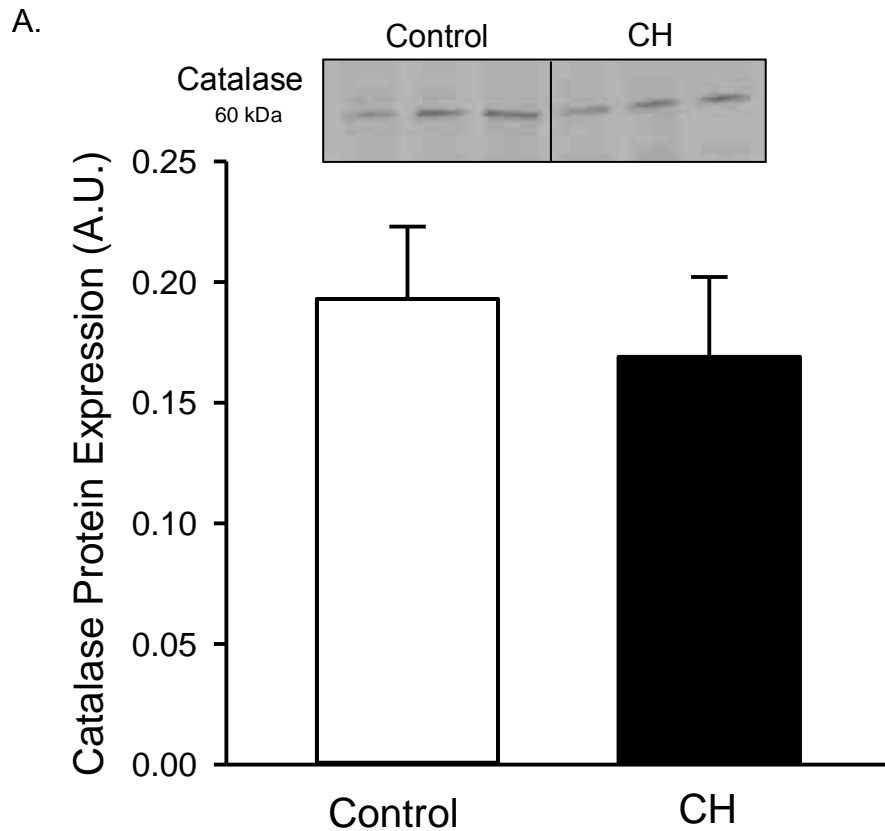


Figure 12: Catalase expression and activity are unchanged following CH. A) Representative western blot and summary data for analysis of catalase protein expression (60kDa) in isolated pulmonary arteries from control rats and rats exposed to CH normalized to Coomassie staining and B) catalase activity in pulmonary arteries from control rats and rats exposed to CH. Values are means \pm SE by *t*-test $n = 6$ /group; * $p < 0.05$ vs. control.

Chapter 4 - Discussion

The overall objective of this project was to examine H_2O_2 levels following CH and mechanisms that contribute to H_2O_2 production and degradation that are potentially altered in rat pulmonary arteries. The major findings of this project are that CH: 1) decreases H_2O_2 levels; 2) decreases SOD expression and activity; and 3) increases H_2O_2 degradation via glutathione peroxidase in pulmonary arteries. We conclude that decreased H_2O_2 levels following CH may contribute to the augmented ASIC1-dependent SOCE observed in pulmonary arteries and PASMC (79, 118).

H_2O_2 Levels and Potential Regulation

Though controversy exists regarding whether pulmonary ROS are elevated following CH (99, 102, 105, 120, 132, 181), our laboratory has previously shown increased O_2^- following CH (81). This should indicate a parallel increase in H_2O_2 levels due to dismutation by SOD. However, many forms of pulmonary hypertension indicate dysfunction in SOD (4, 37, 48, 86, 121, 130, 182). Thus we sought to assess H_2O_2 levels in pulmonary arteries following CH. The finding of decreased H_2O_2 levels (Figure 6) is consistent with previous studies from our Vascular Physiology Group which found decreased levels in pulmonary arteries of mice after 5 days of CH (130) and also consistent with findings from a CH-induced pulmonary hypertension model in piglets (37).

There is inconsistency in the literature regarding the role H_2O_2 serves in HPV and as a signaling molecule in general. Some groups report vasoconstrictor responses (84, 148, 183), while others report dilation with the differences

revolving around concentration (19, 20). In addition, various H₂O₂ constriction induced-mechanisms have been reported (83). Without constriction data, our finding of lower H₂O₂ levels is difficult to place in a larger context in regards to direct effects on vasoreactivity. Further discussion is addressed in the Future Directions section on this topic. As others have found dysregulation in SOD in regards to expression and activity (4, 37, 48, 86, 121, 130, 182), this prompted further investigation as to the regulation of H₂O₂ levels.

Regulation of SOD

The significant reduction in SOD1 and SOD3 protein in pulmonary arteries following CH, and analogous Cu/ZnSOD activity reduction (Figure 7, 8) is consistent with data from a model of CH-induced pulmonary hypertension in piglet pulmonary arteries which found reduced expression of SOD1 and SOD3, reduced SOD1 activity, and no changes in SOD2 expression and activity (37, 48). Similar findings were noted in a fetal lamb model of pulmonary hypertension showing a decrease in total SOD activity and no change in SOD2 expression in pulmonary arteries (15). A decrease in SOD2 expression, which would also mediate lower H₂O₂ levels, has been found in idiopathic forms of pulmonary hypertension including in PASMC from fawn hooded rats (4, 142) and humans with Group I pulmonary hypertension (4, 47).

SOD1 and SOD2 transcriptional activation has been linked to HIF-2 α in HEK cells (144), and a negative regulatory mechanism could be involved following HIF activation although we see no effect in SOD2. Endothelin as well as

NADPH-oxidase have been implicated in decreasing Cu/ZnSOD expression (27), though the mechanism of action is not known.

The transcription factor activating protein-1 (AP-1) has been shown to regulate SOD protein expression. One study showed that the binding of AP-1 mediated a decrease in Cu/ZnSOD expression by blocking another transcription factor, Sp1 (6). Interestingly, the induction of *c-fos* and *c-jun*, components of AP-1, is increased by O_2^- (145), which we have shown to be augmented following CH (81). Studies in cancer cells have shown an increase in AP-1 transcription after sustained hypoxia with a dependence on HIF-1 α (106, 108). In a model of CH using pulmonary artery endothelial cells, AP-1 binding activity is increased and dependent on Ca^{2+} influx via SOCs (43, 100). A decrease in Cu/ZnSOD expression therefore could be due to AP-1 activation.

A potential explanation for the reduction in SOD1 and SOD3 activity following CH is the availability of copper and zinc which act as cofactors for the enzymes. In rats fed a reduced copper diet, Cu/ZnSOD activity is reduced in erythrocytes and the aorta (143). Similarly, in rats and rhesus macaques fed a reduced zinc diet, plasma SOD3 activity is reduced (123). There does not appear to be any research done on copper and zinc levels in pulmonary arteries following CH currently.

Another explanation for decreased SOD1 and SOD3 activity is that the delivery of copper to SOD could be compromised. Copper is shuttled to SOD1 in particular by the copper chaperone for SOD1 (CCS)(26). CCS expression is not altered in pulmonary arteries in a fetal lamb model of pulmonary hypertension

(15). Interestingly, however, CCS requires oxygen (O_2) for the posttranslational activation of SOD1, with no activation under anaerobic conditions but activation with exposure to room air (18, 52). The model of CH-induced pulmonary hypertension used in this thesis represents a partial pressure O_2 of 70 mmHg, versus 122 mmHg in Albuquerque, NM ($P_{IO_2} = F_{IO_2} \cdot (P_B - P_{H_2O})$), with even less O_2 entering into the pulmonary tissue (14). With a hypoxic environment, less O_2 could affect CCS and therefore reduce SOD1 activity. Copper metabolism Murr1 domain containing 1 (COMMD1) protein has also been shown to regulate SOD1 transcription and activity in an inhibitory fashion in situations of excess copper by binding to CCS and preventing final steps of SOD1 activation (166). CH has been shown to increase expression of copper transport proteins in a HIF-1 α dependent manner and increase copper uptake in pulmonary arteries (187). Interestingly, copper is required for HIF-1 α activation (45), so hypoxia could have multiple effects on the system with an end result of decreased SOD1 activity. SOD3 copper is not regulated by CCS, but by Antioxidant-1 (Atox1) which delivers copper to the trans-Golgi network since SOD3 is extracellular (78). Following CH, however, there is no change in Atox1 levels in mouse pulmonary arteries (187).

Lastly, regulation of SOD activity involves a feedback mechanism as SOD1 and SOD3 can initiate a peroxidase reaction which disrupts enzyme activity, without affecting SOD2 (55, 67, 68, 85). This occurs through oxidation at multiple histidine sites, many of which are normally involved in the binding of copper to the enzyme (90, 164). This has been demonstrated in a lamb model of

persistent pulmonary hypertension of the newborn, with H₂O₂ inhibiting SOD3 activity in PASMC (178). This could explain the discrepancy between SOD1 and SOD3 expression (Figure 7) and activity levels (Figure 8). There was not a complete reduction in SOD1 and SOD3 expression and therefore subsequent H₂O₂ levels (Figure 6). Therefore, with some residual H₂O₂ produced, this could oxidize and inactivate SOD1 and SOD3 potentially explaining the complete attenuation of Cu/ZnSOD activity (Figure 8).

Regulation of Glutathione Peroxidase and Catalase

The observed reduction in H₂O₂ levels (Figure 6) could additionally be due to an increase in degradation. To address this question, we utilized several chemical tools. PEG-SOD and the SOD mimetic, tiron, were applied to pulmonary arteries to overcome the SOD1 and SOD3 deficiencies, but still low H₂O₂ levels persisted in pulmonary arteries from rats exposed to CH (Figure 9). Shifting to the degradation portion of the pathway, we utilized the inhibitors 3-amino-1,2,4-triazole and mercaptosuccinic acid to inhibit catalase and glutathione peroxidase, respectively, as they are the two main enzymes responsible for H₂O₂ catalysis. If degradation has a greater contribution to the low H₂O₂ levels observed, inhibiting degradation should normalize H₂O₂ levels between the control and CH groups. This is indeed what was observed (Figure 10a) indicating that enhanced degradation of H₂O₂ is a larger contributing factor to the decreased H₂O₂ levels observed following CH in pulmonary arteries versus production by SOD. When H₂O₂ degradation was assessed, tissue from rats

exposed to CH had enhanced catalysis of H₂O₂ (Figure 10b). However, this analysis does not distinguish which enzymes, catalase or GPx, are contributing to the degradation of H₂O₂.

When the protein expression and activity of these enzymes was assessed, we found a significant increase in only GPx in pulmonary arteries from rats exposed to CH (Figure 11) with no change in catalase (Figure 12). In this study, GPx-1 in particular was examined for expression, which is constitutively expressed and intracellular in location (94). GPx-1 undergoes extensive transcriptional, post-transcriptional, translational, and post-translational regulation some of which is not related to this project such as gender or age specific regulation (126, 127), or regulation specific to a decrease in expression or activity (94).

Enzymes exist with transcriptional regulation that is sensitive to oxidative stress, including the antioxidant response element (ARE)(140) and oxygen response element (ORE)(34, 103), as well as transcriptional responses to ROS that are ARE-like (36). As with SOD, AP-1 transcription factors have been shown to bind the ARE in cells from rodents, most notably NF-E2-related factor 2 (Nrf2) with its effector protein Keap1 (30, 117). In an unstressed scenario, Keap1 prevents Nrf2 from binding the ARE. In various oxidative stress situations ROS and also xenobiotics, thiol modifications to Keap1 release Nrf2 and allow for binding to the ARE (117). PKC also been shown to phosphorylate Nrf2 (117, 119) and is increased in expression in rat pulmonary arteries following CH (150).

The ORE activates under conditions of changing oxygen tension and is regulated by the transcription factor Ku (34, 103).

GPx-2 (7, 29, 131, 153) and GPx-3 (11, 131) consistently reveal ARE responses to oxidative stress, but GPx-1 reveals more of a xenobiotic response to ROS (36, 168). Paraquat, a O_2^- generating drug (33), has been shown to induce GPx-1 transcription via the promoter as well as stimulate GPx activity in lungs of mice (36). Interestingly, the human GPx-1 does contain two OREs (34, 103). In addition, glutamate-cysteine ligase (GCL), the first enzyme in the glutathione biosynthesis pathway (29, 117) and glucose-6-phosphate dehydrogenase (G6PD) have an ARE (29, 131). G6PD has been shown to play a role in HPV and expression is increased in PASMC following hypoxia (28, 60). ROS, via TNF- α , has also been shown to increase GSH via GCL in alveolar epithelial cells (129).

One explanation for the increase in expression and activity of GPx includes, like with Paraquat (36), that the increased O_2^- we observe following CH (81) is stimulating increased GPx expression and activity. Another explanation includes links to a potential ARE with transcription regulation as well as a possible increase in one of the GPx substrates, GSH, via GCL which would increase GPx activity. Several groups have shown elevated GSH following CH (134, 180). In addition, SOD1 knockout mice reveal an increase in GCL mRNA and Nrf2 protein expression (64), which downstream could correspond to increased GSH. Taken together, this would allow for a shift in the redox state of the VSMC to a more reduced state due to higher GSH.

Both GPx and catalase activity can be regulated by the nonreceptor tyrosine kinases c-Abl and Arg. Although there was no change in catalase expression and activity, these data are consistent with a CH-induced pulmonary hypertension model in piglets (37). c-Abl activity in particular can be activated by ROS (159) and so it is possible that following CH there is a change in the phosphorylation status for these two enzymes (21, 22). Studies have shown that ROS levels correlate with catalase activity via c-Abl and Arg association. With an increase in ROS, catalase activity decreases due to c-Abl and Arg dissociating from catalase or targeting it for ubiquitination (23, 24). With a decrease in ROS, c-Abl and Arg associate with catalase activating it through phosphorylation (22, 24). There was a tendency for catalase expression and activity to be decreased in pulmonary arteries following CH, but this was not statistically significant (Figure 12). So, it is possible that the increase in O_2^- following CH (81) affects catalase, but perhaps this is being offset by the reduction in H_2O_2 .

GPx and catalase have very different K_m values, with GPx operating efficiently under low H_2O_2 conditions (25) whereas catalase with its high K_m is not as effective in low H_2O_2 (111, 136). Thus, since we observed higher GPx expression, higher GPx activity, and low H_2O_2 levels all with no changes in catalase and taking into account the K_m values the data indicate that GPx is the main enzyme responsible for H_2O_2 degradation following CH.

Future Directions

Although the present study provides insights regarding H₂O₂ levels and their regulation, further studies are needed to investigate how these levels affect ASIC1 activity and trafficking in VSM. Our laboratory has shown that the breakdown of H₂O₂ elicits a significant increase in ASIC1-dependent SOCE in control PASMC compared to vehicle, whereas there is no effect in PASMC from rats exposed to CH (128). This could indicate that, like observed in pulmonary arteries with Amplex Red (Figure 6), there is less H₂O₂ present in PASMC. With the addition of H₂O₂, ASIC1-dependent SOCE was significantly reduced in PASMC from both groups compared to vehicle (128). These data indicate that H₂O₂ attenuates ASIC1-dependent SOCE and that following CH there is a loss of H₂O₂ inhibition of SOCE. Interestingly, similar data have been found regarding H₂O₂ attenuation of SOCE in thyroid cells (163) and mast cells (160).

H₂O₂ attenuates plasma membrane localization of ASIC1 in transfected Chinese hamster ovary (CHO) cells (185). This could be through interacting with the trafficking protein PICK1 which has been shown to interact with ASICs (40, 72). PICK1 is regulated by redox state, with H₂O₂ causing disulfide bond formation within PICK1 (151). One explanation for the increase in ASIC1-dependent SOCE following CH (79, 118) may be due to lower H₂O₂ levels allowing PICK1 to traffic more channels to the plasma membrane.

Redox could also affect ASIC1 through glutathione levels. Several groups have shown elevated pulmonary GSH following CH (134, 180). This may be possible because of *de novo* synthesis initiated by ROS (129). Glutathione

contains a reactive thiol group which can donate or receive electrons. This allows it to exist in its reduced and oxidized form, GSH and GSSG respectively and also interact with other thiol groups on for example protein cysteines. Thereby, GSH is able to donate electrons and break an existing disulfide bond in a protein into two thiols (156). A higher level of reduced glutathione could act to break inhibitory disulfide bonds that form within ASIC1 subunits (185) and allow for more ASIC1-dependent SOCE. Studies performed in neurons examining ASIC1 current found that GSH enhanced ASIC1 current, whereas the oxidizing agent DTNB reduced current (2, 31, 32) and this was linked to modulation at extracellular cysteine 61 (32). In one particular study, the enhanced ASIC1 current remained despite wash out suggesting redox modulation by GSH at extracellular thiols (31). In addition to GSH, the reducing agent DTT has been shown to slow desensitization of the channel, allowing it to remain open longer (2, 31). Together, this suggests redox regulation of ASIC1 at thiols in the extracellular domain that contribute to channel kinetics.

ASIC1-dependent SOCE could also be affected by H_2O_2 itself. H_2O_2 is a strong oxidizing agent which can form hydroxyl radicals. These ROS can interact with protein thiols to form disulfide bonds (35). The majority of structural disulfide bonds (58) form within proteins designated for the plasma membrane inside the ER of cells (97). However, modifications to thiol groups can also occur in the cytosol (35). Interestingly, the ER maintains an oxidized state due to increased GSSG (73) and H_2O_2 (97, 98) allowing for the production of disulfide bonds within proteins undergoing processing (44). Part of the oxidized environment is

due to the disulfide bond formation process itself with endoplasmic reticulum oxidoreducin 1 protein and protein disulfide isomerase, in which H_2O_2 is a byproduct (146).

ASIC1 has numerous cysteines in its extracellular domain in addition to four within the C-terminal (58, 185). The subunits that form ASIC1 naturally have disulfide bonds linking them together, however H_2O_2 has been shown to increase the number of these bonds (185). Another explanation for how H_2O_2 is affecting ASIC1 is by causing disulfide bond formation within the ER or cytosol, and that following CH there less H_2O_2 present and therefore not as many disulfide bonds. One group has examined the role of H_2O_2 to ASIC1 current as well as plasma membrane localization in ASIC1 transfected CHO cells. Their studies revealed a decrease in ASIC1 current and plasma membrane localization with the addition of H_2O_2 , and both of these responses were C-terminal cysteine dependent (185). Other oxidizing agents can also inhibit ASIC1 current, as mentioned previously (2, 31, 32), and this inhibition has been linked to modulation of the extracellular domain (32). Taken together, this suggests that redox regulation of ASIC1 thiols via oxidants produced by H_2O_2 may contribute to channel trafficking. Although no studies have been performed examining H_2O_2 and channel kinetics, based on studies mentioned previously examining redox agents and the fact that H_2O_2 can be found in the cytosol and diffuse (10), it is also possible that redox modulation by H_2O_2 could affect ASIC1 activity. Therefore the shift in the levels of H_2O_2 following CH may allow for a change in ASIC1 activity.

Summary

In summary (Figure 13), this work reveals decreased pulmonary arterial H_2O_2 levels following CH. This reduction is due in part to lower levels of SOD1 and SOD3 as well as decreased SOD activity. In addition, higher GPx expression and activity contribute to the increased catalysis of H_2O_2 following CH. This work is consistent with previous data from our laboratory, that demonstrates a loss of H_2O_2 inhibition of ASIC1-dependent SOCE in PASMC (128)(Figure 13).

The implications of this work relate to the importance of Ca^{2+} regulation and its pivotal role in vasoconstriction in the broad scheme of pulmonary hypertension. This is especially important as current treatments for Group III PH are not very effective, including traditional Ca^{2+} channel blockers (39, 137). This work implicating an imbalance in ROS as an activator of ASIC1-dependent SOCE (128) is important in the path towards understanding mechanisms and new treatments for PH. In addition, the role of H_2O_2 in regulating vasoreactivity remains unclear (83). Future studies investigating the role of H_2O_2 and oxidizing and reducing agents in general to plasma membrane localization and channel kinetics are necessary to address key questions involving the regulation of ASIC1 especially in disease states such as pulmonary hypertension.

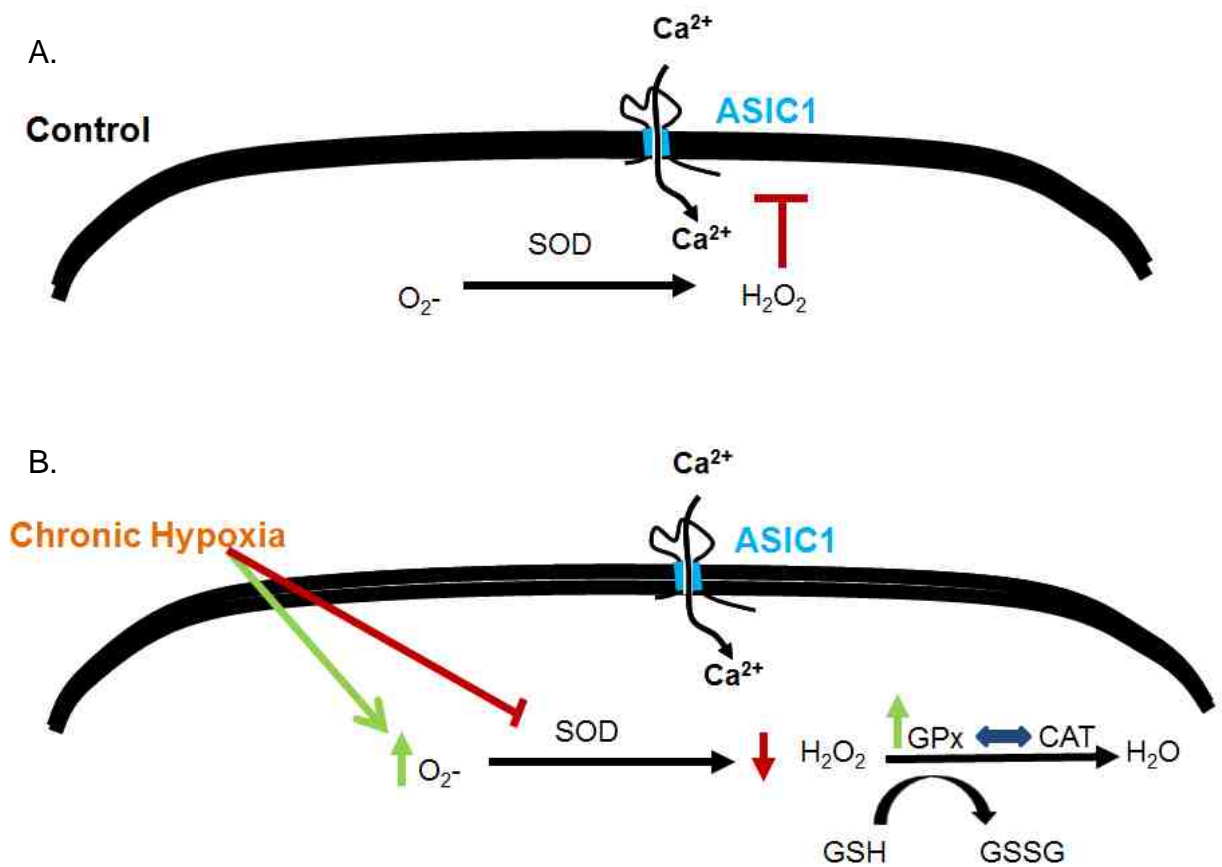


Figure 13: Summary diagram. A) Control pulmonary artery in which H₂O₂ inhibits ASIC1 SOCE and B) pulmonary artery from CH animal in which we have found elevated O₂⁻ and reduced H₂O₂. This reduction in H₂O₂ is due to a decrease in production from diminished SOD1 and SOD3 expression and activity and an increase in catalysis through GPx, allowing for increased ASIC1-dependent SOCE.

Chapter 5 - References

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