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Superoxide anions inhibit intracellular calcium response in porcine airway smooth muscle cells.

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Abstract:
BACKGROUND: Superoxide anions (O2-) have multiple effects on pulmonary parenchyma altering cell proliferation, cellular metabolism, and airway smooth muscle (ASM) contraction. Intracellular Ca2+ concentration ([Ca2+]i) plays a significant role in the regulation of ASM contraction, relaxation, proliferation, and gene expression.
OBJECTIVE: We investigated the effects of O2- on agonist-stimulated changes in [Ca2+]i in ASM cells.
DESIGN/METHODS: Fura-2 AM-loaded, freshly isolated porcine ASM (PASM) cells were used to examine [Ca2+]i release in response to acetylcholine (ACh), histamine, endothelin, caffeine, and thapsigargin in the presence or absence of extracellular calcium.
RESULTS: Exposure of PASM cells to xanthine and xanthine oxidase (X+XO) resulted in a time-dependent generation of O2-, inhibited by superoxide dismutase (SOD). Pre-incubating PASM cells with X+XO for 15- or 45-min inhibited net [Ca2+]i responses to ACh, Histamine, Caffeine, and Thapsigargin compared to control cells. Pretreating PASM cells with SOD for 30 min mitigated the inhibitory effect of X+XO treatment on ACh-induced Ca2+ elevation suggesting role of O2-. X+XO treatment also inhibited caffeine-and thapsigargin-induced Ca2+ elevation suggesting effect of O2- on intracellular calcium release and reuptake mechanisms.
CONCLUSIONS: Superoxide attenuates [Ca2+]i release, reuptake and may interfere with physiological functions of ASM cells.

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Superoxide anions inhibit intracellular calcium response in porcine airway smooth muscle cells.

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Keywords: Superoxide, airway smooth muscle cell, intracellular calcium, superoxide dismutase, oxidative lung injury

Abstract

BACKGROUND: Superoxide anions (O_2^-) have multiple effects on pulmonary parenchyma altering cell proliferation, cellular metabolism, and airway smooth muscle (ASM) contraction. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a significant role in the regulation of ASM contraction, relaxation, proliferation, and gene expression.
OBJECTIVE: We investigated the effects of O_2^- on agonist-stimulated changes in $[Ca^{2+}]_i$ in ASM cells.
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RESULTS: Exposure of PASM cells to xanthine and xanthine oxidase (X+XO) resulted in a time-dependent generation of O_2^-, inhibited by superoxide dismutase (SOD). Pre-incubating PASM cells with X+XO for 15- or 45-min inhibited net $[Ca^{2+}]_i$ responses to ACh, Histamine, Caffeine, and Thapsigargin compared to control cells. Pretreating PASM cells with SOD for 30 min mitigated the inhibitory effect of X+XO treatment on ACh-induced Ca^{2+} elevation suggesting role of O_2^-. X+XO treatment also inhibited caffeine-and

thapsigargin-induced Ca^{2+} elevation suggesting effect of O_2^- on intracellular calcium release and reuptake mechanisms.
CONCLUSIONS: Superoxide attenuates $[Ca^{2+}]_i$ release, reuptake and may interfere with physiological functions of ASM cells.

Introduction

The development of bronchopulmonary dysplasia (BPD) and other chronic newborn lung illnesses has been linked to oxidative stress, which is caused by an excessive buildup of reactive oxygen species, particularly superoxide [1]. Moreover, premature lung exposure to high oxygen levels results in the creation of reactive oxygen species, which in turn causes oxygen toxicity and altered airway smooth muscle reactivity [2]. Reactive oxygen species (ROS) have harmful effects that cause oxidative stress, which may play a role in the etiology of several adult lung diseases include acute respiratory distress syndrome, emphysema, and asthma [3, 4]. Inflammatory mediators of cell and tissue injury include ROS such as superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH-) [5]. It is unclear how ROS affects the physiological control of functions in cells that exist in the airways, such as cells of the airway smooth muscle (ASM). The primary contractile cells of the airways are ASM cells, and during a normal respiratory cycle, ASM cell contraction and relaxation control airway tone. Recent research has shown that ASM cells play a role in airway disorders through immunomodulation and structural remodeling in addition to contraction [6]. As a result, it has been shown that changes in the structure and function of ASM cells play a crucial role in the pathogenesis of many lung illnesses [7].

Calcium is a very versatile second messenger critical in the regulation of ASM cell functions including contraction, proliferation and secretion [8]. Cells keep a minimal basal

level of calcium. Activation of cells with external stimuli such as Gq-coupled G protein-coupled receptor agonists leads to elevation of intracellular calcium levels $[Ca^{2+}]_i$ which is essential for signal transduction in effector cells. Calcium (Ca^{2+}) in ASM cells regulates activities of numerous enzymes such as protein kinases, proteases, phospholipases, and endonucleases that are involved in a variety of cellular functions [9]. Moreover, deregulation of Ca^{2+} homeostasis is seen early in the development of irreversible cell injury [10]. The Ca^{2+} mobilizing pathways of ASM cells are Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs) and Ca^{2+} release from intracellular Ca^{2+} stores evoked by inositol 1,4,5- trisphosphate [Ins(1,4,5)P_3] through IP_3 receptor channels on the sarcoplasmic reticulum [9] . The initial Ca^{2+} release also activates Ca^{2+}-induced calcium release pathway presumably via activation of ryanodine receptors (RyRs) on sarcoplasmic reticulum [11] . Earlier studies from our laboratory and others have demonstrated that release of calcium from intracellular stores such as SR plays a pivotal role in agonist-induced calcium elevation in ASM cells. Alterations and impairments in any of these Ca^{2+}- mobilizing pathways will affect ASM cell functions due to modulation of basal and contractile agonist-induced calcium levels [3]. Oxidative stress is one such modulator that can influence the normal calcium homeostasis in ASM cells and therefore, oxidative stress-induced modulation of Ca^{2+} regulatory pathways in ASM cells needs to be proven.

In this study, we investigated the effect of oxidative stress on ASM using xanthine and xanthine oxidase (X/XO) to generate superoxide and study the effect of these O_2^- anions on intracellular store-mediated regulation of calcium homeostasis using freshly dissociated porcine ASM (PASM) cells.

MATERIALS AND METHODS

Materials

Routinely used reagents, acetylcholine and histamine were obtained from Sigma Chemical Company (St. Louis, MO). Fura-2 AM and dihydrorhodamine were purchased from Molecular Probes (Eugene, OR). Endothelin-1 (ET-1), SOD, caffeine, thapsigargin, xanthine (X), and xanthine oxidase (XO) were obtained from Calbiochem (La Jolla, CA). Cell dissociation kit and other reagents were purchased from Worthington Biochemical (Freehold, NJ).

Airway smooth muscle cell preparation

PASM cells were isolated from the trachea as described previously [12]. Briefly, 6- to 10-wk-old, outbred Yorkshire pigs (~10 -18 kg body weight) were anesthetized with an intramuscular injection of 8 mg/kg tiletamine hydrochloride-zolazepam (Telazol, Fort Dodge Laboratories, Fort Dodge, IA) and 8 mg/kg xylazine. The animals were euthanized by barbiturate overdose following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of University of Minnesota. Isolated tracheas were transferred to ice-cold Hank's balanced salt solution (HBSS) containing 10 mM HEPES, 11 mM glucose, 2.5 mM $CaCl_2$, and 1.2 mM $MgCl_2$ (pH 7.4) and maintained in an oxygenated environment. Following the removal of epithelium from the trachea, the airway smooth muscle layer was dissected and used for cell dissociation. The tissue was initially minced in ice-cold HBSS and transferred to Earle's balanced salt solution containing 20 U/ml papain and 0.005% DNase (Worthington Biochemical, Freehold, NJ), and incubated at 37℃ for 2 hours. After the initial incubation, 0.4 mg/ml type IV collagenase and 0.3 U/ml elastase were added and incubated at 37℃ until the cells were completely dispersed (~15-30 min). Cell dispersion was aided by gentle trituration with a fire-polished glass pipette. The solution was

centrifuged at 2,000 rpm for 5 min, and the pelleted cells were resuspended in HBSS. The cells were placed at 4°C overnight and subsequently prepared for plating. The cell suspension (200 µl) was pipetted onto glass coverslips and allowed to attach at 37°C in 95% O_2 and 5% CO_2 incubator for 30 min. Coverslips with attached cells were placed in HBSS containing 5 µM Fura-2 AM (Molecular Probes, Eugene, OR) and incubated at 37°C for 30 min. Coverslips were washed with HBSS, treated as described in the experimental protocols, and used to determine $[Ca^{2+}]_{i.}$

Digital video fluorescence imaging

Coverslips were mounted on an open slide chamber (Warner Instruments, Hamden, CT) and placed on the stage of a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan). Cells were perfused with HBSS, or agonists as described in the protocol. The cells were visualized using a Nikon Fluor 40X oil immersion objective lens. Fura-2-loaded cells were excited at 340 and 380 nm using a Lambda DG-4 filter changer (Sutter Instrument, Novato, CA), and emissions were collected using a 510 nm barrier filter. Fluorescence excitation, image acquisition, and real-time data analyses were controlled using a Metafluor fluorescence imaging system (Universal Imaging, Bedford Hills, NY). Images were acquired using a Photometric Cool Snap 12-bit digital camera (Roper Scientific, Teledyne Photometrics, AZ) and transferred to a computer for subsequent analysis. The ratio of fluorescence intensities at 340 and 380 nm were calculated approximately every 0.75 s, and $[Ca^{2+}]_i$ was calculated from the ratio of intensities at 340 nm and 380 nm by extrapolation from a calibration curve as described previously [13].

Superoxide generation

Xanthine (X) and Xanthine oxidase (XO) were used to generate superoxide in all the experiments. Superoxide generation was determined fluorometrically using dihydrorhodamine. PASM cells were loaded with 5 μM dihydrorhodamine for 30 min and washed with HBSS to remove excess dye. The cells were resuspended in HBSS containing 100 mM xanthine, and basal fluorescence was measured at 485 nm and 538 nm excitation and emission wavelengths, respectively. Then, xanthine oxidase was added at a final concentration of 10 mU/ml to the cell suspension, and the change in the fluorescence was measured over time. Our findings suggest that treatment of cells with xanthine and xanthine oxidase results in a sustained release of superoxide anions as demonstrated by an increase in rhodamine fluorescence (Figure 1). Further, in a select set of experiments, cells were pre-incubated with HBSS containing superoxide dismutase (SOD) 500 U/ml for 30 min, treated with X/XO as described above and the generation of superoxide was determined. Pretreatment of cells with SOD resulted in a significant ($p<0.05$) inhibition of X/XO-induced generation of superoxide in PASM cells (Figure 1).

Experimental protocols

Agonist-induced intracellular calcium responses:

PASM cells loaded with Fura-2 AM were superfused with HBSS containing calcium and magnesium, and basal $[Ca^{2+}]_i$ was determined as described above. After the baseline $[Ca^{2+}]_i$ reached a stable level, the cells were stimulated with acetylcholine (Ach), histamine, or endothelin-1 (ET-1) for 2 min (concentrations described in the results/figure legends) and change in $[Ca^{2+}]_i$ was determined. Cells were subsequently washed with HBSS for 5-10 min (Figure 2).

Cells loaded with Fura-2 AM were washed and maintained in HBSS with no calcium and containing 1 mM lanthanum chloride ('0' Ca^{2+} HBSS). Basal $[Ca^{2+}]_I$ was determined as described above. The cells were subsequently perfused with '0' Ca^{2+} HBSS containing 100 nM ACh, 1 μM ACh, 50 μM histamine, 200 nM ET-1, 50 nM caffeine, or 3 μM thapsigargin, for at least 1 min. Changes in $[Ca^{2+}]_i$ was monitored during stimulation of cells with agonists followed by which the cells were washed with fresh HBSS. $[Ca^{2+}]_i$ upon agonist stimulation was determined by calculating net $[Ca^{2+}]_i$ by subtracting peak $[Ca^{2+}]_i$ from the basal $[Ca^{2+}]_i$ (Figure 3). In a select set of experiments, calcium data were analyzed by calculating the area under the curve (AUC) for a given time period of agonist stimulation (Figure 4).

Effects of superoxide (O_2^-) on $[Ca^{2+}]_i$ responses:

Cells loaded with Fura-2 AM were perfused with HBSS with or without Ca^{2+}. PASM cells were preincubated with X/XO for 15, 30, or 45 min followed by which the cells were used to measure the basal and agonist-induced increase in $[Ca^{2+}]_i$ as described above. After determining basal $[Ca^{2+}]_i$, the cells were stimulated with 100 nM ACh, 1 μM ACh, 50 μM histamine, or 200 nM ET-1 for at least 1 min, and agonist-induced change in $[Ca^{2+}]_i$ was determined. In a select set of experiments, PASM cells were incubated with 500 U/ml SOD for 30 min following with the cells were treated with X/XO for 30 minutes, and 100 nM Ach-induced change in $[Ca^{2+}]_i$ was determined as described above (Figure 5).

Effect of superoxide on ryanodine receptor-mediated calcium release:

To study the effects of superoxide on ryanodine receptor-mediated calcium release we stimulated the isolated PASM cells with caffeine and thapsigargin. Caffeine is known to induce calcium release by sensitizing ryanodine receptor (RyR) to calcium [20]. We used

thapsigargin (TPG) to decrease the SR ATPase enzyme activity that is essential for the re-uptake of Ca^{2+} into stores, inhibition of which will increase the cytoplasmic Ca^{2+} concentration. The experiments were conducted in zero calcium conditions to prevent the influx and efflux of Ca^{2+} from extracellular space.

Cells loaded with Fura-2 AM were perfused with HBSS containing calcium or '0' Ca^{2+} HBSS. PASM cells were preincubated with X/XO for 30 min followed by which the cells were used to measure the basal and agonist-induced increase in $[Ca^{2+}]_i$ as described above. After determining basal $[Ca^{2+}]_i$, the cells were stimulated with 5 mM caffeine or 3 μM thapsigargin for at least 1 min, and agonist-induced change in $[Ca^{2+}]_i$ was determined. The mean net responses to caffeine and thapsigargin were determined as described (Figure 6).

Statistical analysis

All experiments were repeated in at least 4-5 different cell preparations. Calcium data was analyzed either by determining the net change in $[Ca^{2+}]_i$ by subtracting basal $[Ca^{2+}]_i$ from the peak $[Ca^{2+}]_i$ or by calculating area under curve (AUC) for a specific period of time of agonist stimulation. Statistical significance was determined using Student's *t*-test or one-way analysis of variance (ANOVA) using GraphPad Prism 9 (GraphPad Inc., San Diego, CA) statistical software. The two means were considered significantly different when the *p*-value was less than 0.05.

Results

Generation of superoxide

The overall goal of these studies was to determine the effect of superoxide anion species on the regulation of dynamic intracellular calcium concentration in ASM cells. In

this context, we established an experimental model in which we treated freshly isolated PASM cells with superoxide anions generated by the action of xanthine oxidase on its substrate xanthine. The cells were incubated with 100 mM xanthine prepared in HBSS and the addition of 10 mU/ml xanthine oxidase to the cell suspension demonstrated a sustained release of superoxide as determined using a fluorometer in relative fluorescence unit (RFU) (Figure 1). Furthermore, change in fluorescence of rhodamine by treatment of cells with xanthine and xanthine oxidase was significantly ($p<0.05$, $n=4$) inhibited by pretreating cells with superoxide dismutase for 30 min (Figure 1). These findings suggest that xanthine generates superoxide anions when treated with xanthine oxidase which could be used as an experimental model to study the effect of superoxide anions on intracellular calcium regulatory mechanisms in ASM cells.

Effect of superoxide on an agonist-induced elevation of $[Ca^{2+}]_i$

Ach is the endogenous ligand released from parasympathetic nerve terminals and activates ASM cells. Therefore, we conducted experiments with Ach to assess the effect of superoxide anions on $[Ca^{2+}]_i$ in both regular calcium and zero calcium conditions. Stimulation of PASM cells maintained in regular HBSS and zero calcium HBSS with 100 nM or 1 μM Ach resulted in an increase in $[Ca^{2+}]_i$ (Figure 2A and B). Further, pre-treatment of cells with superoxide generated by the X/XO significantly attenuated this response (Figure 2C and D). Evaluation of baseline calcium concentration suggests that exposing ASM cells to X/XO for 15-45 min does not modulate basal calcium concentration in ASM cells. These data suggest that acute exposure of PASM cells to superoxide anions attenuate Ach-induced elevation of $[Ca^{2+}]_i$.

ASM cells express multiple Gq-coupled receptors and during disease conditions several mediators are released which function as ligands for these receptors. For example, histamine and endothelin-1 are released during airway inflammation and acts of ASM cells via H1 and ET1 receptors, respectively. Therefore, we investigated the effect of superoxide anions on histamine- and endothelin 1-induced elevation of $[Ca^{2+}]_i$. Histamine and endothelin-1 stimulation of PASM cells maintained in regular (Figure 3A) and zero calcium HBSS (Figure 3B) resulted in elevation of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ responses to histamine (Figure 3C) and endothelin 1 (Figure 3D) were attenuated by pre-treatment of PASM cells with X/XO. Studies related to superoxide effect were conducted in cells marinated in zero calcium HBSS.

Previous studies have demonstrated that agonist-induced elevation of $[Ca^{2+}]_i$ in ASM cells is biphasic characterized by an initial elevation that reaches peak within a few seconds of agonist stimulation followed by a steady state elevation which is above basal but below the peak [14]. Additional evaluation of traces obtained from individual regions of interest (ROIs) in PASM cells suggest that agonist stimulation indeed results in a biphasic elevation of $[Ca^{2+}]_i$. We further analyzed the calcium data by assessing AUC for a given time of stimulation. The AUC analysis demonstrates that treatment of PASM cells with X/XO significantly attenuates both peak and steady state $[Ca^{2+}]_i$ upon agonist stimulation (Figure 4).

Effect of superoxide dismutase:

To further confirm the effect of X+XO in attenuating agonist-induced calcium dynamics in PASM cells are due to generation of superoxide anions, we pre-treated cells with SOD for 15 min prior to addition of X/XO and determined changes in $[Ca^{2+}]_{i.}$ Pre-

treatment of cells with SOD significantly ($p<0.05$) mitigated the attenuation of $[Ca^{2+}]_i$ by X/XO in response to 100 nM Ach (Figure 5). These data suggest that the effect of X+XO in attenuating calcium homeostasis in PASM cells is due to generation of superoxide anions.

***Effect of superoxide anions on Caffeine- and Thapsigargin-induced calcium elevation*:**

$[Ca^{2+}]_i$ responses to 5 mM caffeine and 3 µM thapsigargin were studied in zero calcium HBSS. Stimulation of cells with caffeine and thapsigargin increased $[Ca^{2+}]_i$ in control cells which were attenuated in cells treated with X+XO (Figure 6).

Discussion

Studies presented above suggest that ROS such as superoxide anions modulate calcium homeostasis in ASM cells. Our findings also suggest that the superoxide anions attenuate both influx of calcium from extracellular space and release and reuptake from intracellular stores. A change in calcium dynamics effected by superoxide would have a considerable influence on the normal physiological functions of ASM cells.

The formation of the neonatal lung involves a variety of cell types and intricate signaling pathways working in concert. Lung development is affected by extended use of mechanical ventilation and high oxygen levels, which results in changes to the architecture of the lungs known as airway remodeling and altered ASM responses to pathogenic mediators. Only a portion of these intricate processes is understood [15]. ASM cells control the tone and contraction of the airways [16]. Recent research has revealed that ASM plays a hyperproliferative and hypersecretory role in pathological situations. Numerous investigations have shown how important ROS is to lung pathologies. ROS impact on

calcium transmission and ASM operations, however, is not well understood. The studies presented herein advance our understanding of signaling modulatory role of ROS in ASM cells that is critical in multiple lung diseases [17, 18].

As mentioned above, ROS has been implicated in lung pathologies. Our findings suggest that inhibiting ROS generation or preventing the effect of ROS species on lung cells could be an attractive therapeutic approach. In fact, the use of recombinant superoxide dismutase was evaluated as a newer potential therapeutic agent in recent years for the treatment of BPD. Several animal studies have demonstrated that intravenous, intraperitoneal, or intratracheal (IT) administration of SOD (native or encapsulated in surfactant liposomes) significantly ameliorates lung damage and improves survival from prolonged hyperoxia and mechanical ventilation [19, 20].

Our findings suggest that superoxide anions impair intracellular calcium dynamics. $[Ca^{2+}]_i$ is regulated by influx through ion channel and release of calcium from intracellular stores via activation of Gq-coupled receptors signaling [21]. We studied the agonists Ach, histamine, and endothelin-1 based on previous studies showing their ability to induce calcium release in smooth muscle cells [22, 23]. Our findings demonstrate that calcium inhibitory effect of ROS stems from mechanisms downstream of the receptors for individual agonists as the calcium responses by all the three agonists were equally affected by superoxide. The downstream regulatory processes include ion channels and intracellular stores. Our studies using thapsigargin and caffeine as well as studies conducted in zero calcium HBSS asserted the effect of ROS on intracellular stores. Future studies are needed to address the biochemical changes at the molecular level by ROS species in ASM cells that could contribute to altered calcium homeostasis.

Oxidative stress and associated oxidative damage are mediators of vascular injury and inflammation in many cardiovascular diseases, including hypertension, hyperlipidemia, and diabetes[24]. Increased generation of ROS has been demonstrated in experimental and human hypertension. Antioxidants and agents that interrupt NAD(P)H oxidase driven superoxide production regress vascular remodeling, improve endothelial function, reduce inflammation, and decrease blood pressure in hypertensive models.[24]

Previously researchers showed that bronchial asthma is significantly associated with increased oxidative stress expressed by the increased markers of oxidative damage [25]. The finding of reduced SOD activity in lung cells of patients with asthma suggests that diminished SOD activity serves as a marker of the inflammation characterizing asthma. [26].

In our experiment series, we generated superoxide with an in vitro system using X/XO. We previously showed O_2- attenuates agonist-induced $[Ca^{2+}]_i$ mobilizing pathways [27]. SOD reversed the effects in our experiments (Fig.5). Cholinergic receptors activated by the endogenous agonist Ach elevate $[Ca^{2+}]_i$ in many cell types. Muscarinic ACh receptors, found on glands, smooth muscle, cardiac muscle, and neurons, elevate $[Ca^{2+}]_i$ by stimulating release from intracellular stores. Our PASM cells were perfused with HBSS containing no calcium and 1 mM lanthanum chloride ('0' Ca^{2+} HBSS) to prevent the entry of extracellular calcium. Thus, we speculate that generated O_2- influenced molecular mechanisms potentially involved in calcium release from intracellular stores. O_2- exerted similar effects in vascular smooth muscle cells [28]. In this study, Ach induced $[Ca^{2+}]_i$ release in a concentration-dependent manner, which was quenched in a time-dependent manner by incubating ASM cells in O_2- generated by the X/XO system (Figure

2, 5). Calcium release from the SR through RyR is an important component of the intracellular calcium response after activation of G protein–coupled receptors (GPCRs) in ASM cells [27]. Ach is an endogenous agonist released from parasympathetic nerve terminals. Histamine and endothelin are released during airway diseases due to inflammatory processes [29]. Of note, calcium is a common second messenger at which signaling from multiple Gq-coupled receptors converge in ASM cells. Since the calcium regulatory pathways activated by multiple agonists are equally affected to superoxide anions, our data suggest direct effect on calcium regulatory processes rather than the modulation of upstream signaling elements (e.g., phospholipase C, G proteins) or cell surface receptors.

Ach is an endogenously occurring smooth muscle stimulant. In our experimental designs, we studied the response of $[Ca^{2+}]_i$ to different agonists that were chosen based on their anticipated target response based on previous studies. [30-32] .

Exposure of ASM cells to contractile agonists results in biphasic elevation of $[Ca^{2+}]_i$ characterized by a rapid, transient rise in Ca^{2+}, followed by a decline to a lower steady-state concentration sustained above the basal level [33, 34]. This biphasic $[Ca^{2+}]_i$ response results from calcium influx from the extracellular space and release of calcium from intracellular stores (i.e., the sarcoplasmic reticulum [SR]).

In airway epithelial cells, voltage-dependent Ca^{2+}channels are absent, and mobilization of Ca^{2+} is controlled mainly by Ca^{2+} release from storage sites and CRAC [35]. The generation of ROS participates in normal cell signaling, but oxidative stress can damage cellular macromolecules such as lipids, proteins, and DNA. These effects may contribute to the pathogenesis of severe lung disease in premature newborns and adults

[36]. In our invitro experiment, superoxide induced attenuation of calcium response in the smooth muscle cell, indicate impaired reactivity to agonist stimulation and thereby impairing smooth muscle bronchoconstriction. Superoxide is not freely diffusible but can cross membranes via ion channels. Extracellular superoxide enters the cell via anion blocker-sensitive chloride channel 3 [37]. Here, we showed superoxide could suppress Ca^{2+} release from intracellular storage sites, while the addition of SOD reversed these effects. Airway hyperreactivity as shown in bronchial asthma and BPD, may be mediated by multiple other factors including release of proinflammatory cytokines and chemokines, which were not studied in our experiment. The attenuated calcium response could be explained by cell viability as we well. Exposure to superoxide leads to loss of cell viability and incubation of the isolated PASM cells in an oxidative environment may be a contributing factor that remains to be studied. We plan to include cell viability studies in our future experiments. Superoxide dismutase (SOD) is an important antioxidant known to reduce free radical damage. Intracellular free calcium in smooth muscle can change rapidly and many cellular enzymes can be affected by SOD. We demonstrated the effects of superoxide-mediated calcium response and its ability to reverse some of the calcium responses. Major calcium release channels from the sarcoplasmic/endoplasmic reticulum (SR/ER) are ryanodine receptors (RyR) in excitable cells and inositol 1,4,5-trisphosphate receptors (IP_3R) in non-excitable cells. ROS can directly modulate RyR activity by oxidizing redox-sensing thiol groups [38]. In future experiments, we intend to study some of the physiologic effects of smooth muscle contractility upon exposure to superoxide under different oxidative conditions.

ABBREVIATIONS

PASM: Porcine Airway Smooth Muscle

O_2^-: Superoxide anion

Ach: Acetylcholine.

ET-1: Endothelin 1

$[Ca^{2+}]_i$: intracellular calcium

ROS: Reactive oxygen species

SOD: Superoxide dismutase

X/XO: Xanthine / Xanthine oxidase

HBSS: Hanks' balanced salt solution

RyR: Ryanodine receptor

TPG: Thapsigargin

DECLARATIONS

ETHICS AND ANIMAL USE APPROVAL

The Institutional Animal Care and Use Committees of the University of Minnesota approved the study protocols for the animal care, harvest, and sampling of pig (*Sus scrofa*) tissues.

AVAILABILITY OF DATA AND MATERIALS

The authors are willing to share the raw data and details of experimental materials used as per appropriate requests.

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None of the other authors have financial relationships to disclose.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

RK: performed experiments, analyzed data and prepared figures and manuscript.

MK: contributed study design, data statistical analysis, interpretation, and manuscript writing and editing.

DD: contributed to study methods, data analysis, and manuscript writing and editing.

REFERENCES:

1. Perez, M., et al., *Oxygen radical disease in the newborn, revisited: Oxidative stress and disease in the newborn period.* Free radical biology & medicine, 2019. **142**: p. 61-72.
2. Choi, Y., et al., *Oxygen Toxicity to the Immature Lung-Part I: Pathomechanistic Understanding and Preclinical Perspectives.* International journal of molecular sciences, 2021. **22**(20): p. 11006.
3. Liochev, S.I., *Reactive oxygen species and the free radical theory of aging.* Free Radic Biol Med, 2013. **60**: p. 1-4.
4. Bast, A., G.R. Haenen, and C.J. Doelman, *Oxidants and antioxidants: state of the art.* Am J Med, 1991. **91**(3c): p. 2s-13s.
5. Cochrane, C.G., *Cellular injury by oxidants.* Am J Med, 1991. **91**(3c): p. 23s-30s.
6. Amrani, Y. and R.A. Panettieri, *Airway smooth muscle: contraction and beyond.* The International Journal of Biochemistry & Cell Biology, 2003. **35**(3): p. 272-276.
7. An, S.S., et al., *Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma.* 2007. **29**(5): p. 834-860.
8. Deshpande, D.A., et al., *Modulation of Calcium Signaling by Interleukin-13 in Human Airway Smooth Muscle.* 2004. **31**(1): p. 36-42.
9. Kuriyama, H., et al., *Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels.* Physiol Rev, 1998. **78**(3): p. 811-920.
10. Trump, B.F. and I.K. Berezesky, *Calcium-mediated cell injury and cell death.* The FASEB Journal, 1995. **9**(2): p. 219-228.

11. Cully, T.R., J.N. Edwards, and B.S. Launikonis, *Activation and propagation of Ca2+ release from inside the sarcoplasmic reticulum network of mammalian skeletal muscle.* 2014. **592**(17): p. 3727-3746.
12. White, T.A., M.S. Kannan, and T.F. Walseth, *Intracellular calcium signaling through the cADPR pathway is agonist specific in porcine airway smooth muscle.* Faseb j, 2003. **17**(3): p. 482-4.
13. Deshpande, D.A., et al., *CD38/cyclic ADP-ribose-mediated Ca2+ signaling contributes to airway smooth muscle hyper-responsiveness.* Faseb j, 2003. **17**(3): p. 452-4.
14. Jude, J.A., et al., *Calcium signaling in airway smooth muscle.* Proceedings of the American Thoracic Society, 2008. **5**(1): p. 15-22.
15. Warburton, D., *Overview of Lung Development in the Newborn Human.* Neonatology, 2017. **111**(4): p. 398-401.
16. Prakash, Y.S., *Airway smooth muscle in airway reactivity and remodeling: what have we learned?* Am J Physiol Lung Cell Mol Physiol, 2013. **305**(12): p. L912-33.
17. Prakash, Y.S., *Emerging concepts in smooth muscle contributions to airway structure and function: implications for health and disease.* Am J Physiol Lung Cell Mol Physiol, 2016. **311**(6): p. L1113-l1140.
18. Vogel, E.R., et al., *Moderate hyperoxia induces extracellular matrix remodeling by human fetal airway smooth muscle cells.* Pediatr Res, 2017. **81**(2): p. 376-383.
19. O'Brodovich, H.M. and R.B. Mellins, *Bronchopulmonary dysplasia. Unresolved neonatal acute lung injury.* Am Rev Respir Dis, 1985. **132**(3): p. 694-709.
20. Padmanabhan, R.V., et al., *Protection against pulmonary oxygen toxicity in rats by the intratracheal administration of liposome-encapsulated superoxide dismutase or catalase.* Am Rev Respir Dis, 1985. **132**(1): p. 164-7.
21. Vetter, I. and R.J. Lewis, *Characterization of endogenous calcium responses in neuronal cell lines.* Biochem Pharmacol, 2010. **79**(6): p. 908-20.
22. Nicholls, J.A., J.R. Greenwell, and J.I. Gillespie, *Agonist concentration influences the pattern and time course of intracellular Ca2+ oscillations in human arterial smooth muscle cells.* Pflugers Arch, 1995. **429**(4): p. 477-84.
23. Korbmacher, C., et al., *Endothelin depolarizes membrane voltage and increases intracellular calcium concentration in human ciliary muscle cells.* Biochem Biophys Res Commun, 1989. **164**(3): p. 1031-9.
24. Touyz, R.M. and E.L. Schiffrin, *Reactive oxygen species in vascular biology: implications in hypertension.* Histochem Cell Biol, 2004. **122**(4): p. 339-52.
25. Comhair, S.A., et al., *Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity.* Am J Pathol, 2005. **166**(3): p. 663-74.
26. Smith, L.J., et al., *Reduced superoxide dismutase in lung cells of patients with asthma.* Free Radic Biol Med, 1997. **22**(7): p. 1301-7.
27. Jude, J.A., et al., *Calcium signaling in airway smooth muscle.* Proc Am Thorac Soc, 2008. **5**(1): p. 15-22.
28. Touyz, R.M., et al., *Vascular smooth muscle contraction in hypertension.* Cardiovasc Res, 2018. **114**(4): p. 529-539.
29. Fernandes, L.B. and R.G. Goldie, *The single mediator approach to asthma therapy: is it so unreasonable?* Current Opinion in Pharmacology, 2003. **3**(3): p. 251-256.

30. Markwardt, K.L., P.E. Magnino, and I.H. Pang, *Effect of histamine on phosphoinositide turnover and intracellular calcium in human ciliary muscle cells.* Exp Eye Res, 1996. **62**(5): p. 511-20.
31. Matsumoto, S., et al., *Endothelin-induced changes of second messengers in cultured human ciliary muscle cells.* Invest Ophthalmol Vis Sci, 1996. **37**(6): p. 1058-66.
32. Shirahata, M., R.S. Fitzgerald, and J.S. Sham, *Acetylcholine increases intracellular calcium of arterial chemoreceptor cells of adult cats.* J Neurophysiol, 1997. **78**(5): p. 2388-95.
33. Sims, S.M., Y. Jiao, and Z.G. Zheng, *Intracellular calcium stores in isolated tracheal smooth muscle cells.* Am J Physiol, 1996. **271**(2 Pt 1): p. L300-9.
34. Murray, R.K. and M.I. Kotlikoff, *Receptor-activated calcium influx in human airway smooth muscle cells.* J Physiol, 1991. **435**: p. 123-44.
35. Muallem, S., et al., *Regulation of intracellular calcium in epithelial cells.* Semin Cell Biol, 1990. **1**(4): p. 305-10.
36. Thannickal, V.J. and B.L. Fanburg, *Reactive oxygen species in cell signaling.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2000. **279**(6): p. L1005-L1028.
37. Hawkins, B.J., et al., *Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling.* Mol Biol Cell, 2007. **18**(6): p. 2002-12.
38. Kourie, J.I., *Interaction of reactive oxygen species with ion transport mechanisms.* Am J Physiol, 1998. **275**(1): p. C1-24.

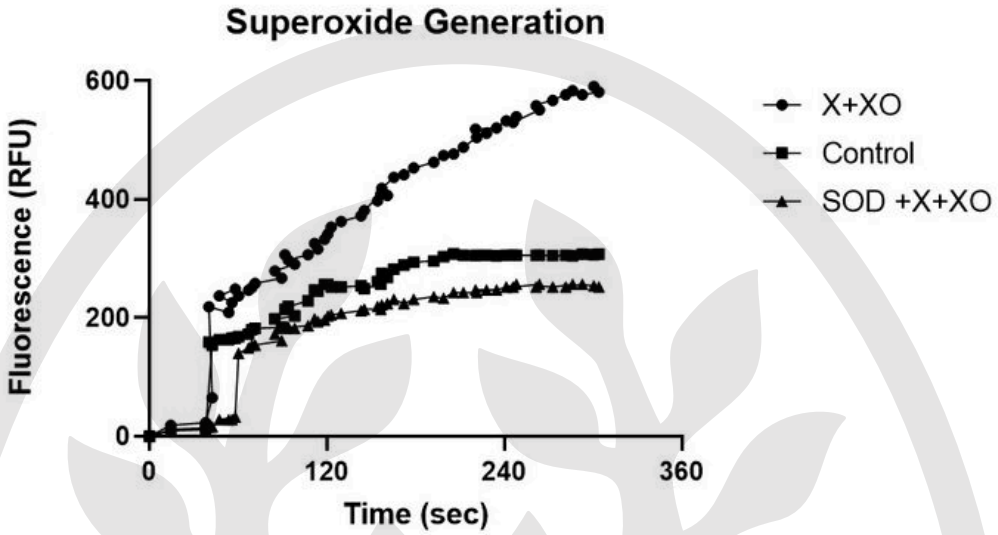

Figure 1

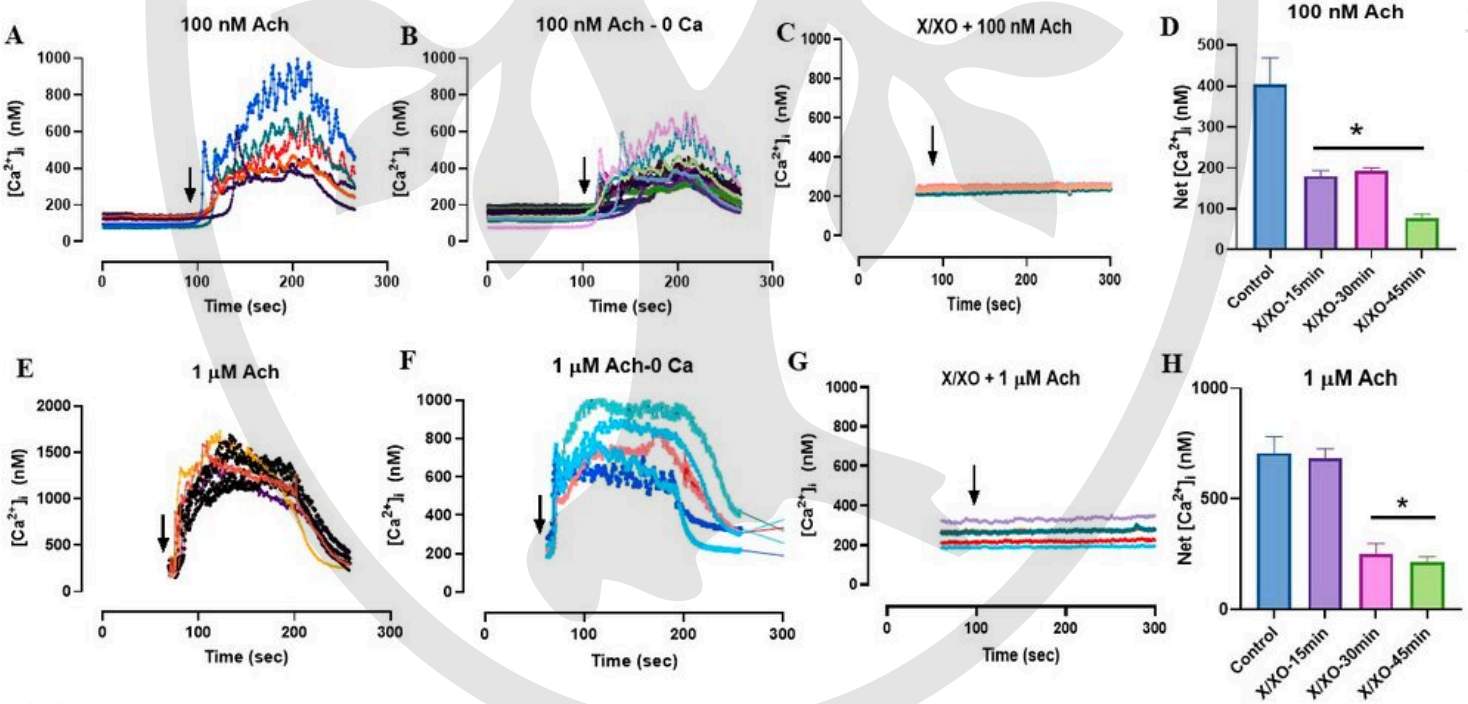

Figure 2

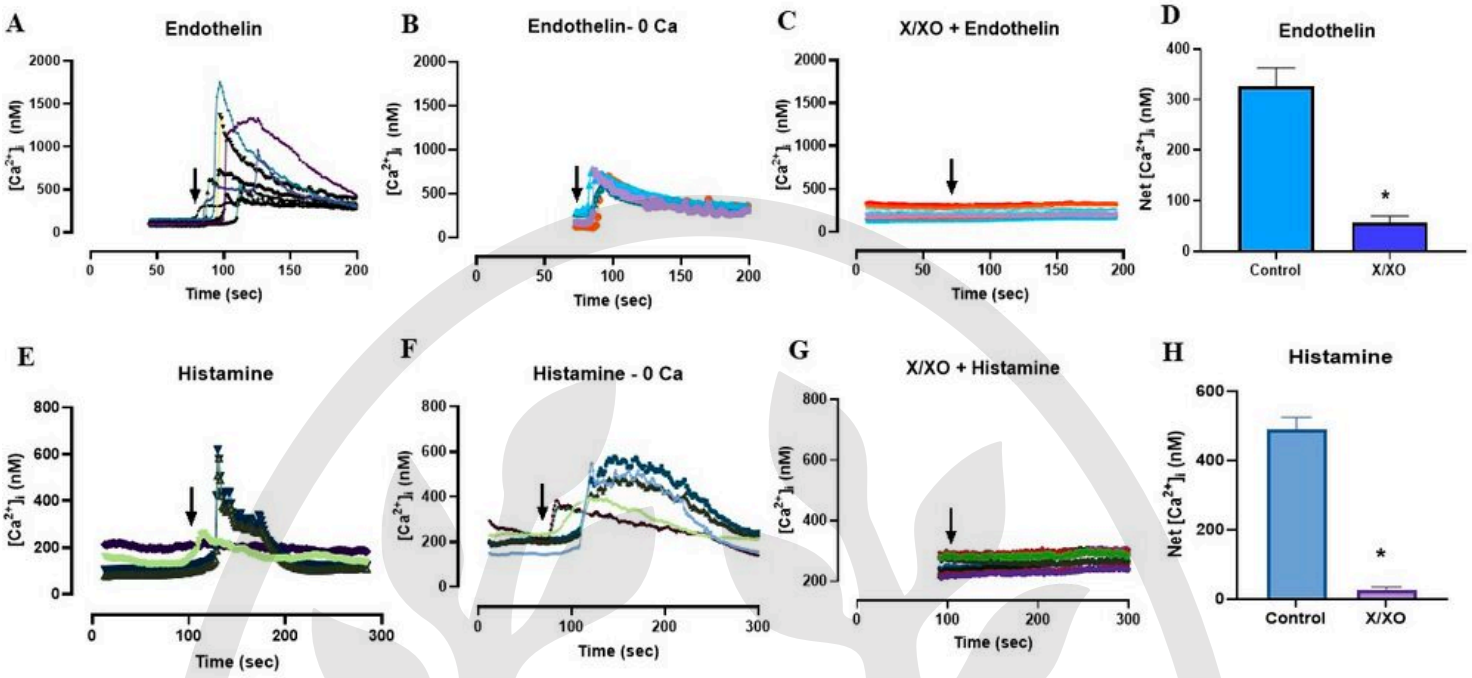

Figure 3

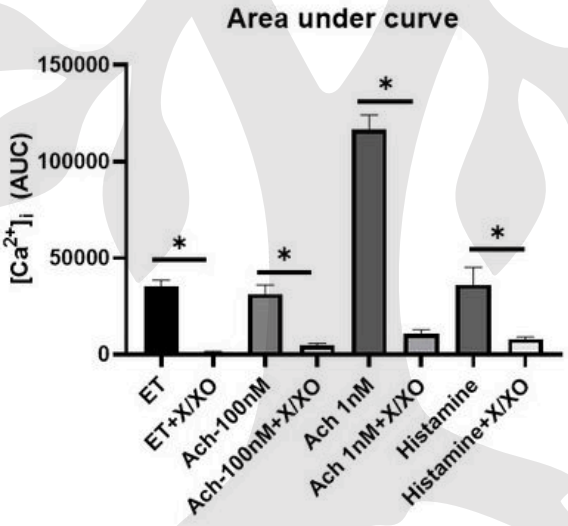

Figure 4

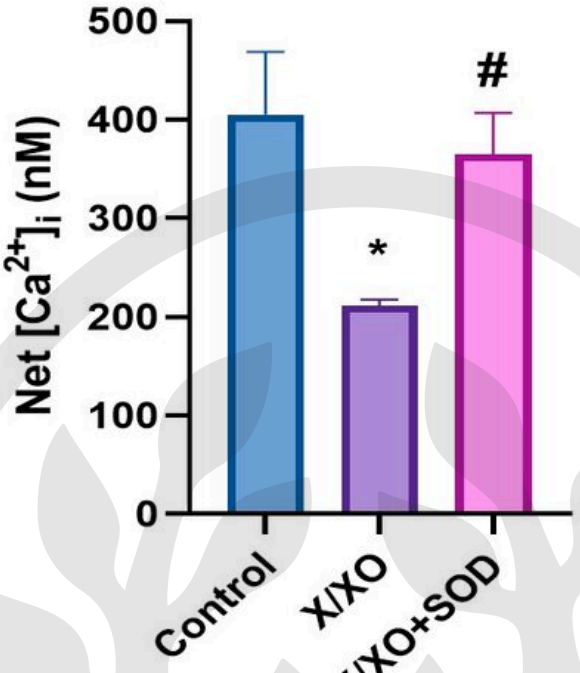

Figure 5

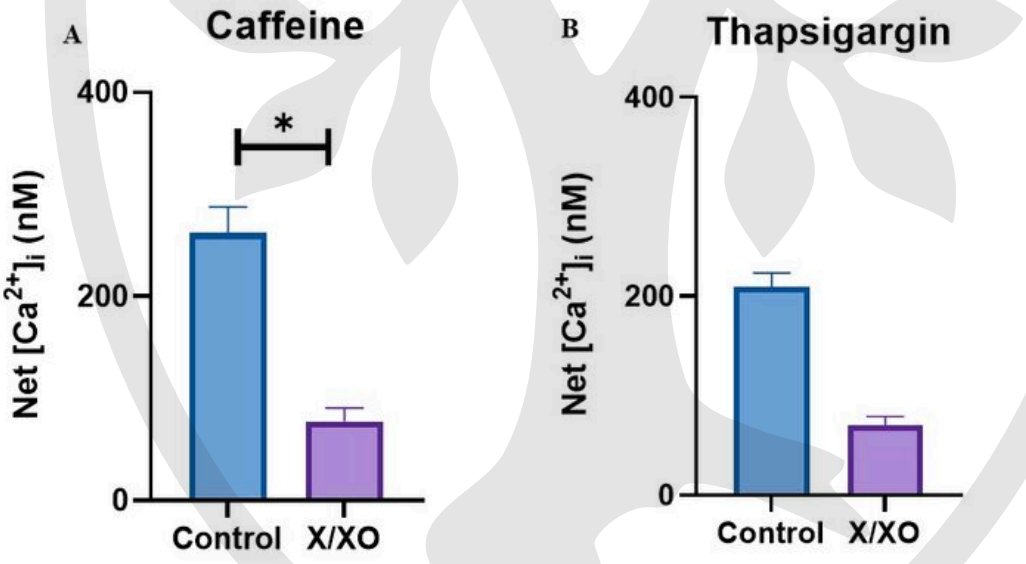

Figure 6