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Superoxide Anions Inhibit Intracellular Calcium Response in Porcine Airway Smooth Muscle Cells

Ramesh Krishnan, MD[1] Mathur S. Kannan, PhD[2] Deepak A. Deshpande, PhD[3]

[1] Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee
[2] Departments of Pediatrics and Veterinary Pathobiology, University of Minnesota, Minneapolis, Minnesota
[3] Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania

Address for correspondence Ramesh Krishnan, MD, Division of Neonatology, 853 Jefferson Ave. Suite 201, Memphis, TN 38163, Tennessee (e-mail: rkrishn4@uthsc.edu).

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Abstract

Background Superoxide anions (O_2^-) have multiple effects on pulmonary parenchyma altering cell proliferation, cellular metabolism, and airway smooth muscle (ASM) contraction. Intracellular calcium ($[Ca^{2+}]_i$) concentration 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.

Design/Methods Fura-2 AM-loaded, freshly isolated porcine ASM (PASM) cells were used to examine $[Ca^{2+}]_i$ release in response to acetylcholine (ACh), histamine, endothelin, caffeine, and thapsigargin (TPG) in the presence or absence of extracellular Ca^{2+}.

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). Preincubating PASM cells with X + XO for 15- or 45-minute inhibited net $[Ca^{2+}]_i$ responses to ACh, histamine, caffeine, and TPG compared with control cells. Pretreating PASM cells with SOD for 30 minutes 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 TPG-induced Ca^{2+} elevation suggesting effect of O_2^- on $[Ca^{2+}]_i$ release and reuptake mechanisms.

Conclusion Superoxide attenuates $[Ca^{2+}]_i$ release, reuptake, and may interfere with physiological functions of ASM cells.

Keywords
- superoxide
- airway smooth muscle cell
- intracellular calcium
- superoxide dismutase
- oxidative
- lung illnesses

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 (ROS), particularly superoxide.[1] Moreover, premature lung exposure to high oxygen levels results in the creation of ROS, which in turn causes oxygen toxicity and altered airway smooth muscle (ASM) reactivity.[2] ROS have harmful effects that cause oxidative stress, which may play a role in the etiology of several adult lung diseases such as 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

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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 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 (Ca^{2+}) 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 Ca^{2+}. Activation of cells with external stimuli such as Gq-coupled G protein–coupled receptor (GPCR) agonists leads to elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ levels which is essential for signal transduction in effector cells. 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 $[Ca^{2+}]_i$ stores evoked by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) through IP_3 receptor channels on the sarcoplasmic reticulum (SR).[9] The initial Ca^{2+} release also activates Ca^{2+}-induced Ca^{2+} release pathway presumably via activation of ryanodine receptors (RyRs) on SR.[11] Earlier studies from our laboratory and others have demonstrated that release of Ca^{2+} from intracellular stores such as SR plays a pivotal role in agonist-induced Ca^{2+} 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 Ca^{2+} levels.[3] Oxidative stress is one such modulator that can influence the normal Ca^{2+} 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 Ca^{2+} homeostasis using freshly dissociated porcine ASM (PASM) cells.

Materials and Methods

Materials

Routinely used reagents, acetylcholine (ACh) 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), superoxide dismutase (SOD), caffeine, thapsigargin (TPG), X, and 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-week-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 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 ASM 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), and incubated at 37°C for 2 hours. After the initial incubation, 0.4 mg/mL type IV collagenase and 0.3 U/mL elastase was added and incubated at 37°C until the cells were completely dispersed (~15–30 minutes). Cell dispersion was aided by gentle trituration with a fire-polished glass pipette. The solution was centrifuged at 2,000 rpm for 5 minutes, 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 minutes. Coverslips with attached cells were placed in HBSS containing 5 µM Fura-2 AM (Molecular Probes) and incubated at 37°C for 30 minutes. 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 was calculated approximately every 0.75 seconds, and $[Ca^{2+}]_i$ was calculated from the ratio of intensities at 340 and 380 nm by extrapolation from a calibration curve as described previously.[13]

Superoxide Generation

X and XO were used to generate superoxide in all the experiments. Superoxide generation was determined

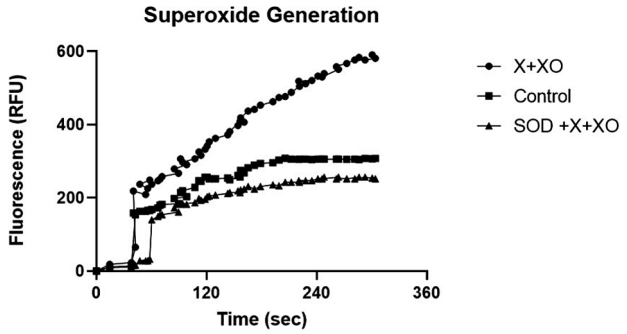

Fig. 1 The X/XO system used to generate superoxide in all the experiments. Superoxide generation was determined fluorometrically using dihydrorhodamine fluorescence (RFU). The figure represents sustained superoxide generation over time (seconds) by X/XO system (shaded circle), control (shaded square), quenching of superoxide production by addition of SOD to X/XO system (shaded triangle). RFU, relative fluorescence unit; SOD, superoxide dismutase; X, xanthine; XO, xanthine oxidase.

fluorometrically using dihydrorhodamine. PASM cells were loaded with 5 µM dihydrorhodamine for 30 minutes and washed with HBSS to remove excess dye. The cells were resuspended in HBSS containing 100 mM X, and basal fluorescence was measured at 485 and 538 nm excitation and emission wavelengths, respectively. Then, XO 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 X and XO results in a sustained release of superoxide anions as demonstrated by an increase in rhodamine fluorescence (►**Fig. 1**). Further, in a selected set of experiments, cells were preincubated with HBSS containing SOD 500 U/mL for 30 minutes, treated with X/XO as described earlier 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 (►**Fig. 1**).

Experimental Protocols

Agonist-Induced Intracellular Calcium Responses

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

Cells loaded with Fura-2 AM were washed and maintained in HBSS with no Ca^{2+} and containing 1 mM lanthanum chloride ("0" Ca^{2+} HBSS). Basal $[Ca^{2+}]_i$ was determined as described earlier. 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 TPG, for at least 1 minute. 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 $[Ca^{2+}]_i$ (►**Fig. 3**). In a selected set of experiments, Ca^{2+} data were analyzed by calculating the area under the curve (AUC) for a given time period of agonist stimulation (►**Fig. 4**).

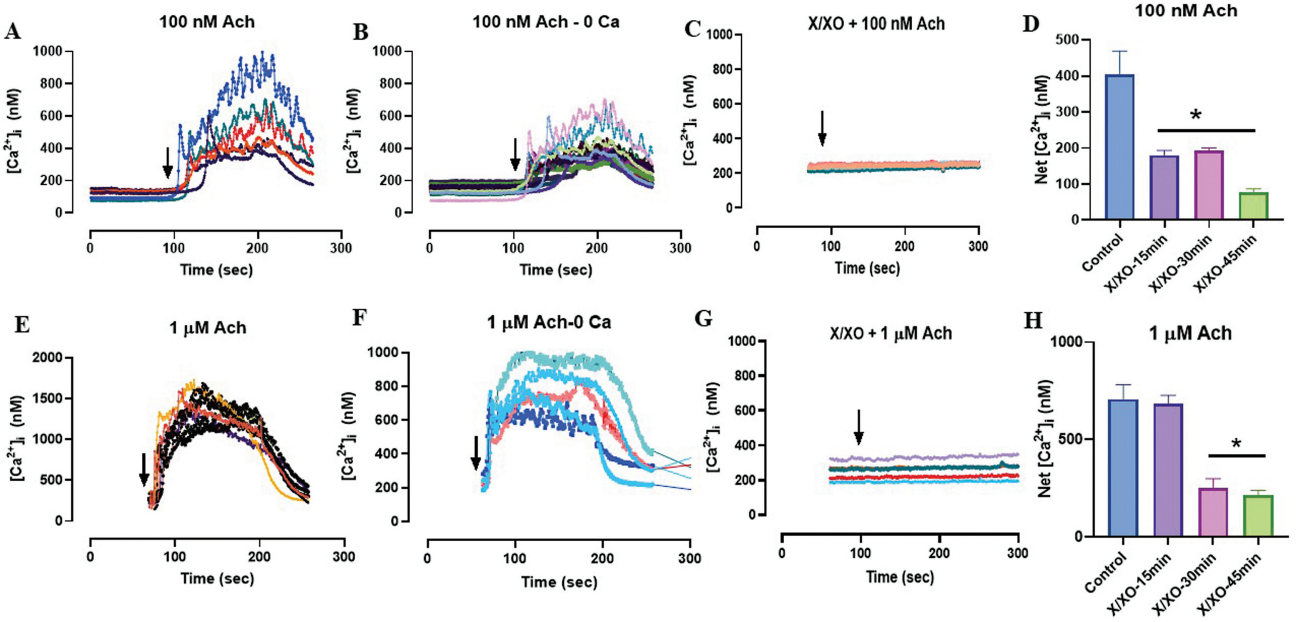

Fig. 2 Effect of O^{2-} anions on net $[Ca^{2+}]_i$ responses to 100 nM ACh (top panel) and1 mM ACh in a time-dependent manner over 15, 30, and 45 minutes exposure to X/XO system (bottom panel). The net $[Ca^{2+}]_i$ responses in PASM cells exposed to X/XO were compared with those in control cells exposed to HBSS. Net $[Ca^{2+}]_i$ response was significantly reduced after 15, 30, and 45 minutes ($p < 0.05$) preincubation with X/XO system, when compared with controls (D, H). Data represent mean ± SEM. ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+}; HBSS, Hank's balanced salt solution; PASM, porcine airway smooth muscle; SEM, standard error of the mean; X, xanthine; XO, xanthine oxidase.

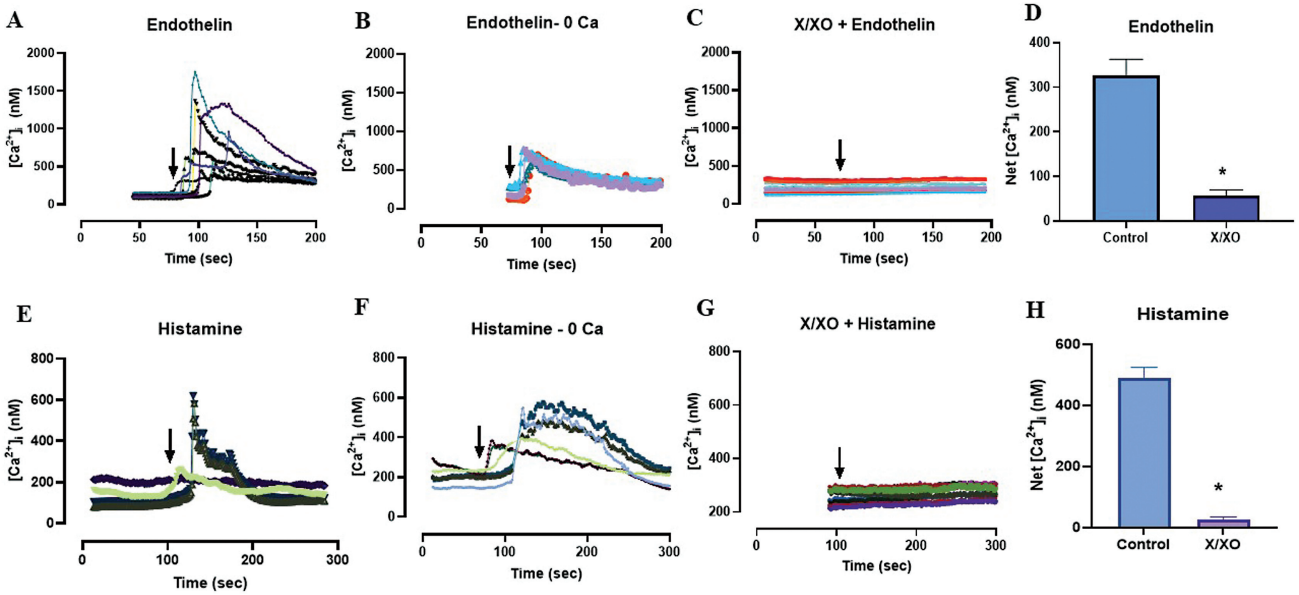

Fig. 3 Effect of O^{2-} anions on the net $[Ca^{2+}]_i$ responses to endothelin (top panel) and histamine (bottom panel). Preincubation with X/XO system for 30, diminished net $[Ca^{2+}]_i$ response ($p < 0.05$) in the cells when compared with controls. Data represent mean ± SEM. $[Ca^{2+}]_i$, intracellular Ca^{2+}; SEM, standard error of the mean; X, xanthine; XO, xanthine oxidase.

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 minutes followed by which the cells were used to measure the basal and agonist-induced increase in $[Ca^{2+}]_i$ as described earlier. 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 minute, and agonist-induced change in $[Ca^{2+}]_i$ was determined. In a selected set of experiments, PASM cells were incubated with 500 U/mL SOD for 30 minutes 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 earlier (►**Fig. 5**).

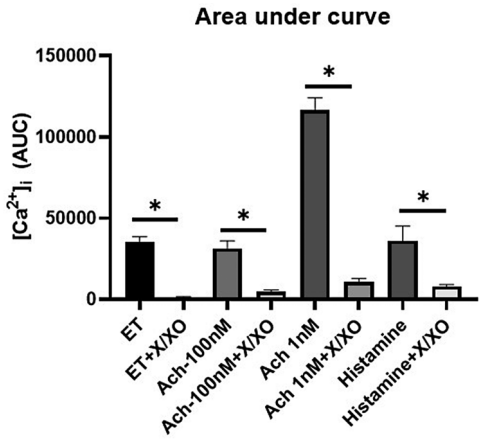

Fig. 4 Area under the curve for net $[Ca^{2+}]_i$ responses to different agonists studied. Data represent mean ± SEM ($^*p < 0.05$). $[Ca^{2+}]_i$, intracellular Ca^{2+}; SEM, standard error of the mean.

Effect of Superoxide on Ryanodine Receptor-Mediated Calcium Release

To study the effects of superoxide on RyR-mediated Ca^{2+} release, we stimulated the isolated PASM cells with caffeine and TPG. Caffeine is known to induce Ca^{2+} release by sensitizing RyR to Ca^{2+}.[14] We used TPG to decrease the SR

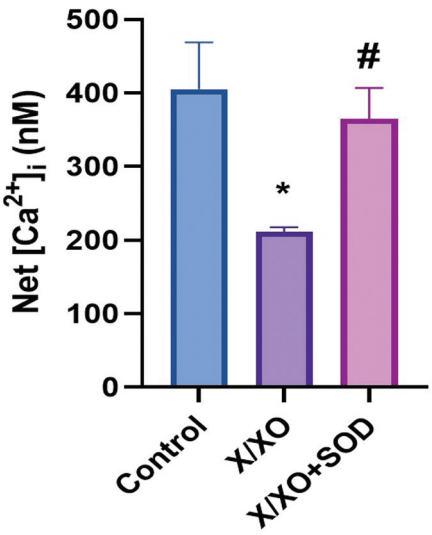

Fig. 5 Effect of O^{2-} anions on the net $[Ca^{2+}]$i responses to 100 nM ACh. The net $[Ca^{2+}]$i responses in PASM cells exposed to X/XO were compared with those in control cells exposed to HBSS. The net $[Ca^{2+}]$i response was significantly reduced after 30 minutes ($^*p < 0.05$) preincubation with X/XO system, when compared with controls. The addition of SOD was able to reverse this agonist-induced attenuation with reversal of net $[Ca^{2+}]$i levels to near baseline ($^\#p < 0.05$). Data represent mean ± SEM. ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular Ca^{2+}; HBSS, Hank's balanced salt solution; PASM, porcine airway smooth muscle; SEM, standard error of the mean; SOD, superoxide dismutase; X, xanthine; XO, xanthine oxidase.

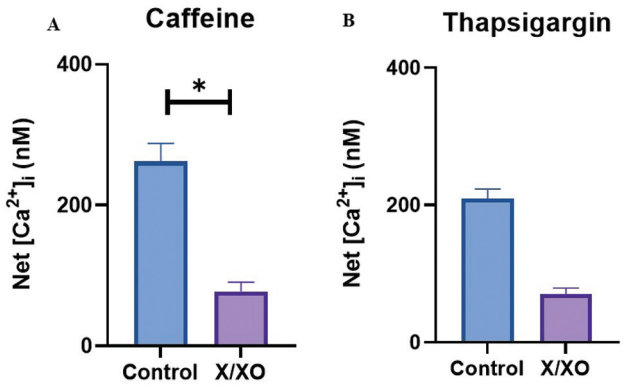

Fig. 6 Effect of O^{2-} anions on the net $[Ca^{2+}]$i responses to 5 mM caffeine (**A**) and 3 µM thapsigargin (**B**). The net $[Ca^{2+}]_i$ responses in PASM cells exposed to X/XO were compared with those in control cells exposed to HBSS. Net $[Ca^{2+}]$i response was significantly reduced after 30 minutes ($^*p < 0.05$) preincubation with X/XO system, when compared with controls upon exposure to caffeine. Data represent mean ± SEM. $[Ca^{2+}]_i$, intracellular Ca^{2+}; HBSS, Hank's balanced salt solution; SEM, standard error of the mean; X, xanthine; XO, xanthine oxidase.

ATPase enzyme activity that is essential for the reuptake of Ca^{2+} into stores, inhibition of which will increase the cytoplasmic Ca^{2+} concentration. The experiments were conducted in zero Ca^{2+} conditions to prevent the influx and efflux of Ca^{2+} from extracellular space.

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

Statistical Analysis

All experiments were repeated in at least four to five different cell preparations. Ca^{2+} data were 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 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 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 $[Ca^{2+}]_i$ 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 XO on its substrate X. The cells were incubated with 100 mM X prepared in HBSS and the addition of 10 mU/mL XO to the cell suspension demonstrated a sustained release of superoxide as determined using a fluorometer in relative fluorescence unit (►**Fig. 1**). Furthermore, change in fluorescence of rhodamine by treatment of cells with X and XO was significantly ($p < 0.05$, $n = 4$) inhibited by pretreating cells with SOD for 30 minutes (►**Fig. 1**). These findings suggest that X generates superoxide anions when treated with XO which could be used as an experimental model to study the effect of superoxide anions on $[Ca^{2+}]_i$ 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 Ca^{2+} and zero Ca^{2+} conditions. Stimulation of PASM cells maintained in regular HBSS and zero Ca^{2+} HBSS with 100 nM or 1 µM ACh resulted in an increase in $[Ca^{2+}]_i$ (►**Fig. 2A, B**). Further, pretreatment of cells with superoxide generated by the X/XO significantly attenuated this response (►**Fig. 2C, D**). Evaluation of baseline Ca^{2+} concentration suggests that exposing ASM cells to X/XO for 15 to 45 minutes does not modulate basal Ca^{2+} 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 ET-1 are released during airway inflammation and acts of ASM cells via H1 and ET-1 receptors, respectively. Therefore, we investigated the effect of superoxide anions on histamine- and ET-1-induced elevation of $[Ca^{2+}]_i$. Histamine and ET-1 stimulation of PASM cells maintained in regular (►**Fig. 3A**) and zero Ca^{2+} HBSS (►**Fig. 3B**) resulted in elevation of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ responses to histamine (►**Fig. 3C**) and ET-1 (►**Fig. 3D**) were attenuated by pretreatment of PASM cells with X/XO. Studies related to superoxide effect were conducted in cells marinated in zero Ca^{2+} 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.[15] Additional evaluation of traces obtained from individual regions of interest in PASM cells suggests that agonist stimulation indeed results in a biphasic elevation of $[Ca^{2+}]_i$. We further analyzed the Ca^{2+} 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 (►**Fig. 4**).

Effect of Superoxide Dismutase

To further confirm the effect of X + XO in attenuating agonist-induced Ca^{2+} dynamics in PASM cells is due to generation of superoxide anions, we pretreated cells with SOD for

15 minutes prior to addition of X/XO and determined changes in $[Ca^{2+}]_i$. Pretreatment of cells with SOD significantly ($p < 0.05$) mitigated the attenuation of $[Ca^{2+}]_i$ by X/XO in response to 100 nM ACh (►**Fig. 5**). These data suggest that the effect of X + XO in attenuating Ca^{2+} 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 TPG were studied in zero Ca^{2+} HBSS. Stimulation of cells with caffeine and TPG increased $[Ca^{2+}]_i$ in control cells which were attenuated in cells treated with X + XO (►**Fig. 6**).

Discussion

Studies presented earlier suggest that ROS such as superoxide anions modulate Ca^{2+} homeostasis in ASM cells. Our findings also suggest that the superoxide anions attenuate both influx of Ca^{2+} from extracellular space and release and reuptake from intracellular stores. A change in Ca^{2+} 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.[16] ASM cells control the tone and contraction of the airways.[17] 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 Ca^{2+} 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.[18,19]

As mentioned earlier, ROS has been implicated in lung pathologies. Our findings suggest that inhibiting ROS generation or preventing the effect of ROS on lung cells could be an attractive therapeutic approach. In fact, the use of recombinant SOD 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 administration of SOD (native or encapsulated in surfactant liposomes) significantly ameliorates lung damage and improves survival from prolonged hyperoxia and mechanical ventilation.[14,20]

Our findings suggest that superoxide anions impair $[Ca^{2+}]_i$ dynamics. $[Ca^{2+}]_i$ is regulated by influx through ion channel and release of Ca^{2+} from intracellular stores via activation of Gq-coupled receptors signaling.[21] We studied the agonists ACh, histamine, and ET-1 based on previous studies showing their ability to induce Ca^{2+} release in smooth muscle cells.[22,23] Our findings demonstrate that Ca^{2+} inhibitory effect of ROS stems from mechanisms downstream of the receptors for individual agonists as the Ca^{2+} responses by all the three agonists were equally affected by superoxide. The downstream regulatory processes include ion channels and intracellular stores. Our studies using TPG and caffeine as well as studies conducted in zero Ca^{2+} HBSS asserted the effect of ROS on intracellular stores. Future studies are needed to address the biochemical changes at the molecular level by ROS in ASM cells that could contribute to altered Ca^{2+} 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.[15] 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 Ca^{2+} and 1 mM lanthanum chloride ("0" Ca^{2+} HBSS) to prevent the entry of extracellular Ca^{2+}. Thus, we speculate that generated O_2^- influenced molecular mechanisms potentially involved in Ca^{2+} release from intracellular stores. O_2^- exerted similar effects in vascular smooth muscle cells.[27] 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 (►**Figs. 2** and **5**). Ca^{2+} release from the SR through RyR is an important component of the $[Ca^{2+}]_i$ response after activation of GPCRs in ASM cells.[15] ACh is an endogenous agonist released from parasympathetic nerve terminals. Histamine and endothelin are released during airway diseases due to inflammatory processes.[28] Of note, Ca^{2+} is a common second messenger at which signaling from multiple Gq-coupled receptors converge in ASM cells. Since the Ca^{2+} regulatory pathways activated by multiple agonists are equally affected to superoxide anions, our data suggest direct effect on Ca^{2+} 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.[29–31]

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.[32,33] This biphasic $[Ca^{2+}]_i$ response results from Ca^{2+} influx from the extracellular space and release of Ca^{2+} from intracellular stores (i.e., the SR).

In airway epithelial cells, VDCCs are absent, and mobilization of Ca^{2+} is controlled mainly by Ca^{2+} release from storage sites and calcium release-activated channel.[34] 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.[35] In our in vitro experiment, superoxide induced attenuation of Ca^{2+} response in the smooth muscle cell, indicating 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.[36] 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 Ca^{2+} response could be explained by cell viability as 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. SOD is an important antioxidant known to reduce free radical damage. Free $[Ca^{2+}]_i$ in smooth muscle can change rapidly and many cellular enzymes can be affected by SOD. We demonstrated the effects of superoxide-mediated Ca^{2+} response and its ability to reverse some of the Ca^{2+} responses. Major Ca^{2+} release channels from the SR/endoplasmic reticulum are RyRs in excitable cells and Ins $(1,4,5)P_3$ receptors in nonexcitable cells. ROS can directly modulate RyR activity by oxidizing redox-sensing thiol groups.[37] In future experiments, we intend to study some of the physiologic effects of smooth muscle contractility upon exposure to superoxide under different oxidative conditions.

Conclusion

Superoxide anions inhibit $[Ca^{2+}]_i$ release, reuptake, and may interfere with physiological functions of ASM cells. Further studies are needed to explore physiologic functions.

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.

Authors' Contribution

R.K. performed experiments, analyzed data, and prepared figures and manuscript. M.K. contributed to study design, data statistical analysis, interpretation, and manuscript writing and editing. D.D. contributed to study methods, data analysis, and manuscript writing and editing.

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Conflict of Interest

None declared.

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