

# Superoxide Anion Inhibits Intracellular Calcium Response in Porcine Airway Smooth Muscle Cells

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## Research

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

**Posted Date:** February 23rd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-223666/v1>

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# Abstract

**BACKGROUND:** Superoxide is implicated in lung disease, injury, and transplantation. In lung, many defense mechanisms, especially superoxide dismutase (SOD) and metallothionein, neutralize superoxide. Superoxide anions ( $O_2^-$ ) have multiple effects on pulmonary parenchyma, altering cell proliferation, redox enzyme activation and smooth muscle contraction. Airway smooth muscle (ASM) contraction requires elevated intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ).  $[Ca^{2+}]_i$  release from intracellular stores also participates in contractile responses to multiple agonists.

**OBJECTIVE:** We investigated the effects of  $O_2^-$  on agonist-stimulated  $[Ca^{2+}]_i$  responses in ASM cells.

**DESIGN/METHODS:** Porcine ASM (PASM) cells were dissociated using collagenase and papain. Fura-2 AM-loaded PASM cells were used to examine  $[Ca^{2+}]_i$  release in response to acetylcholine (ACh), histamine, endothelin combined with lanthanum, and no  $Ca^{2+}$ .

**RESULTS:** In PASM cells, agonist exposure generated a biphasic  $Ca^{2+}$  response. Dihydrorhodamine-loaded cells exposed to xanthine and xanthine oxidase showed time-dependent generation of ( $O_2^-$ ), which was inhibited by SOD. Pre-incubation with xanthine and xanthine oxidase for 15 or 45 min revealed significant inhibition of net  $[Ca^{2+}]_i$  responses to 100 nM and 1 M ACh and 50 M histamine. However, basal  $[Ca^{2+}]_i$  was similar in cells exposed to  $O_2^-$  and controls. Multiple agonists inhibited  $Ca^{2+}$  release in the presence of  $O_2^-$ .

**CONCLUSIONS:** Superoxide impairs  $[Ca^{2+}]_i$  release and may interfere with the contractile mechanism in ASM cells. Alteration of a common signaling pathway may be involved in  $[Ca^{2+}]_i$  regulation. The effects of  $O_2^-$  were not likely due to cell damage since basal  $[Ca^{2+}]_i$  was unchanged. We need further experiments to identify the molecular targets of  $O_2^-$  in  $Ca^{2+}$  homeostasis.

## Introduction

Oxidative stress resulting from the toxic effects of reactive oxygen species (ROS) may contribute to the pathogenesis of various pulmonary disorders, such as acute respiratory distress syndrome, emphysema, and asthma [1], [2]. ROS, including superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^-$ ), are inflammatory mediators of cell and tissue injury [3]. ROS exert their biological effects through lipid peroxidation of the cell membrane, alteration of enzyme activities, and breakage of DNA strands. Airway epithelial cells are vulnerable to ROS from endogenous and exogenous sources because in addition to their own oxygen metabolites, these cells are exposed to oxidant air pollutants, catalase-negative bacteria, and inflammatory cells [4]. Various oxidants induce epithelial damage and mucus hypersecretion, characteristics of asthma. In fact, ample evidence indicates ROS stimulate signal

transduction related to epithelial cell dysfunction or injury, potentially resulting in the above pathophysiology [5], [6]. However, little is known about the intracellular regulatory mechanisms involved.

Elevated intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) is an initial step in smooth muscle contraction. The main  $\text{Ca}^{2+}$  mobilizing pathways of smooth muscle cells are  $\text{Ca}^{2+}$  entry through voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs), agonist-induced  $\text{Ca}^{2+}$  entry, and  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores evoked by *D-myoinositol* 1,4,5- trisphosphate [ $\text{Ins}(1,4,5)\text{P}_3$ ] (10).  $\text{Ca}^{2+}$  release also activates  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  entry (CRAC) in vascular smooth muscle cells. Impairments in any of these  $\text{Ca}^{2+}$ -[1] mobilizing pathways in smooth muscle cells will affect vascular contraction, so oxidative stress-induced inhibition of  $\text{Ca}^{2+}$  pathways should be considered a potential contributor to vascular diseases. In bovine aortic endothelial cells (BAEC), superoxide inhibits CRAC and  $\text{Ca}^{2+}$  extrusion and accelerates  $\text{Ca}^{2+}$  leakage from intracellular  $\text{Ca}^{2+}$  stores [7]. However, reports on the effects of oxidative stress, especially the mobilizing properties of  $\text{Ca}^{2+}$  in airway smooth muscle (ASM) cells, are lacking.

$[\text{Ca}^{2+}]_i$  is essential in signal transduction and regulates numerous enzyme activities, such as proteases, phospholipases, and endonucleases [8]. Moreover, altered intracellular  $\text{Ca}^{2+}$  homeostasis occurs early in the development of irreversible cell injury [9]. In airway epithelium,  $[\text{Ca}^{2+}]_i$  mediates cell functions including ion transport [10] and mucus secretion [11]. Extracellularly applied ROS increase  $[\text{Ca}^{2+}]_i$  in other cell types, such as vascular endothelial cells [12] and renal tubular epithelial cells [13]. Nevertheless, the effect of ROS on  $\text{Ca}^{2+}$  dynamics in airway epithelium remains unexplored. The mechanisms underlying  $[\text{Ca}^{2+}]_i$  oscillations are diverse and may be driven entirely by influx of extracellular  $\text{Ca}^{2+}$  across the plasma membrane, by the release of  $\text{Ca}^{2+}$  from intracellular stores, or by an interaction between intra- and extracellular sources of  $\text{Ca}^{2+}$ . Therefore, to determine whether ROS alter airway epithelial  $\text{Ca}^{2+}$  signaling properties and if so, to elucidate the mechanism, we studied freshly dissociated porcine ASM (PASM) cells in vitro using a hypoxanthine (HX)–xanthine oxidase (XO) system. We also examined the ability of superoxide dismutase (SOD) to reverse the effects of superoxide.

## 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), SOD, xanthine (X) and XO were obtained from Calbiochem (La Jolla, CA).

### Airway smooth muscle cell preparation

PASM cells were isolated from the trachea as previously described ([14]). Briefly, 6- to 10-wk-old, outbred Yorkshire pigs (~10 -18 kg body weight) were anesthetized with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol, 8 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) combined with xylazine (8 mg/kg). The animals were euthanized by barbiturate overdose in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC), University of Minnesota. Isolated tracheas were transferred to ice-cold HBSS containing 10 mM HEPES, 11 mM glucose, 2.5 mM CaCl<sub>2</sub>, and 1.2 mM MgCl<sub>2</sub> (pH 7.4) and maintained in an oxygenated environment. Following removal of the epithelium from the trachea, the 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°C for 2 hours. After the initial incubation, 0.4 mg/ml type IV collagenase and 0.3 U/ml elastase (Worthington Biochemical) were added and incubated at 37°C 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 beaded onto glass coverslips and allowed to attach at 37°C in 95% O<sub>2</sub> and 5% CO<sub>2</sub> 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 in HBSS, treated as described in the experimental protocols, and used to determine [Ca<sup>2+</sup>]<sub>i</sub>.

## Digital video fluorescence imaging

Coverslips were mounted on a 150-µl 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 ×40 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 video fluorescence imaging system (Metafluor; Universal Imaging, Bedford Hills, NY). Images were acquired using a Photometric Cool Snap 12-bit digital camera (Roper Scientific, Teledyne Photometrics, Tucson, 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<sup>2+</sup>]<sub>i</sub> was calculated from the ratio of intensities at 340 nm and 380 nm by extrapolation from a calibration curve as previously described [15].

## Superoxide generation

X/XO were used to generate superoxide in all the experiments. XO (10 mU/ml) was incubated with 100 mM X prepared in HBSS. Superoxide generation was determined fluorometrically using dihydrorhodamine. PASM cells were loaded with 5 mM dihydrorhodamine for 30 min 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 nm and 538 nm excitation and emission wavelengths, respectively. Then, XO was added to the cell suspension, and the change in the fluorescence was measured. In different experiments, cells were preincubated with HBSS containing SOD, followed by the addition of X/XO. The generation of superoxide with and without preincubation with SOD was determined.

## Experimental protocols

### ***Agonist-induced intracellular calcium responses:***

PASM cells were perfused with HBSS containing no calcium and 1 mM lanthanum chloride ('0' Ca<sup>2+</sup> HBSS). Basal [Ca<sup>2+</sup>]<sub>i</sub> was determined as described above. The cells were subsequently perfused with '0' Ca<sup>2+</sup> HBSS containing 100 nM ACh, 1 mM ACh, 50 mM histamine, or 200 nM ET-1 for at least 1 min. Changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored during stimulation of cells with agonists, and peak [Ca<sup>2+</sup>]<sub>i</sub> was determined. The net intracellular Ca<sup>2+</sup> response to each agonist was calculated by subtracting basal from peak [Ca<sup>2+</sup>]<sub>i</sub>.

### ***Effects of superoxide (O<sub>2</sub><sup>-</sup>) on [Ca<sup>2+</sup>]<sub>i</sub> responses:***

Unless otherwise noted, cells were perfused with '0' Ca<sup>2+</sup> HBSS. PASM cells were incubated with X/XO for 15, 30 or 45 min. The X/XO system generated superoxide anions over time as described above. The cells were washed with HBSS and used for [Ca<sup>2+</sup>]<sub>i</sub> measurements as described above. After determining basal [Ca<sup>2+</sup>]<sub>i</sub>, the cells were stimulated with 100 nM ACh, 1 mM ACh, 50 mM histamine, or 200 nM ET-1 for at least 1 min. The net [Ca<sup>2+</sup>]<sub>i</sub> responses to each of the agonists were calculated by subtracting basal from peak [Ca<sup>2+</sup>]<sub>i</sub>. The net intracellular Ca<sup>2+</sup> responses in PASM cells exposed to X/XO were compared to those in control cells exposed to HBSS.

### ***Effects of superoxide dismutase on superoxide (O<sub>2</sub><sup>-</sup>)-mediated inhibition of [Ca<sup>2+</sup>]<sub>i</sub> responses:***

Cells were preincubated with 250 or 500 U/ml SOD for 30 min before exposure to X/XO. These experiments were repeated to determine the net [Ca<sup>2+</sup>]<sub>i</sub> responses to agonists as described above. The net

[Ca<sup>2+</sup>]<sub>i</sub> responses of PASM cells to multiple agonists upon exposure to X/XO with or without preincubation with SOD were compared.

## Statistical analysis

All experiments were repeated in at least 4-5 different cell preparations. Data were analyzed using one-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., San Diego, CA) statistical software. Two means were considered significantly different when the *p* value was less than 0.05.

## Results

### Generation of superoxide:

The X/XO system generated superoxide in a time-dependent manner (Figure 1).

### Response to agonists:

[Ca<sup>2+</sup>]<sub>i</sub> responses to agonists were attenuated with superoxide generated by the X/XO system (*p*<0.05), (Figure 2-5). This attenuation of [Ca<sup>2+</sup>]<sub>i</sub> responses to agonists was also time dependent and varied with agonist concentrations (Figure 2-3).

### Effect of superoxide dismutase:

SOD reversed the attenuation of [Ca<sup>2+</sup>]<sub>i</sub> in a concentration-dependent manner (*p*<0.05) (Figure 6).

## Discussion

Fetal lung development involves multiple cell types and complex interaction of signaling mechanisms. Exposure to mechanical ventilation and high oxygen concentrations alter lung development. These complex processes are only partially understood [16]. ASM cells regulate airway muscle tone and contractility throughout life [17]. We are improving our understanding of [Ca<sup>2+</sup>]<sub>i</sub> in ASM cells and the development of airway hyperreactivity and fibrosis after hyperoxia exposure in the developing lung [18, 19].

Identifying the mechanisms underlying ASM structure and function early in development will help determine potential targets in disease. XO may be a source of biological free radical generation. This enzyme generates the superoxide radical and is widely used to obtain O<sub>2</sub><sup>-</sup>. Superoxide and XO also contribute to myocardial reperfusion injury [20, 21].

In our experiments, we generated superoxide with an in vitro system using X/XO. We previously showed  $O_2^-$  attenuates agonist-induced  $[Ca^{2+}]_i$  mobilizing pathways. SOD reversed these effects in a time-dependent manner. 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 entry of extracellular calcium. Thus,  $O_2^-$  influences molecular mechanisms potentially involved in calcium release from intracellular stores.  $O_2^-$  exerts similar effects in vascular smooth muscle cells [22]. 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.

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 [23, 24]. 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]). Real-time confocal imaging also revealed calcium oscillations originate from one end of the cell and propagate toward the other end [25].

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 [26]. The generation of ROS participates in normal cell signaling, but oxidative stress can damage cellular macromolecules such as lipids, protein, and DNA. These effects may contribute to the pathogenesis of severe lung disease in premature newborns and adults [27].

Superoxide is not freely diffusible but can cross membranes via ion channels. Extracellular superoxide enters the cell via anion blocker-sensitive chloride channel 3 [28]. Here, we showed superoxide could suppress  $Ca^{2+}$  release from intracellular storage sites, while the addition of SOD reversed these effects. 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<sub>3</sub>R) in non-excitabile cells. ROS can directly modulate RyR activity by oxidizing redox-sensing thiol groups [29]. In future experiments, we intend to study the effects of caffeine-induced IP<sub>3</sub>R-mediated release and plan further experiments to identify the molecular targets of  $O_2^-$  in  $Ca^{2+}$  homeostasis.

## Abbreviations

PASM: Porcine Airway Smooth Muscle

ACh: Acetylcholine

ET-1: Endothelin 1

[Ca<sup>2+</sup>]<sub>i</sub> : intracellular calcium

ROS: Reactive oxygen species

SOD: Superoxide dismutase

X/XO: Xanthine / Xanthine oxidase

## **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 pigs (*Sus scrofa*) tissue.

## **AVAILABILITY OF DATA AND MATERIALS**

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

## **FUNDING**

This study was supported in part by a grant from Academic Health Center, University of

Minnesota, and a grant from the National Institutes of Health to Mathur Kannan, PhD.

None of the other authors have financial relationships to disclose.

## **COMPETING INTERESTS**

The authors declare that they have no competing interests.

## **AUTHOR CONTRIBUTIONS**

RK: contributed to study design, methods, data and statistical analysis, interpretation, and manuscript writing.

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

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

# ACKNOWLEDGMENTS

The authors would like to acknowledge Dr. Courtney Bricker-Anthony, Scientific Editor at Le Bonheur Children's Foundation Research Institute, for her help with scientific editing of this manuscript.

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## Figures

Figure 1

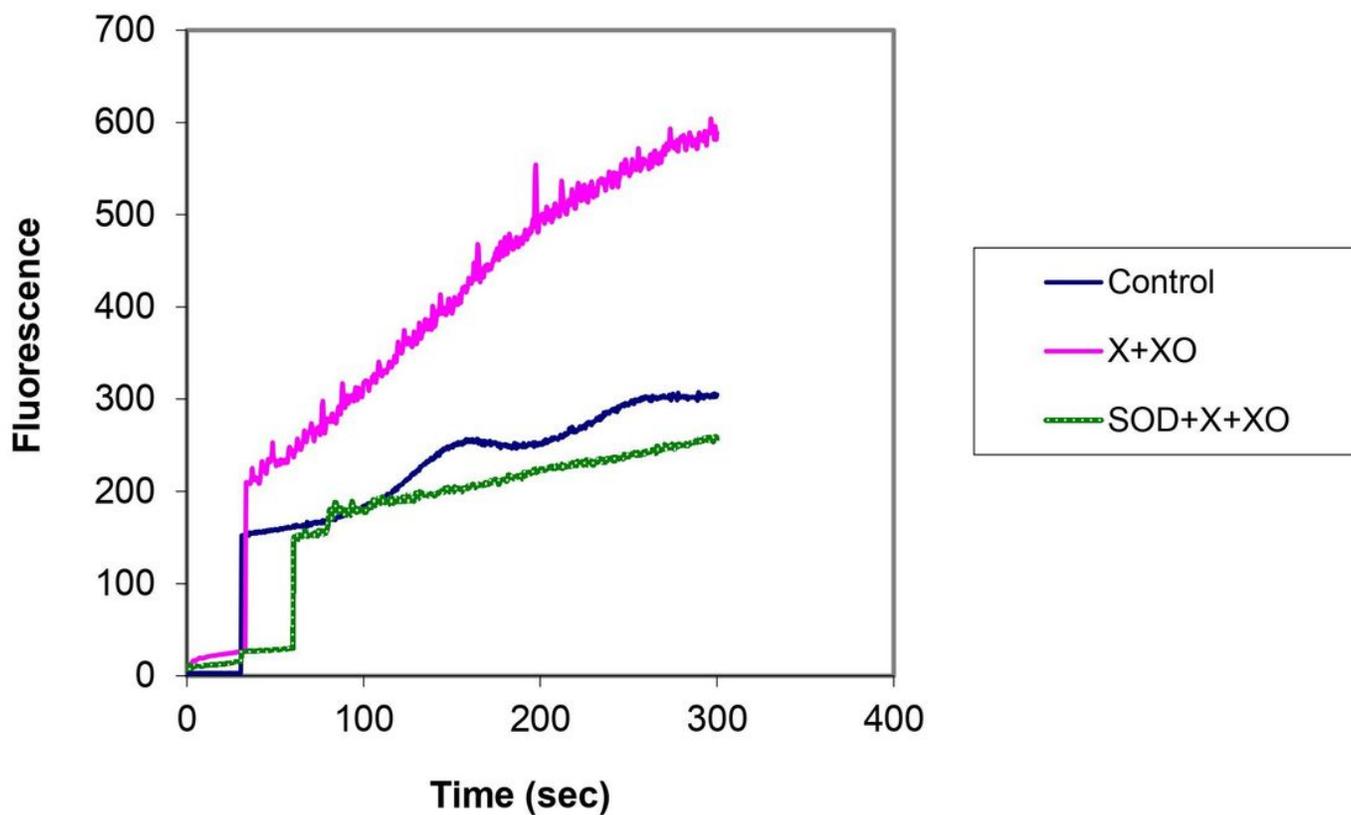


Figure 1

X/XO were used to generate superoxide in all the experiments. Superoxide generation was determined fluorometrically using dihydrorhodamine. The figure represents superoxide generation by X/XO system (pink), control (blue), quenching of superoxide production by addition of SOD to X/XO system (green)

Figure 2

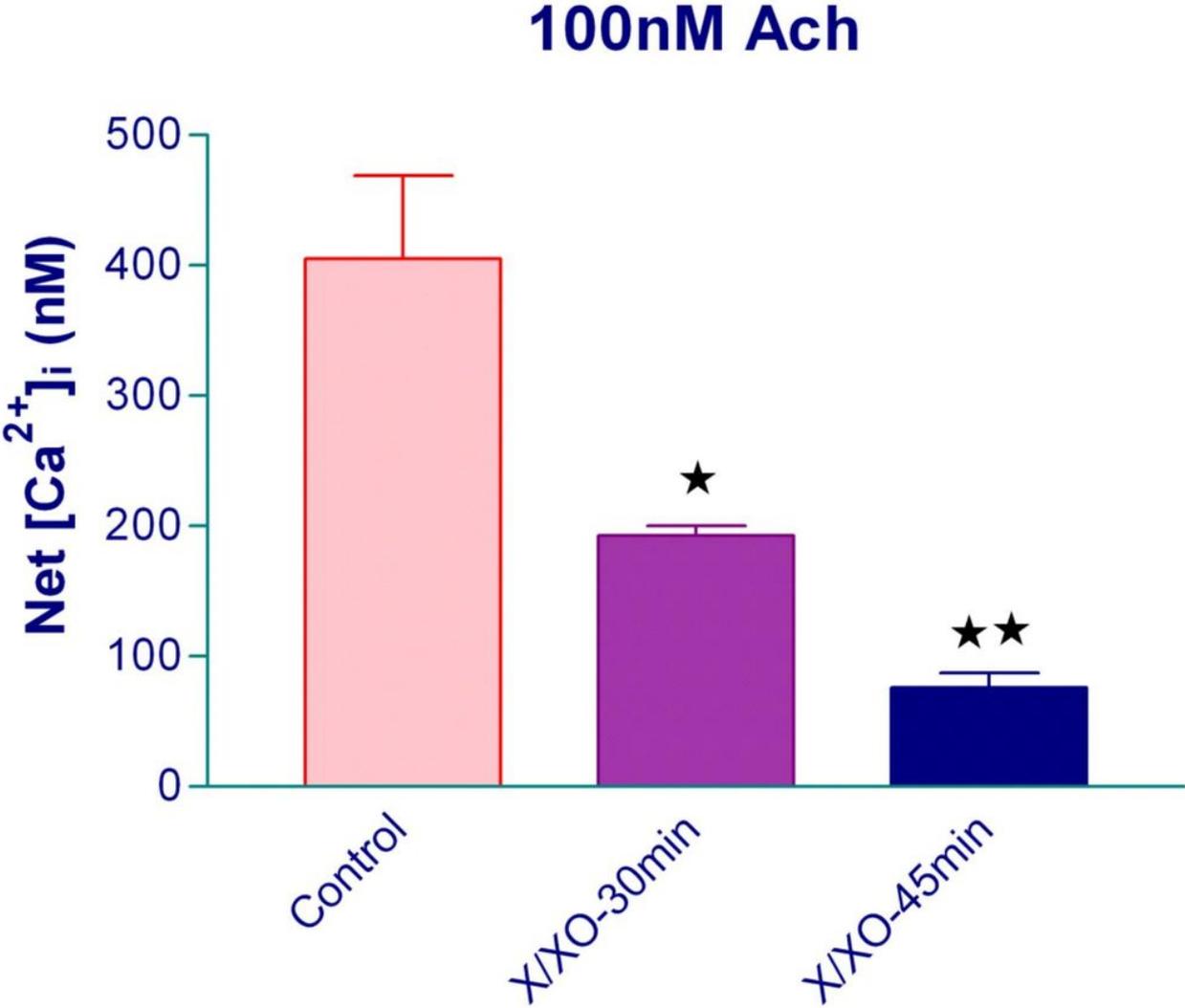


Figure 2

Effect of O<sub>2</sub><sup>-</sup> anions on net [Ca<sup>2+</sup>]<sub>i</sub> responses to 100 nM Ach. The net intracellular Ca<sup>2+</sup> responses in PASM cells exposed to X/XO were compared to those in control cells exposed to HBSS

Figure 3

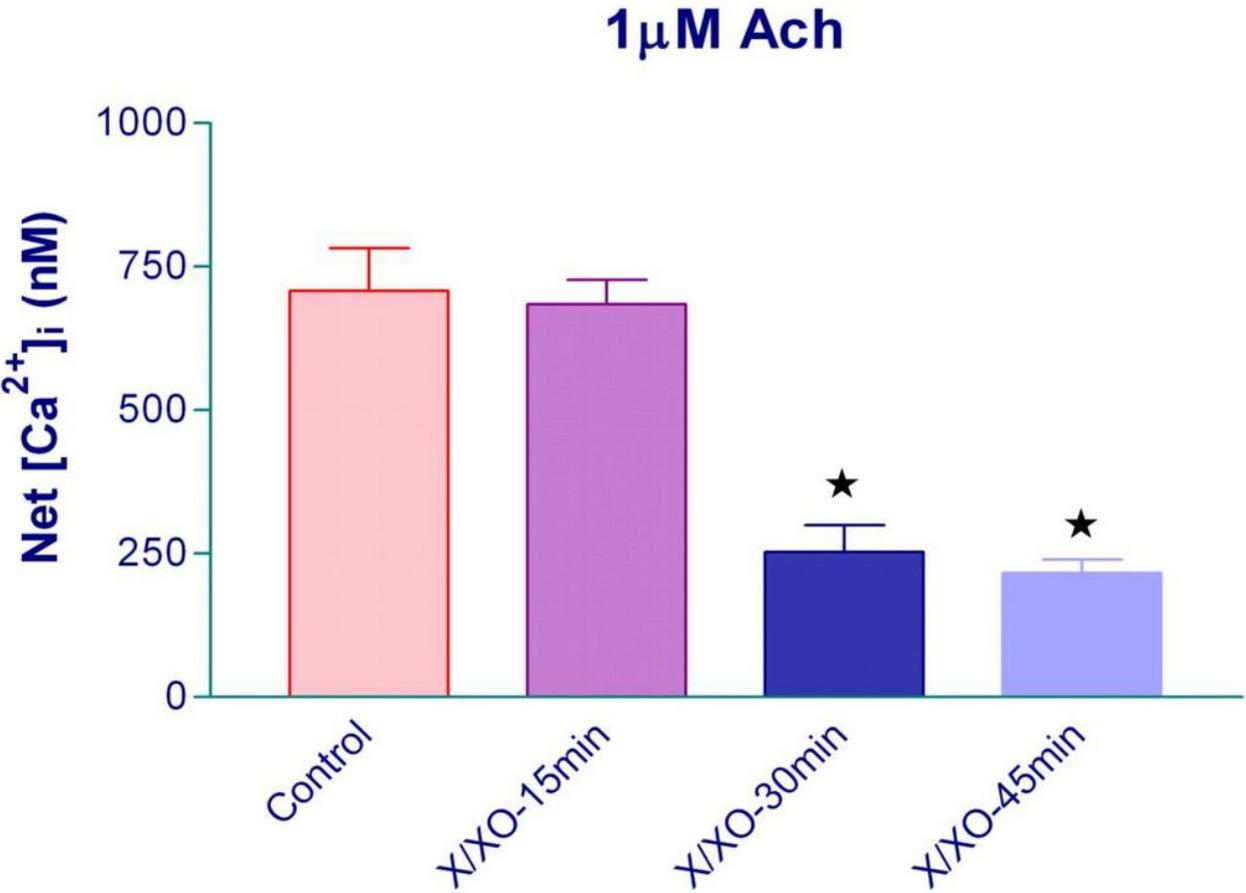


Figure 3

Effect of O<sub>2</sub><sup>-</sup> anions on net [Ca<sup>2+</sup>]<sub>i</sub> responses to 1 mM ACh

Figure 4

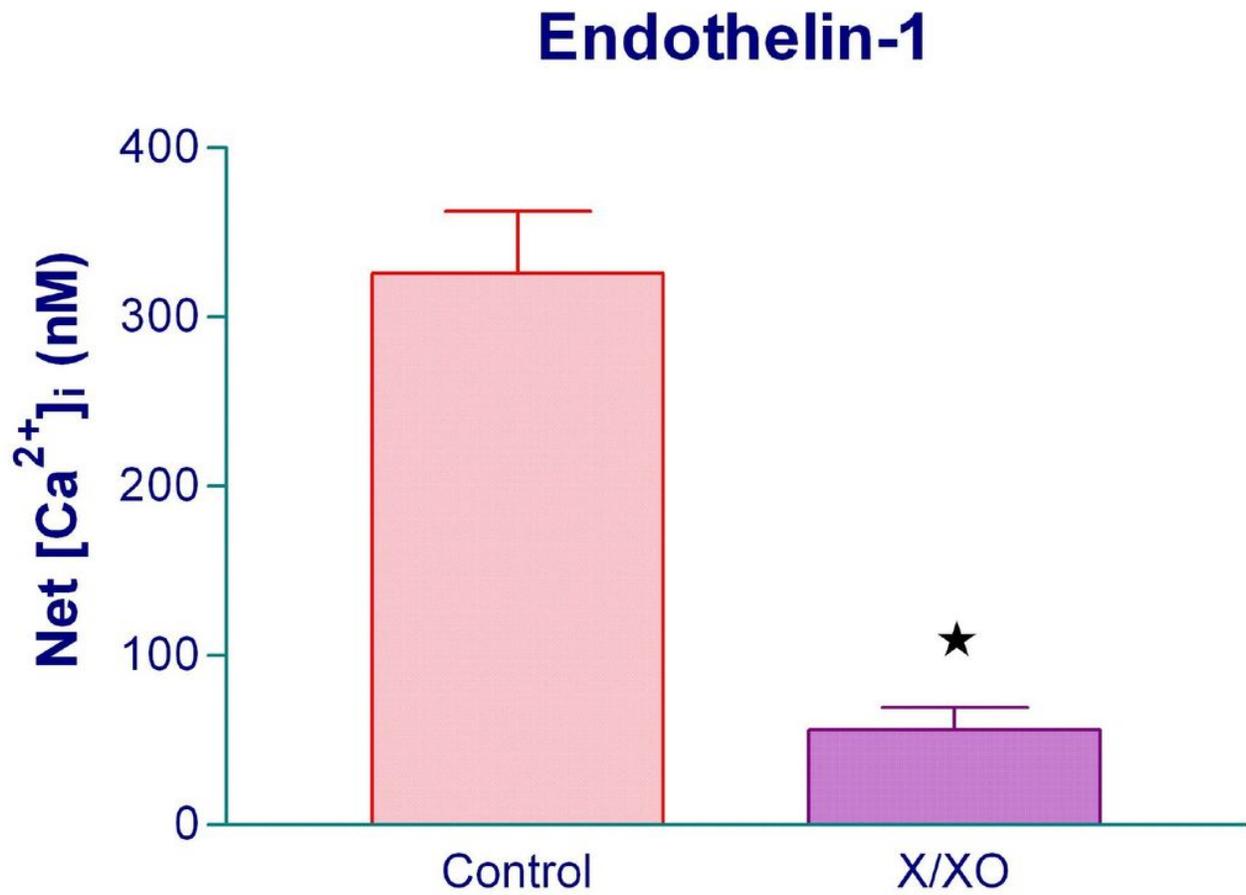


Figure 4

Effect of O<sub>2</sub><sup>-</sup> anions on net  $[Ca^{2+}]_i$  responses to endothelin-1

Figure 5

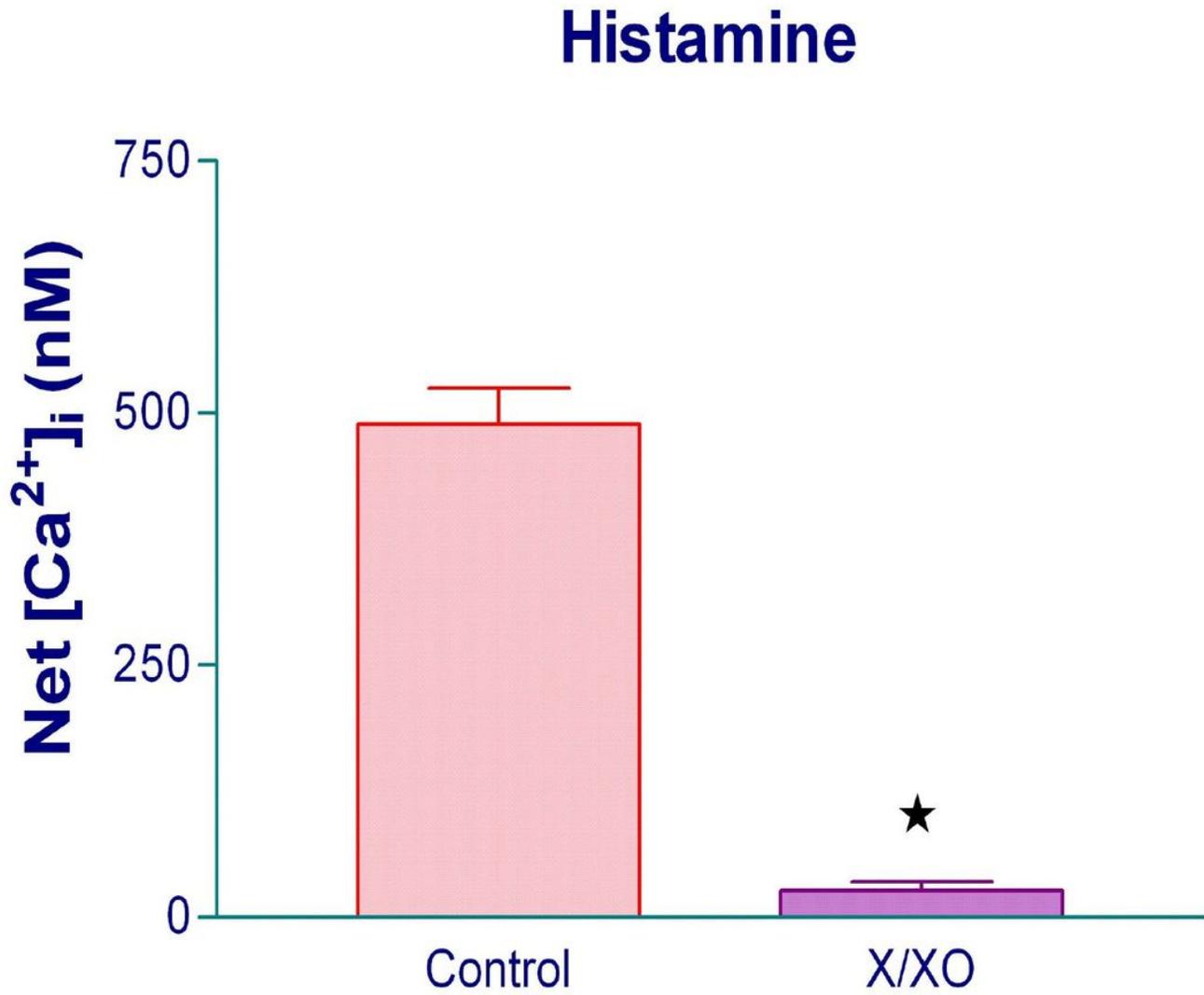


Figure 5

Effect of O<sub>2</sub><sup>-</sup> anions on net  $[Ca^{2+}]_i$  responses to histamine

Figure 6

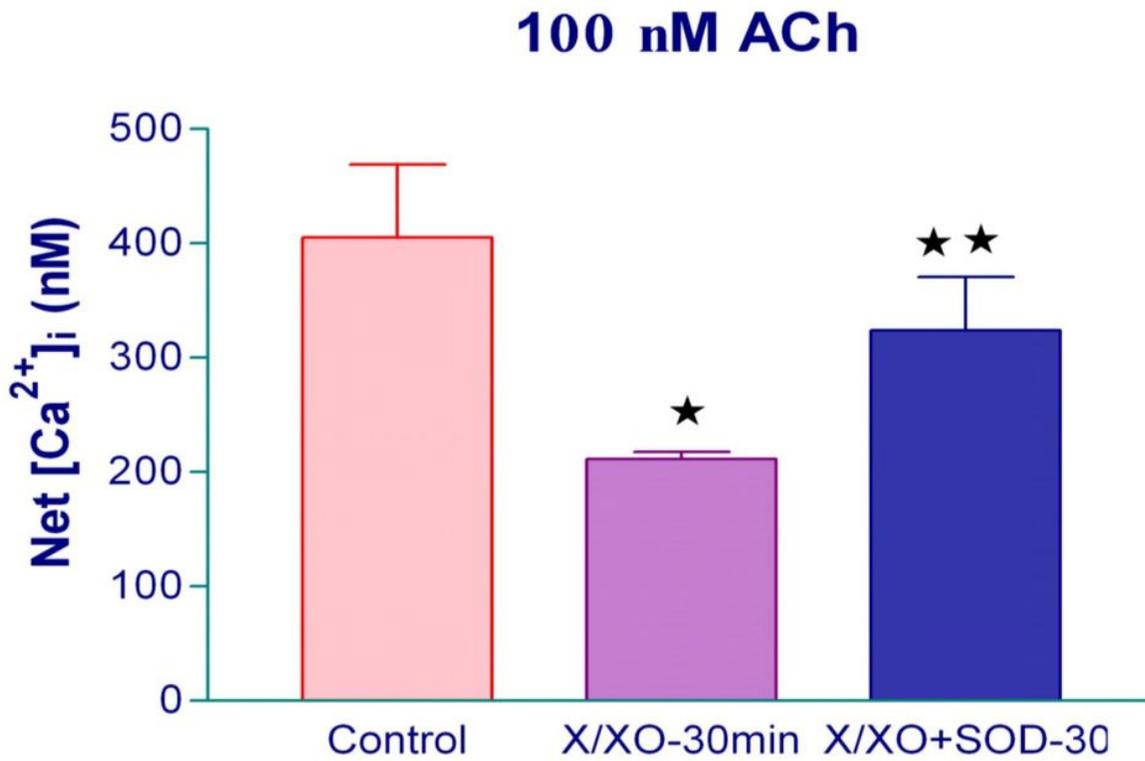


Figure 6

Effect of superoxide dismutase on ACh-induced O<sub>2</sub><sup>-</sup> anion release and net [Ca<sup>2+</sup>]<sub>i</sub> responses.

Figure 7

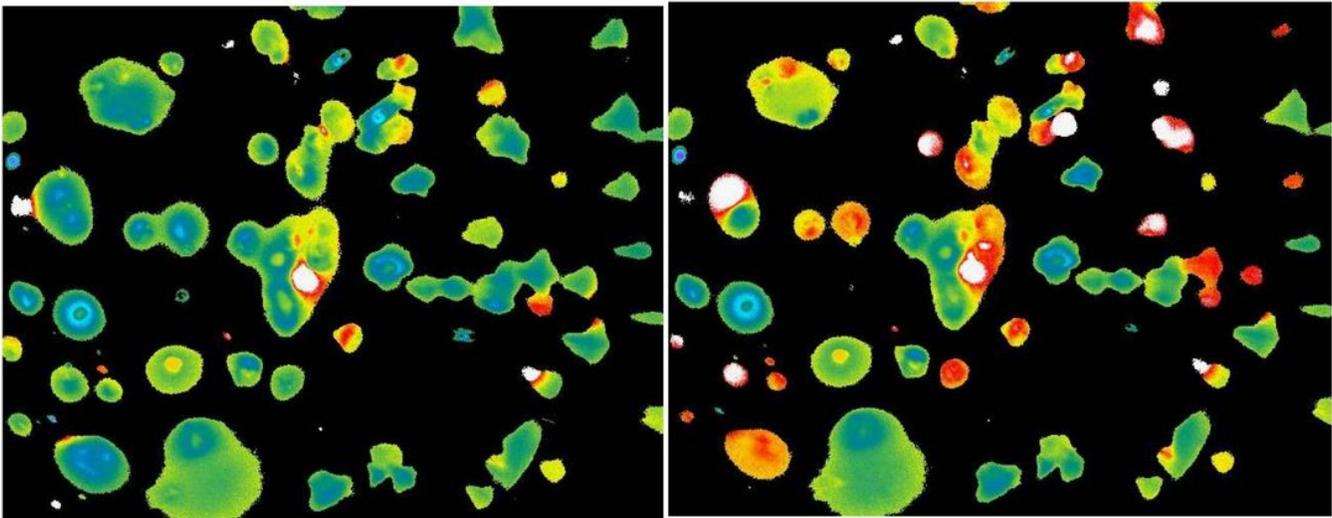


Figure 7

Effects of different agonists on intracellular calcium release with conversion of Fura-2 AM to fluorescent Fura as visualized under video fluorescent microscopy