Acetazolamide prevents hypoxia-induced reactive oxygen species generation and calcium release in pulmonary arterial smooth muscle

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Abstract

Upon sensing a reduction in local oxygen partial pressure, pulmonary vessels constrict, a phenomenon known as hypoxic pulmonary vasoconstriction. Excessive hypoxic pulmonary vasoconstriction can occur with ascent to high altitude and is a contributing factor to the development of high-altitude pulmonary edema. The carbonic anhydrase inhibitor, acetazolamide, attenuates hypoxic pulmonary vasoconstriction through stimulation of alveolar ventilation via modulation of acid–base homeostasis and by direct effects on pulmonary vascular smooth muscle. In pulmonary arterial smooth muscle cells (PASMCs), acetazolamide prevents hypoxia-induced increases in intracellular calcium concentration ([Ca2+]), although the exact mechanism by which this occurs is unknown. In this study, we explored the effect of acetazolamide on various calcium-handling pathways in PASMCs. Using fluorescent microscopy, we tested whether acetazolamide directly inhibited store-operated calcium entry or calcium release from the sarcoplasmic reticulum, two well-documented sources of hypoxia-induced increases in [Ca2+], in PASMCs. Acetazolamide had no effect on calcium entry stimulated by store-depletion, nor on calcium release from the sarcoplasmic reticulum induced by either phenylephrine to activate inositol triphosphate receptors or caffeine to activate ryanodine receptors. In contrast, acetazolamide completely prevented Ca2+-release from the sarcoplasmic reticulum induced by hypoxia (4% O2). Since these results suggest the acetazolamide interferes with a mechanism upstream of the inositol triphosphate and ryanodine receptors, we also determined whether acetazolamide might prevent hypoxia-induced changes in reactive oxygen species production. Using roGFP, a ratiometric reactive oxygen species-sensitive fluorescent probe, we found that hypoxia caused a significant increase in reactive oxygen species in PASMCs that was prevented by 100 μM acetazolamide. Together, these results suggest that acetazolamide prevents hypoxia-induced changes in [Ca2+], by attenuating reactive oxygen species production and subsequent activation of Ca2+-release from sarcoplasmic reticulum stores.

Keywords

pulmonary vascular smooth muscle, carbonic anhydrase, acetazolamide, Ca2+-release, intracellular Ca2+, hypoxia, vascular smooth muscle

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Introduction

Reductions in alveolar oxygen concentration can occur under a variety of conditions, including with ascent to high altitude or in the setting of acute and chronic lung diseases. As oxygen tensions fall, pulmonary blood vessels respond with hypoxic pulmonary vasoconstriction (HPV) in an attempt to divert blood flow away from poorly oxygenated regions of the lung. When excessive, HPV can cause...
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high-altitude pulmonary edema (HAPE).\textsuperscript{1,2} Although studied extensively, the exact cellular mechanisms by which hypoxia induces HPV are still being defined. In general, HPV is believed to require Ca\textsuperscript{2+} influx into the cytoplasm of pulmonary arterial smooth muscle cells (PASMCs), as HPV is attenuated by the removal of extracellular Ca\textsuperscript{2+} and by antagonists of voltage-gated Ca\textsuperscript{2+} channels\textsuperscript{3–9} and Ca\textsuperscript{2+}-permeable non-selective cation channels (NSCC).\textsuperscript{10–12} Other studies have demonstrated a role for Ca\textsuperscript{2+} release from internal stores in the sarcoplasmic reticulum (SR) in permeable non-selective cation channels (NSCC).\textsuperscript{10–12} However, the mechanism behind HPV in spontaneously breathing dogs.\textsuperscript{32,33}

Institutional Animal Care and Use Committee.

Methods

All procedures and protocols in this study were conducted in accordance with NIH guidelines for the proper care and use of animals in research and were approved by the Institutional Animal Care and Use Committee.

Isolation of PASMCs

PASMCs were isolated following protocols described previously by Shimoda et al.\textsuperscript{34} Briefly, intrapulmonary arteries (200–600 μm outer diameter) were isolated from adult male Wistar rats (250–350 g) under a dissecting microscope and cleaned of adventitial tissue. Only male animals were used in this study due to known sex-dependent differences in pulmonary responses to acute hypoxia.\textsuperscript{35–37} The endothelium was removed by gently rubbing the lumen with a cotton swab after which the arteries were allowed to recover for at least 30 min in N-[2-hydroxyethyl]piperazine-N\textsuperscript{-2-ethanesulfonic acid] (HEPES)-buffered saline solution (HBSS) containing (in mM): 130 NaCl, 5 KCl, 1.2 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 10 HEPES and 10 glucose on ice. The pH of the solution was adjusted to 7.2 using 5 M NaOH. The arteries were then transferred to reduced-Ca\textsuperscript{2+} (20 μM CaCl\textsubscript{2}) HBSS at room temperature for at least 20 min. The tissue was digested for 15–20 min at 37°C in reduced-Ca\textsuperscript{2+} HBSS to which was added collagenase (type I; 1750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml) and dithiothreitol (1 mM). Single PASMCs were obtained by gentle trituration in Ca\textsuperscript{2+}-free HBSS. Cells were transiently cultured on uncoated 25 mm glass cover slips in Ham’s F-12 media supplemented with 0.5% fetal bovine serum and 1% penicillin/streptomycin for 24-48 h. For each isolation, PASMCs were validated by using one coverslip of cells to measure change in [Ca\textsuperscript{2+}]\textsubscript{i} in response to 80 mM KCl (Fig. 1a) and one coverslip fixed and incubated in primary antibodies against smooth muscle-specific actin and smooth muscle specific myosin heavy chain, and counterstained with DAPI (Fig. 1b). Only isolations where >85% of cells stained positive for smooth muscle-specific actin and myosin and responded with >50 nM increase in [Ca\textsuperscript{2+}]\textsubscript{i} were used for experiments.

Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}

Coverslips containing PASMCs incubated with Fura-2 AM (5–7 μM for 60 min at 37°C) were mounted in a laminar flow cell chamber and perfused with modified Krebs bicarbonate (KRB) solution that contained (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO\textsubscript{4}, 25 NaHCO\textsubscript{3}, 11 glucose and 1.2 KH\textsubscript{2}PO\textsubscript{4}. Cells were perfused with KRB solution for 15 min at 37°C to remove extracellular dye and allow...
equilibration to a stable baseline prior to beginning protocols. Ratiometric measurement of Fura-2 fluorescence was performed on a workstation (Intracellular Imaging Inc., Cincinnati, OH) consisting of an inverted microscope with epi-fluorescence attachments. Light from a xenon arc lamp was filtered at 340 and 380 nm using an electronic filter wheel, and focused onto the PASMCs under examination via a 20x fluorescence objective (Super Fluor 20, Nikon). Emitted light from the cells was filtered at 510 nm using a band pass filter cube, returned through the objective and detected by a CCD imaging camera. Unless otherwise stated, images were captured at a rate of 5/min. Photobleaching of Fura-2 was minimized by use of a shutter within the filter wheel. Protocols were executed and data collected online with InCytim 2 software (Intracellular Imaging Inc.). 

[Ca²⁺]i was estimated from in vitro calibration curves generated using solutions consisting of Fura-2 and known concentrations of free Ca²⁺ ranging from 0 to 1350 nM. Up to five reservoirs containing experimental solutions and gassed with either 16% O₂; 5% CO₂ (control) or 4% O₂; 5% CO₂ (hypoxia) were connected via stainless steel tubing to a manifold and solutions changed via stopcocks, allowing rapid switching between experimental conditions.

For experiments measuring store-operated calcium entry (SOCE) through NSCCs, PASMCs were briefly perfused with Ca²⁺-free KRB solution containing 1 mM EGTA to chelate any residual free Ca²⁺ in the system and nifedipine (5 μM) to inhibit voltage-gated (L-type) Ca²⁺ channels. After 10 min, cells were exposed to cyclopiazonic acid (CPA; 10 μM) for 5 min to deplete intracellular Ca²⁺ stores. Cells were then perfused with Ca²⁺-containing KRB solution containing nifedipine and CPA, and the change in [Ca²⁺], recorded as a measure of SOCE. These experiments were complimented by measuring the quenching of Fura-2 fluorescence when extracellular Ca²⁺ was replaced with Mn²⁺. Under these conditions, Mn²⁺ enters the cells in place of Ca²⁺ and quenches Fura-2 dye fluorescence, the rate of which is a direct estimate of Ca²⁺ influx through NSCCs. Fura-2 fluorescence was monitored after exciting the cells at 360 nm (F₃₆₀) at 30 s intervals. Fluorescence excited at 360 nm is the same for Ca²⁺-bound and Ca²⁺-free Fura-2; therefore, changes in fluorescence are assumed to be caused by Mn²⁺ alone. PASMCs were treated with nifedipine in the absence and presence of CPA, and F₃₆₀ recorded before and after addition of MnCl₂ (200 μM) to the perfusate. SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn²⁺.

In a subset of experiments, AZ (100 μM) was added to all the perfusates. To measure Ca²⁺ release from SR stores, PASMCs were perfused with Ca²⁺-free KRB solution for 10 min. This duration of perfusion with Ca²⁺-free solution was previously shown to not deplete intracellular stores.16 Cells were then exposed to phenylephrine (PE; 100 μM) or caffeine (1 mM) for 10 min. For cells where the effect of AZ on store release was tested, AZ (100 μM) was added to all solutions beginning with perfusion with the Ca²⁺-free solution. For Ca²⁺ release measurements, images were captured at a rate of 10/min.

**DCF measurements of ROS generation**

In a subset of early experiments, changes in ROS were measured using the cell-permeant fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Upon cleavage by intracellular oxidases and oxidation, H₂DCFDA is converted to 2',7'-dichlorofluorescein.
(DCF). H₂DCFDA-loaded PASMCs were excited with light filtered at 490 nm, and emitted light was detected at 510 nm. Cells were incubated with H₂DCFDA by continuous perfusion with Krebs solution containing 500 nM H₂DCFDA for at least 15 min before beginning measurements to allow stable uptake of dye. Baseline DCF fluorescence was monitored for at least 5 min prior to beginning drug exposures to verify that a stable baseline had been established. PASMCs were then exposed to control KRB solution or KRB solution containing AZ (100 µM) for 10 min. At the end of this period, cells were challenged with endothelin-1 (ET-1; 10⁻¹₀ M). Since DCF fluorescence is detected at a single wavelength, any change in the concentration of the dye in the perfusion medium (i.e. differing concentrations between perfusion reservoirs) could result in a change in fluorescence that is not due to a change in ROS. To minimize the possibility of this type of error, all experiments were performed by perfusing from a single reservoir into which AZ and/or ET-1 were dissolved directly.

**roGFP measurements of ROS generation**

Because DCF is a single wavelength dye and emission values can be altered by differences in light intensity, amount of dye and photobleaching, we performed experiments exploring the effect of hypoxia on ROS generation with roGFP following the methods described by Suresh et al.³⁸ In brief, the same experimental equipment as described for [Ca²⁺]ᵢ measurements was used. PASMCs cultured on glass coverslips were infected with a baculovirus containing roGFP-Grx1 (Premo redox sensor, Invitrogen) at 80 MOI. After 24 h, cells were washed and changed to basal media for 48 h prior to experimental protocols. PASMCs were mounted in the flow chamber and washed for 15 min prior to data collection. Cells were excited at 380 nm and 490 nm, and emission was observed at 510 nm. F₃₈₀/F₄₉₀ was measured at baseline for 5 min, followed by perfusion with KRB solution bubbled with 4% O₂ for 10 min and then returned to perfusion with 16% O₂-containing KRB solution. In some experiments, cells were perfused with AZ (100 µM) for 15 min prior to challenge with KRB solution containing AZ and gassed with 4% O₂. Images were acquired at 5 images/min. Regions of interest containing individual cells were selected following background subtraction, and the F₃₈₀/F₄₉₀ ratio was calculated using ImageJ software.

**Drugs and reagents**

Endothelin-1 was obtained from American Peptides. roGFP was purchased from Life Biotechnologies. H₂DCFDA-DA and Fura-2 AM were purchased from Molecular Probes/ThermoFisher Scientific. Collagenase was purchased from Worthington. All other drugs were purchased from Sigma Aldrich.

**Statistical analysis**

All data are presented as scatter plots with bars representing mean ± SEM. For each experiment, cells from different animals were used; thus, “n” refers to both the number of biological replicates (i.e. number of animals from which cells were derived) and number of distinct experiments. Each experimental condition was performed on a different coverslip, and data from 10–30 cells per coverslip were measured and averaged to obtain a single value. Data were tested for normality and statistical comparisons were performed using Student’s t-test (paired or unpaired as appropriate).

**Results**

**Effect of AZ on store-operated Ca²⁺ entry**

Previous reports demonstrated that Ca²⁺ influx through both L-type Ca²⁺ channels and NSCCs contributes to the hypoxia-induced increase in [Ca²⁺]ᵢ, in rat PASMCs.¹⁰ Previous work also showed that AZ did not directly inhibit L-type Ca²⁺ channels.²⁹ Thus, as a first step in this study, we tested the ability of AZ to directly block SOCE occurring through NSCCs. In the presence of 0 mM external Ca²⁺, application of CPA caused a transient increase in [Ca²⁺]ᵢ (Fig. 2a), consistent with store depletion. Restoration of extracellular Ca²⁺ caused a rapid increase in [Ca²⁺]ᵢ, that was sustained for the duration of CPA exposure. The peak change in [Ca²⁺]ᵢ was 210 ± 26 nM (Fig. 2b). Application of AZ did not alter baseline [Ca²⁺]ᵢ, (117.4 ± 17.1 nM for control and 96.2 ± 15.5 nM for AZ; n = 5 each) nor the change in [Ca²⁺]ᵢ, induced by CPA (142.0 ± 21.0 nM for control and 134.2 ± 19.9 nM for AZ; n = 5 each). Similarly, AZ had no effect on either the peak or sustained change in SOCE (Fig. 2a and b). Because the increase in [Ca²⁺]ᵢ, measured with Ca²⁺ restoration is a combination of both influx and efflux mechanisms, we also measured SOCE using Mn²⁺ quenching of Fura-2 fluorescence. In the absence of extracellular Ca²⁺, the addition of extracellular Mn²⁺ traverses open Ca²⁺ channels and binds Fura-2, reducing fluorescence. It was previously demonstrated that when L-type Ca²⁺ channels are inhibited, the main entry pathway in PASMCs following exposure to CPA is SOCE via NSCC.³⁹ Under these conditions, the rate of decrease in fluorescence at the 360 nm wavelength provides an estimate of Ca²⁺ entry through NSCCs. Mn²⁺-quenching of F₃₆₀ was present in control cells treated with CPA to induce SOCE and was not different in the presence of AZ (Fig. 2c and d).

**AZ and hypoxia-induced Ca²⁺ release**

Since AZ had no direct effect on SOCE and did not appear to be directly inhibiting the pathways of Ca²⁺ influx from the extracellular space that are known to be involved in the hypoxia-induced increase in PASMC [Ca²⁺], we next tested whether the compound could be inhibiting hypoxia-induced...
release of Ca\(^{2+}\) from the SR. In control PASMCs perfused with Ca\(^{2+}\)-free external solution, switching to KRB solution gassed with 4% O\(_2\) resulted in rapid, transient increase in [Ca\(^{2+}\)]\(_i\) (Fig. 3). However, in PASMCs pretreated with AZ, the change in [Ca\(^{2+}\)]\(_i\) in response to hypoxia was abolished in most experiments, suggesting that AZ was preventing hypoxia-induced changes in [Ca\(^{2+}\)]\(_i\) by inhibiting Ca\(^{2+}\) release from intracellular stores.

**Effect of AZ on Ca\(^{2+}\) release from internal stores**

Given the striking effect of AZ on hypoxia-induced Ca\(^{2+}\) release from intracellular stores, we speculated that the compound might act as a direct inhibitor of IP\(_3\)Rs or RyRs. To test this hypothesis, we measured the effect of AZ on the change in [Ca\(^{2+}\)]\(_i\), induced by activation of SR receptors by agonists. In the absence of extracellular Ca\(^{2+}\), activating either RyRs (Fig. 4a–c) with caffeine (1 mM) or IP\(_3\)Rs (Fig. 4b and d) with PE (100 \(\mu\)M) caused a rapid and pronounced, but transient, increase in [Ca\(^{2+}\)]\(_i\). Pretreatment with AZ did not inhibit the peak change in [Ca\(^{2+}\)]\(_i\), induced by either PE or caffeine, suggesting that agonist-induced activation of SR receptors is not sensitive to AZ.

**Effect of AZ on ET-1-induced ROS production**

Because AZ blocked hypoxia – but not agonist-induced Ca\(^{2+}\) release, we next tested whether AZ might be interfering with a pathway upstream of Ca\(^{2+}\) release. Several studies have shown a role for hypoxia-induced ROS production in initiating the change in [Ca\(^{2+}\)]\(_i\), observed in response to hypoxia.\(^{14,18,40-44}\) In order to test whether AZ might act as a general antioxidant, we measured the ability of AZ to alter ROS levels at baseline and/or prevent ROS production in response to an agonist known to induce ROS: the vasoactive peptide, endothelin-1 (ET-1), which has been shown to induce ROS production via activation of NADPH oxidase.\(^{45,46}\) In the absence of AZ, we found...
that ET-1 caused a significant increase in DCF fluorescence in PASMCs (Fig. 5). Addition of AZ alone had no significant effect on baseline DCF fluorescence after 5 min (3.3 ± 8.9% change; n = 5; p = 0.446). In the presence of AZ, the effect of ET-1 on DCF fluorescence was not diminished, still averaging over 50% increase from baseline. These data suggest the compound was not acting as a general ROS scavenger.
Although we initially used DCF as a means to detect ROS production, this approach has a major limitation with respect to hypoxia. As a single wavelength dye, DCF is only appropriate for measurement of changes in ROS within a sample and with perfusion from a single reservoir. However, eliciting hypoxic responses in PASMCs requires rapid reduction in oxygen levels, which cannot be achieved by changing the gas within a reservoir and instead requires two reservoirs with pre-gassed solution. Thus, we chose to explore other newly developed ROS-detecting tools, i.e. roGFP sensors, to directly determine whether AZ might be preventing hypoxia-induced Ca\(^{2+}\) release by altering ROS production. Using cells infected with a baculovirus containing the roGFP construct, we measured changes in cellular ROS during challenge with hypoxia in the absence and presence of AZ. In untreated cells, reducing oxygen levels to 4% O\(_2\) resulted in a rapid increase in roGFP fluorescence (Fig. 6a), consistent with production of ROS. With re-oxygenation, roGFP fluorescence rapidly returned to original levels. However, in cells pretreated with AZ, no change in roGFP fluorescence was observed in response to hypoxia (Fig. 6b and c). The lack of response to hypoxia in AZ-treated cells could not be attributed to a direct effect of AZ on basal roGFP fluorescence, as perfusion with AZ alone for 15 min resulted in no significant change in fluorescence (3.5 ± 12.0% change in fluorescence (n = 3; p = 0.67)).
**Discussion**

While the inhibiting effects of AZ on HPV have been known for some time, the mechanism by which AZ affords protection from HPV and hypoxia-induced changes in PASMCs (Ca^{2+}) remains incompletely understood. Previous experiments ruled out changes in intracellular acidification, membrane potential or direct effects on voltage-gated Ca^{2+} channels. In this study, we found that AZ does not directly inhibit Ca^{2+} influx through NSCCs induced by store-depletion nor does it block agonist-induced Ca^{2+} release from receptors (IP_{3}R or RyRs) on the SR. Rather, during hypoxia AZ prevented production of ROS, which was associated with a lack of Ca^{2+} release from the SR.

It is widely accepted that generation of HPV requires an elevation in [Ca^{2+}]_i. The sources of Ca^{2+} comprising the response to hypoxia in PASMCs have been extensively studied, with general consensus that release from internal stores and influx through voltage-gated (L-type) Ca^{2+} channels and NSCCs are contributing factors.

Interestingly, previous work showed that AZ had no effect on basal [Ca^{2+}]_i levels and did not alter changes in [Ca^{2+}]_i induced by potassium chloride (KCl), indicating that the drug did not act as a Ca^{2+} chelator and did not block depolarization-driven Ca^{2+} influx through L-type Ca^{2+} channels. The differential inhibitory effect of AZ on the increases in [Ca^{2+}]_i in response to hypoxia and KCl corroborated data reporting that hypoxia can cause Ca^{2+} influx through pathways other than L-type Ca^{2+} channels. For example, NSCCs, which include Ca^{2+} permeable channels that can be activated when intracellular stores are depleted, have been implicated in hypoxia-induced Ca^{2+} responses in PASMCs and in HPV measured in whole animals and intact lung preparations. Thus, a first step in the current study was to evaluate the ability of AZ to directly block Ca^{2+} influx through NSCCs induced by store-depletion (SOCE). Under these conditions, capacitive Ca^{2+} entry following store depletion is believed to act as a mechanism to refill SR stores and can be visualized as a large increase in [Ca^{2+}]_i when extracellular Ca^{2+} levels are returned to normal. As expected, restoration of extracellular Ca^{2+} levels following store depletion resulted in a large and sustained increase in [Ca^{2+}]_i, consistent with previous reports. Because the increase in [Ca^{2+}]_i, measured in this type of Ca^{2+}-restoration experiment reflects a composite response including both influx and efflux mechanisms, we also measured the effect of AZ on Mn^{2+} quenching, a more direct measure of Ca^{2+} entry. Surprisingly, AZ at a concentration and time of treatment sufficient to block hypoxia-induced changes in [Ca^{2+}]_i had no effect on SOCE measured by either method. Since AZ blocks the overall Ca^{2+} response to hypoxia, which several labs have shown requires SOCE, our main intent with these experiments was to demonstrate whether AZ had a direct effect on SOCE (i.e. directly inhibited this channel). Thus, we only performed experiments under non-hypoxic conditions. Overall, our data suggest that AZ does not directly inhibit Ca^{2+} influx through NSCCs.

Given the inability of AZ to directly inhibit the activation of NSCCs and L-type Ca^{2+} channels, the major Ca^{2+} influx pathways responsible for hypoxia-induced changes in PASMCs [Ca^{2+}]_i, we next determined whether AZ might be blocking hypoxia-induced Ca^{2+} release from intracellular stores. In control PASMCs, we observed a large, transient increase in [Ca^{2+}]_i in response to hypoxia in the absence of extracellular Ca^{2+}, which has previously been shown to be due to release from both RyRs and IP_{3}Rs. We found that AZ completely prevented hypoxia-induced Ca^{2+} release and speculated that the lack of response might be due to a direct inhibitory action of AZ on either SR receptor type. To our surprise, Ca^{2+} release in response to PE, which stimulates release from IP_{3}Rs, or caffeine, which stimulates release from RyRs, was unaltered by AZ. The lack of inhibition of Ca^{2+} release in response to PE or caffeine indicates that AZ was not directly inhibiting these channels and is consistent with the lack of effect of AZ on CPA-induced Ca^{2+} responses measured in the capacitive Ca^{2+} entry experiments, since CPA increases [Ca^{2+}]_i by inhibiting Ca^{2+} reuptake into the SR following passive leak from IP_{3}Rs and RyRs. These results also confirm that the effect of AZ on PASMC Ca^{2+}-signaling during hypoxia was not due to toxicity or a non-specific inability to mobilize intracellular Ca^{2+}. Rather, our results suggested that AZ may be interfering with hypoxia signaling pathways upstream of IP_{3}Rs and RyRs.

The exact mechanism by which hypoxia triggers Ca^{2+} release from the SR is still being investigated. Several possibilities have been proposed (reviewed in Sylvester et al.), including ROS-dependent activation of Ca^{2+} release. While it is clear that ROS production changes with hypoxia in PASMCs, whether hypoxia causes an increase or decrease in ROS has been a matter of some debate (see literature for review). In our current study, roGFP fluorescence increased in response to hypoxia, consistent with other reports demonstrating hypoxia induced an increase in ROS production. Surprisingly, AZ completely prevented the hypoxia-induced increase in roGFP fluorescence.

Interestingly, Waypa et al. showed that hypoxia caused an increase in mitochondrial ROS production in PASMCs that was required for the hypoxia-induced increase in [Ca^{2+}]_i. Similar results were observed in mouse lung slices, where blockade of mitochondrial ROS production or removal of extracellular Ca^{2+} reduced HPV. While the exact source of the increased [Ca^{2+}]_i in those studies was not explored, other work has linked activation of Ca^{2+} release from SR stores to ROS generation from NADPH oxidase and mitochondria. Our results showing that inhibition of hypoxia-induced Ca^{2+} release by AZ was associated with suppression of hypoxia-induced ROS generation support the idea that ROS promote Ca^{2+} release in PASMCs during hypoxia; however, we acknowledge that
since we did not specifically test whether production of ROS was required for the hypoxia-induced increase in [Ca\(^{2+}\)], in this study, it remains possible that AZ prevented the hypoxia-induced Ca\(^{2+}\) release by interference with a non-ROS-dependent mechanism.

The mechanism by which AZ prevented hypoxia-induced increases in ROS remains unclear. To test whether AZ might be acting as a general ROS scavenger, we examined whether basal ROS levels were altered by addition of AZ, finding no effect on baseline fluorescence of either DCF or roGFP. Thus, AZ itself was not oxidizing or reducing our ROS sensors. We next tested whether AZ could diminish ROS production by a different agonist: ET-1. ET-1 is a known pulmonary vasoconstrictor believed to modulate HPV\(^{52,63}\) and has been shown to produce ROS in PASMCs\(^{64-66}\), likely via activation of NADPH oxidase.\(^{45,46}\) However, in our experiments, AZ had no effect on ROS production in response to ET-1, refuting the possibility that AZ was simply acting as a general antioxidant, which has been reported for other compounds containing a similar thiadiazole ring.\(^{67}\) These results would also appear to suggest that AZ does not inhibit NADPH oxidase, which has been proposed as part of the mechanism by which ROS generation and [Ca\(^{2+}\)] are increased during hypoxia.\(^{18,41}\)

Instead, it is likely that AZ targets mitochondrial ROS. In several studies, the site of hypoxia-induced ROS production in PASMCs has been localized to Complex III in mitochondria,\(^{43,44,61,62}\) whereas other studies have demonstrated the requirement for Complex II.\(^{68}\) While our data point to AZ interfering with ROS production during hypoxia, whether this occurs at Complex III, at a different site in mitochondria, or via different ROS generating pathways will require further exploration.

One limitation of this study was that we used two different fluorescent dyes to measure ET-1- and hypoxia-induced changes in ROS. While DCF, a single wavelength dye, could easily be used to examine the effects of ET-1 on ROS where perfusion occurred from a single reservoir, exploring the effect of hypoxia was more uncertain since experiments using hypoxia required perfusion of cells from different pre-gassed reservoirs, which could introduce errors due to differences in DCF concentration between solutions. With the development of roGFP,\(^{69}\) a genetically encoded construct that allows ratiometric measures of ROS levels, we were able to more accurately measure hypoxia-induced changes in ROS. Although our experiments utilized different ROS sensors, we nonetheless believe that the results obtained point to a specific role of AZ in inhibiting hypoxia-induced ROS.

In previous experiments, the effect of AZ on HPV and hypoxia-induced Ca\(^{2+}\) responses was shown to be independent of carbonic anhydrase inhibition as an AZ analogue with the sulfonamide group substituted with a methyl group (N-meth-AZ) was similarly potent in inhibiting HPV and hypoxia-induced elevations in PASMC [Ca\(^{2+}\)].\(^{29,33}\)

While we did not perform experiments using N-meth-AZ in this study, the fact that Ca\(^{2+}\) release is required for the global hypoxia-induced increase in [Ca\(^{2+}\)], in PASMCs, which was blocked by N-meth-AZ in earlier studies,\(^{29}\) strongly supports the likelihood that the effects of AZ on ROS and Ca\(^{2+}\) release are independent of carbonic anhydrase.

In addition to attenuating acute HPV, AZ has also been shown to reduce the development of pulmonary hypertension in response to chronic hypoxia.\(^{70}\) Although the exact extent to which contractile mechanisms involved in HPV contribute to enhanced contraction and increased pulmonary arterial pressure during chronic hypoxia are unclear, it is tempting to speculate that reducing pulmonary arterial contraction could contribute to the effect of AZ on pulmonary hypertension. Along these lines, an elevation in [Ca\(^{2+}\)], via NSCCs maintains elevated baseline tone in pulmonary vascular smooth muscle from chronically hypoxic rats.\(^{71}\) Moreover, enhanced production of ROS in PASMCs from chronically hypoxic rats, contributing to augmented contraction, has been reported,\(^{65}\) raising the possibility that AZ may mediate effects on hypoxia-induced pulmonary hypertension via modulation of ROS and Ca\(^{2+}\) signaling.

In summary, we found that AZ blocks the rise in PASMC [Ca\(^{2+}\)], that occurs in response to hypoxia by preventing hypoxia-induced Ca\(^{2+}\) release. Given the previously reported links between ROS and [Ca\(^{2+}\)], and the requirements of Ca\(^{2+}\) release for elevated [Ca\(^{2+}\)] during hypoxia, it seems likely that AZ exerts its inhibitory effect on HPV and the hypoxia-mediated increase in [Ca\(^{2+}\)] in PASMCs by preventing hypoxia-induced ROS production and subsequent triggering of Ca\(^{2+}\) release. Thus, this study sheds new light on the mechanism of action of AZ with respect to repression of hypoxia-induced Ca\(^{2+}\) signaling, and points to future areas of investigation to further refine our understanding of how this compound modulates HPV.

**Authors’ contribution**

LAS, CU, XY, JTS, KS and ERS designed experiments, LAS, CU, XY, HJ and KS performed experiments, LAS and CU analyzed results, LAS, CU, KS, XY, HJ, JTS and ERS drafted article and approved final version.

**Conflict of interest**

The author(s) declare that there is no conflict of interest.

**Ethical approval**

Procedures and protocols were approved by the Johns Hopkins University Animal Care and Use Committee.

**Guarantor**

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