Redox Biology

Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation

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Abstract: Zinc (Zn) has antioxidant, anti-inflammatory and anti-proliferative actions, with Zn dysregulation associated with coronary ischemia/reperfusion injury and smooth muscle cell dysfunction. As the majority of studies concerning Zn have been conducted under non-physiological hyperoxic conditions, we compare the effects of Zn chelation or supplementation on total intracellular Zn content, antioxidant NRF2 targeted gene transcription and hypoxia/reoxygenation-induced reactive oxygen species generation in human coronary artery smooth muscle cells (HCASMC) pre-adapted to hyperoxia (18kPa O2) or normoxia (5kPa O2). Expression of the smooth muscle marker SM22-α was unaffected by lowering pericellular O2, whereas calponin-1 was significantly upregulated in cells under 5kPa O2, indicating a more physiological contractile phenotype under 5kPa O2. Inductively coupled plasma mass spectrometry established that Zn supplementation (10μM ZnCl2 + 0.5μM pyrithione) significantly increased total Zn content in HCASMC under 18 but not 5kPa O2. Zn supplementation increased metallothionein mRNA expression and NRF2 nuclear accumulation in cells under 18 or 5kPa O2. Notably, NRF2 regulated HO-1 and NQO1 mRNA expression in response to Zn supplementation was only upregulated in cells under 18 but not 5kPa O2. Zn supplementation increased intracellular glutathione (GSH) in cells pre-adapted to 18 but not 5kPa O2. Furthermore, whilst hypoxia increased intracellular glutathione (GSH) in cells pre-adapted to 18 but not 5kPa O2, reoxygenation had negligible effects on GSH or total Zn content. Reoxygenation-induced superoxide generation in cells under 18kPa O2 was abrogated by PEG-superoxide dismutase but not by PEG-catalase, and Zn supplementation, but not Zn chelation, attenuated reoxygenation-induced superoxide generation in cells under 18 but not 5kPa O2, consistent with a lower redox stress under physiological normoxia. Our findings highlight that culture of HCASMC under normoxia recapitulates an in vivo contractile phenotype and that effects of Zn on NRF2 signaling are altered by oxygen tension.

Suggested Reviewers: Patricia Oteiza, PhD
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Expertise in zinc modulation of NRF2 signaling

Christiane Ott, PhD
German Institute of Human Nutrition Potsdam-Rehbruecke
### Opposed Reviewers:

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### Response to Reviewers:
June 5, 2023

Professor Tilman Grune
Editor-in-Chief - Redox Biology

Dear Tilman,

Re: REDOX-23-01036R1 – Yang et al.

Thank you for the opportunity to resubmit a revised manuscript.

We have submitted our revised manuscript entitled ‘Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation’ by Fan Yang, Matthew J Smith, Alexander Griffiths, Alexander Morrell, Sarah J Chapple, Richard C Siow, Theodora Stewart, Wolfgang Maret and Giovanni E Mann. We appreciate the Reviewers’ positive comments and that our ‘work is novel, well designed and the manuscript clearly written with appropriate figures and results properly discussed’ and importantly that ‘vascular protection afforded by zinc under physiological oxygen levels is interesting’.

Although we are not able to conduct further experiments within the timeline for resubmission, we have included additional experimental data in the revised manuscript (see Fig. 1 and Supplementary Fig. 1). We hope that this additional data and our response the Reviewers clarifies their queries.

The revised manuscript has been approved by all authors, and all changes to the revision have also been ‘marked-up’ in a supplemental Word file with text highlighted in blue font.

We hope that our revised manuscript will be acceptable for publication in Redox Biology.

Best wishes,

Giovanni E. Mann, Ph.D.
Professor of Vascular Physiology
REDOX-D-23-01036R1 – Yang et al. Revised MS

Authors Responses to Editor-in-Chief and Reviewers

Editor:
We thank the Editor for the opportunity to submit a revised manuscript and have addressed the Reviewers comments. Additions or edits to the manuscript are highlighted in our comments below and in the ‘marked-up’ revision of the manuscript.

Reviewer #1:
This paper investigated how hypoxia/reoxygenation can affect zinc homeostasis and redox regulation (Nrf2) in HCASMC cells. The work is novel, well designed and suitable for the journal, it is also clearly written, figures are appropriate and results properly discussed. The reviewer has only minor comments.

1. “Treatment of cells with the Zn chelator TPEN had negligible effects on Zn content under either 18 or 5kPa O₂”. Is it possible that although zinc cell content is not affected when measured by ICP-MS there is less bioavailable zinc given that this method would also detect the zinc bound to TPEN?

We thank the Reviewer for this insightful comment and concur.
We have added a sentence concerning this point to the revised manuscript (see p. 10, para. 9, lines 7-9):

'Total Zn content may not be altered following treatment of HCASMC with TPEN, since ICP-MS analysis would also detect Zn chelated by TPEN. As reported in many other studies [53], TPEN would be expected to decrease bioavailable Zn levels.'

2. How was the concentration of TPEN selected? There is no effect of TPEN on any of the parameters measured. Did authors consider that the used concentration may not be effective?

In selecting the concentration of TPEN for treatment of HCASMC, we initially conducted MTT viability assays of cells treated for 16 h with different concentrations TPEN (0.5, 0.75, 1.0, 1.25 or 1.5µM). We selected a non-damaging concentration of 1.25 µM, within the concentration range used in many other studies in vitro. We were concerned that cells would not tolerate higher concentrations.

3. TPEN can bind to zinc pools that would not necessarily be decreased when cellular zinc decreases physiologically. It would be important to include a discussion on this.

As the basal Zn concentration in the media for static monolayer cell culture is quite low, it may in part explain the lack of a statistical difference between vehicle and TPEN treated cells. We have rephrased the relevant text in the Discussion (see p. 10, para. 2, lines 6-8):

'The Zn chelator TPEN (1.25µM) had negligible effects on total intracellular Zn content. It is possible that the low basal Zn concentration in the media for HCASMC may in part explain the lack of a statistical difference between vehicle and TPEN treated cells.'

4. It would be helpful to include one sentence in methods explaining how the probe L-012 detect ROS? Does it have any specificity for reactive oxygen species?

To further clarify whether L-012 exhibits specificity for different reactive oxygen species, we have added the following text to the Methods in Section 2.7:

‘L-012 is widely used to measure superoxide (O₄⁻) and other reactive oxygen species (ROS) in biological systems (Zielonka et al., 2013). Although O₄⁻ alone does not react with L-012 to emit luminescence, oxidation of the probe to its radical and reaction of the luminol radical with self-generated O₄⁻ during oxidation of L-012 leads to the emission of blue light, which can be inhibited by superoxide dismutase (SOD) (Zielonka et al., 2013).

Reviewer #2:
The manuscript by Yang et al. describes the vascular protection by zinc in human coronary artery smooth muscle cells through NRF2 signaling under hypoxia/reoxygenation. Thereby, authors analyzed the impact of zinc by comparing the effects of zinc chelation and supplementation on total intracellular zinc level, NRF2 targeted gene transcription and ROS formation induced by hypoxia/reoxygenation in HCASMCs in hyperoxic (18kPa O₂) and normoxic (5kPa O₂) conditions. While expression of smooth
muscle marker SM22-a was unaffected, calponin-1 was significantly upregulated under 5kPa O₂. Further, total zinc content after zinc supplementation was significantly increased in HCASMCs under 18kPa but not in 5kPa O₂. While zinc supplementation also increased metallothionein mRNA expression and NRF2 nuclear accumulation but in both oxygen concentrations, NRF2 regulated HO-1 and NQO1 mRNA expression was only upregulated under 18kPa. In addition, reoxygenation-induced superoxide generation in cells under 18kPa O₂ was abrogated by PEG-superoxide dismutase but not by PEG-catalase and only zinc supplementation, not zinc chelation, attenuated superoxide formation in cells under 18kPa but not in 5kPa O₂. Authors showed that HCASMCs under 5kPa mimic an in vivo contractile phenotype and that effects of zinc on NRF2 signaling are altered under modulated oxygen tension. Since the majorities of the studies addressing cell culture approaches, have been performed under atmospheric oxygen (18kPa), analysis of zinc in vascular protection at normoxic, physiological conditions, is quite interesting and suits well to the journal. I recommend publishing the manuscript after clarification of a few points.

1. Under results 3.1 authors mentioned 3 markers of contractile phenotype early, mid-term and late. In Fig. 1 only data for early and mid-term are shown. Is there a particular reason for not showing the late marker? And does a hyperoxic state have an effect on cell shape?

We appreciate the Reviewer’s important queries. In vitro cell culture is a process that involves loss of contractile markers and restoration of contractile markers after confluence. Previous studies have reported that late markers (e.g. smooth muscle myosin heavy chain (SMMHC)-1 and -2 and smoothelin) are the first to disappear during phenotype modulation (Babij et al., 1992a; van Eys et al., 2007; vanderLoop et al., 1996). For example, the cytoskeletal protein Smoothelin is lost in primary or long-term SMC culture and is only found in fully differentiated contractile SMC (van Eys et al., 2007; vanderLoop et al., 1996). SMαA is markedly decreased during the proliferative phase of cell growth in culture but post-confluence quiescent cells exhibit enhanced expression of SMMHC-1 and SMMHC-2 and SMαA (Fatigati and Murphy, 1984; Reusch et al., 1996; Rovner et al., 1986).

As differences in late marker expression in HCASMC at around 80% confluency under different O₂ levels may not have been detectable, we focused on immunoblotting early and mid-term markers.

Moreover, examining cell shape using an Etaluma microscope (LS720 Microscopes, USA) at ×10 magnification revealed negligible changes HCASMC shape under 18 or 5kPa O₂.

2. Fig. 2 Regarding proliferation, I recommend to additionally measure proliferation marker Ki67 or PCNA, since impedance analysis can also be affected by cell size, shape and attachment quality.

We concur with the Reviewer that impedance measurements can be affected by cell size, shape and attachment quality and agree that it would be meaningful to measure proliferation markers Ki67 or PCNA.

In further support of our finding that adaptation to physiological normoxia (5 kPa O₂) increases HCASMC proliferation, we include additional experimental data for total protein content as another index of cell proliferation. As in the case of cell number (Fig. 2A), HCASMC total protein content was significantly increased after 5 days culture under 5 kPa O₂ (see revised Fig. 2B below). The significant differences observed in cell number and total protein together with a decreased cell doubling time (Fig. 2C) strongly support our conclusion that culture under 5 kPa O₂ enhances HCASMC proliferation. We are unfortunately not able within the timeframe of resubmission to conduct additional experiments to measure Ki67 or PCNA.
Fig. 2 Proliferation of HCASMC during long-term culture under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to either 18 or 5kPa O₂. A-B, Cells seeded at 3500 cells/well into 96-well plates and cell number counted and total protein content measured over 1 – 5 days in culture. C, Cells seeded at 7000 cells/well in E-Plates® and doubling time monitored continuously over 6 days using an iCELLigence platform. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded), two-way ANOVA followed by a Bonferroni Post Hoc test analysis, *P<0.05, **P<0.01, ***P < 0.001.

3. - Fig. 4. Please explain how the Nrf2 ratio between nucleus and cytoplasm was calculated. Was a comparison with DAPI performed? Please include the DAPI staining (as in Fig. 1) or another appropriate control.

Co-staining of cell nuclei was conducted in the present study, and we have accordingly revised Fig. 4 to include DAPI stained nuclei under the different oxygen and experimental conditions.

Fig. 4 Zn supplementation induces NRF2 nuclear accumulation in HCASMC under 18 or 5kPa O₂
A, Representative NRF2 positive immunofluorescence and DAPI stained nuclei in HCASMC pre-adapted to 18 or 5kPa O₂ and then treated for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM), respectively. B, Quantification of NRF2 nuclear:cytoplasmic ratio in HCASMC treated with Veh, TPEN or ZnCl₂+Py. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded) with 20-30 cells analyzed in each culture, two-way ANOVA followed by a Bonferroni Post Hoc test analysis, **P<0.01, ***P<0.001. Scale bar = 20μm.
**Highlights**

- Physiological normoxia upregulates calponin-1 expression in coronary artery smooth muscle cells
- Physiological normoxia attenuates zinc accumulation following Zn supplementation
- Zinc supplementation activates NRF2 signaling and HO-1, NQO1 and MT-1 mRNA expression
- Zinc supplementation attenuates hypoxia/reoxygenation induced superoxide generation
Graphical Abstract

**Hyperoxia (18 kPa O₂)**
- More 'synthetic' phenotype
- High ROS level
- Confers Zn cardioprotective role against high ROS
- High Zn content
- High expression of HO-1
- NRF2
- ARE

**Physioxia (5 kPa O₂)**
- More 'contractile' phenotype
- Physiological ROS level
- Low Zn content
- Low expression of HO-1
- NRF2
- ARE
Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation

Fan Yang1*, Matthew J. Smith1, Dr, Alexander Griffiths2, Alexander Morrell2, Sarah J. Chapple1, Richard C.M. Siow1, Theodora Stewart1, Wolfgang Maret4 and Giovanni E. Mann1*

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Short Title: Zinc supplementation protects against hypoxia/reoxygenation

Keywords
Coronary artery smooth muscle cells; Metals; Metallomics; Metallothionein; Zinc; NRF2; Redox status; Physiological normoxia; Hyperoxia, Hypoxia, Oxygen, Hypoxia-reoxygenation

Abbreviations
GCLM, glutamate cysteine ligase modifier subunit; GSK3β, glycogen synthase kinase 3β; HCASMC, human coronary artery smooth muscle cells; HO-1, heme oxygenase; ICP-MS, inductively coupled plasma mass spectrometry; Keap1, Kelch-like ECH-associated protein 1; MT, metallothionein; NQO1, NAD(P)H:quinone oxidoreductase-1, NRF2, nuclear factor-erythroid 2 p45-related factor 2; ROS, reactive oxygen species; TPEN, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine; ZnT1, zinc transporter 1

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Highlights

- Physiological normoxia upregulates calponin-1 expression in coronary artery smooth muscle cells
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- Zinc supplementation activates NRF2 signaling and HO-1, NQO1 and MT-1 mRNA expression
- Zinc supplementation attenuates hypoxia/reoxygenation induced superoxide generation

Graphical Abstract
Abstract

Zinc (Zn) has antioxidant, anti-inflammatory and anti-proliferative actions, with Zn dysregulation associated with coronary ischemia/reperfusion injury and smooth muscle cell dysfunction. As the majority of studies concerning Zn have been conducted under non-physiological hyperoxic conditions, we compare the effects of Zn chelation or supplementation on total intracellular Zn content, antioxidant NRF2 targeted gene transcription and hypoxia/reoxygenation-induced reactive oxygen species generation in human coronary artery smooth muscle cells (HCASMC) pre-adapted to hyperoxia (18kPa O₂) or normoxia (5kPa O₂). Expression of the smooth muscle marker SM22-α was unaffected by lowering pericellular O₂, whereas calponin-1 was significantly upregulated in cells under 5kPa O₂, indicating a more physiological contractile phenotype under 5kPa O₂. Inductively coupled plasma mass spectrometry established that Zn supplementation (10μM ZnCl₂ + 0.5μM pyrithione) significantly increased total Zn content in HCASMC under 18 but not 5kPa O₂. Zn supplementation increased metallothionein mRNA expression and NRF2 nuclear accumulation in cells under 18 or 5kPa O₂. Notably, NRF2 regulated HO-1 and NQO1 mRNA expression in response to Zn supplementation was only upregulated in cells under 18 but not 5kPa. Furthermore, whilst hypoxia increased intracellular glutathione (GSH) in cells pre-adapted to 18 but not 5kPa O₂, reoxygenation had negligible effects on GSH or total Zn content. Reoxygenation-induced superoxide generation in cells under 18kPa O₂ was abrogated by PEG-superoxide dismutase but not by PEG-catalase, and Zn supplementation, but not Zn chelation, attenuated reoxygenation-induced superoxide generation in cells under 18 but not 5kPaO₂, consistent with a lower redox stress under physiological normoxia. Our findings highlight that culture of HCASMC under normoxia recapitulates an in vivo contractile phenotype and that effects of Zn on NRF2 signaling are altered by oxygen tension.
1. Introduction

Zinc (Zn) has emerged as a valuable biomarker in diagnosis and therapy in coronary artery and heart disease associated with oxidative stress and redox dysregulation [1-3]. Nuclear factor E2-related factor 2 (NRF2) is a well-known antioxidant transcription factor [4-8] and plays a critical role in the maintenance of cellular redox homeostasis in oxidative stress and ischemia-reperfusion injury [9, 10]. Although Zn supplementation has been reported to reduce superoxide (O$_2^-$) generation, lower apoptotic indices, restore ATP levels and attenuate NADPH oxidase mediated oxidative stress [2, 11-14], there is evidence that Zn supplementation at the onset of reperfusion enhances the severity of myocardial infarction (MI) [15] and increases reactive oxygen species (ROS) generation in cardiomyocytes and aortic smooth muscle cells [16-18]. We recently established that NRF2 activation regulates total Zn content in coronary vascular cells in a cell-type specific manner under physiological oxygen levels [19]. Based on these findings and conflicting reports on Zn supplementation, further investigation of the relationship between Zn and NRF2 targeted cellular antioxidant defenses in IR and hypoxia/reoxygenation is warranted.

As a redox-inactive metal, Zn plays an important role in oxidative stress by affording some protection of thiol groups against reactive oxygen species (ROS) damage [20, 21]. NRF2 transcriptional activity is increased by ROS interacting zinc coordination site in Keap1 [22], leading to inhibition of NRF2 ubiquitination and proteasomal degradation [23, 24]. In human renal tubular cells, Zn supplementation induced inhibition of GSK3β has been linked to an inhibition of NRF2 nuclear export and enhanced transcriptional activity [25]. Zn has also been shown to regulate expression of the NRF2 target glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis [26]. Moreover, Zn can activate the metal regulatory element binding transcription factor 1 (MTF-1), which plays an important role in regulating antioxidant responses and maintaining metal homeostasis [27]. Activation of MTF-1 upregulates expression of metallothioneins (MT) and the selenoprotein1 gene, which encodes an antioxidant glutathione-binding protein known to scavenge free radicals [27]. MTF-1 and NRF2 transcription factors are thus linked through a pool of free Zn$^{2+}$ modulated by both MT and transcriptional machinery [28].

We recently highlighted cell-type differences in redox signaling and total intracellular Zn content in human coronary artery endothelial and smooth muscle cells (HCASMC) upon lowering pericellular O$_2$ levels from standard cell culture hyperoxia (18kPa) to physiological normoxia (5kPa) and hypoxia (1kPa) [19]. In the present study, we investigate whether Zn chelation or supplementation affect intracellular Zn content and NRF2 redox signaling differentially in HCASMC cultured long-term (5 days) under hyperoxia or physiological normoxia in an O$_2$-controlled workstation. In the context of hypoxia/reoxygenation, we report the first evidence that Zn supplementation significantly attenuates reoxygenation induced ROS generation in HCASMC under 18 but not 5kPa O$_2$. In support of our recent recommendations [29, 30], the present study further highlights the importance of assessing redox signaling, Zn metabolism and the effects of Zn supplementation in cells cultured under pericellular O$_2$ levels encountered in vivo.
2. Methods and Materials

2.1 HCASMC culture under defined pericellular O\textsubscript{2} levels

As previously described [19], primary human coronary artery smooth muscle cells (HCASMC, PromoCell, Germany) were cultured in Smooth Muscle Cell Basal Medium 2 (PromoCell), supplemented with growth medium 2 supplement pack (PromoCell) and 1% penicillin (100U/ml)/streptomycin (100µg/ml). Cells were treated with medium containing 2µM Zn or with medium supplemented with a Zn chelator (TPEN, 1.25µM) or ZnCl\textsubscript{2} (10µM) + pyrithione (Zn ionophore, 0.5µM). HCASMC were pre-adapted for 5d in a dual Scitive O\textsubscript{2}-controlled workstation (Baker, USA) under 18kPa O\textsubscript{2} (hyperoxia) or 5kPa O\textsubscript{2} (physiological normoxia) and 5% CO\textsubscript{2} at 37°C. All protocols and experiments were conducted within the O\textsubscript{2}-controlled workstation and/or plate reader (CLARIOstar, BMG Labtech, Germany) [31-34].

2.2 Measurement of cell proliferation using real time cell analysis (RTCA) platform

Cell proliferation was assessed using an RTCA (iCELLigence\textsuperscript{TM}, Acea Biosciences) platform [31], which uses non-invasive electrical impedance to quantify cell proliferation label-free in real-time. HCASMC adapted to 18 or 5kPa O\textsubscript{2} were seeded into E-Plates\textsuperscript{®} in triplicate at a concentration of 7000 cells/well. Adherent cells at the electrode-solution interface impede electron flow, and the magnitude of impedance (Cell Index, CI) serves as an index of cell proliferation. Cell adhesion was measured over the first 2h to achieve a baseline CI and proliferation then recorded over 6 days with media changed every 2d.

2.3 Inductively coupled plasma mass spectrometry analysis of total intracellular Zn content in HCASMC

We previously described ICP-MS protocols for measuring total intracellular Zn content in HCASMC lysates collected in purified trace metal free water with a resistivity ≥18.2 MΩcm obtained from a Milli-Q system (Merck Millipore, USA) [19]. Cell lysates were introduced to an ICP-QMS via a Cetac ASX-520 autosampler (Teledyne, USA) coupled to a SeaSpray glass nebulizer fitted to a quartz cyclonic spray chamber. Zn concentrations were normalised to cell protein measured using the bicinchoninic acid (BCA) assay.

2.4 NRF2 nuclear accumulation assessed by immunofluorescence

To assess nuclear translocation of NRF2 after treatment with a Zn chelator or Zn supplementation, HCASMC were pre-adapted for 5d to 18 or 5kPa O\textsubscript{2} and then seeded into 8-chambered coverslips (Ibidi, Germany) for 48h. Cells were then treated for 16h with (i) vehicle (Veh, 0.01% dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK), (ii) N,N,N′,N′-tetraakis (2-pyridylmethyl) ethylenediamine (Zn chelator, TPEN, 1.25µM, Sigma-Aldrich, UK) [35] or (iii) ZnCl\textsubscript{2} (10µM, Sigma-Aldrich, UK) and 2-mercaptopypyridine N-oxide sodium salt (Zn\textsuperscript{2+} ionophore pyrithione, 0.5µM, Sigma-Aldrich, UK). Immunofluorescence analysis was performed using a primary anti-NRF2 antibody (Santa Cruz, USA) and donkey anti-Rabbit DyLight\textsuperscript{®} 488 conjugated secondary antibody (Bethyl Laboratories, USA). Fluorescence was visualized at ×40 magnification using a fluorescence microscope (Etaluma LS720, USA), with images quantified as the ratio of nuclear:cytoplasmic NRF2 immunofluorescence, with cell nuclei stained with DAPI [36].
2.5 Immunoblotting

Whole cell lysates were collected using SDS lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, UK), separated by gel electrophoresis, electro-transferred onto polyvinylidene difluoride membranes (Millipore, Sigma, USA) and probed with primary and HRP-conjugated secondary antibodies (Millipore, Sigma, USA): NQO1 (Santa Cruz, USA), HO-1 (BD Biosciences, USA), ZnT1 (Abcam, UK) and β-actin (Sigma-Aldrich, UK) and analyzed by enhanced chemiluminescence (Millipore, Sigma, USA). Images were captured using a G:Box system (Syngene, UK) and densitometric analysis conducted using ImageJ software (National Institutes of Health, USA), as previously described [19].

2.6 Quantitative RT-PCR

HCASMC RNA was isolated using a RNeasy® Mini Kit (Qiagen, Germany) and RNA content and purity assessed using a spectrophotometer (NanoDrop Technologies, USA) [31]. Total RNA was reverse–transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). HO-1, NQO1 and MT-1 mRNA were assessed by real-time qPCR (Applied Biosystems, USA) and normalized to the geometric mean of ribosomal protein lateral stalk subunit P0 (RPLP0), TATA-binding protein (TBP) and succinate dehydrogenase complex, subunit A (SDHA) (primer sequences in Supplementary Table 1).

2.7 L-012 chemiluminescence measurements of ROS generation in HCASMC under hypoxia/reoxygenation

HCASMC were seeded into white clear bottomed 96-well plates in quadruplicate and adapted for 5d under 18 or 5kPa O2. As previously described [34], cell monolayers were incubated in the absence or presence of polyethylene glycol superoxide dismutase (pSOD, 20U/ml, Sigma-Aldrich, UK) or polyethylene glycol catalase (pCAT, 200U/ml, Sigma-Aldrich, UK) to scavenge superoxide or H2O2 respectively. To determine the effects of Zn chelation or supplementation on ROS generation, cells were pre-adapted for 5d to 18 or 5kPa O2 and then treated for 16h with (i) vehicle (Veh, 0.01% DMSO), (ii) TPEN (1.25μM) or (iii) ZnCl2 (10μM ZnCl2 + pyrithione 0.5μM). After incubation with the chemiluminescent luminol analogue L-012 (8-amino-5-chloro-7-phenylpyridol[3,4-d] pyridazine-1,4-(2H,3H)dione sodium salt, 10μM, Tocris Bioscience, UK), cells were rapidly transferred from the O2-controlled workstation to an O2-controlled plate reader (CLARIOstar, BMG Labtech, UK) at 37°C [34]. Cells were then exposed to hypoxia (1kPa O2) for 1h and reoxygenation under either 18 or 5kPa O2, respectively, and chemiluminescence measured at 60s intervals over 3h and expressed as mean light units×10⁶ or 10⁴/mg protein.

L-012 is widely used to measure superoxide (O2−) and other reactive oxygen species (ROS) in biological systems [37]. Although O2− alone does not react with L-012 to emit luminescence, oxidation of the probe to its radical and reaction of the luminol radical with self-generated O2− during oxidation of L-012 leads to the emission of blue light, which can be inhibited by superoxide dismutase (SOD) [37].

2.8 Effects of hypoxia/reoxygenation on intracellular glutathione levels

HCASMC were pre-adapted for 5d to 18 or 5kPa O2 in an O2-regulated workstation and then exposed to hypoxia for 1h by lowering O2 in the workstation to 1kPa and reoxygenation for 1h or 24h under 18 or 5kPa O2, respectively (see Supplementary Fig. 3). GSH levels were determined using a fluorometric assay [38].

6
Luminescence and fluorescence were measured in a plate reader (CLARIOstar, BMG Labtech, Germany) and expressed as nmol/mg protein, as previously described [31].

2.9 Effects of ZnCl₂ and pyrithione co-treatment on cell viability
Cell viability was assessed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, U.K.), as previously described [39]. HCASMC were co-treated for 16h with ZnCl₂ (10, 12 or 14µM) and the Zn ionophore pyrithione (0.25, 0.5, 0.75 or 1µM) and then incubated with 5μg/ml MTT for 3h at 37°C. Insoluble formazan salts were dissolved in DMSO and absorbance at 570nm measured in a plate reader (CLARIOstar, BMG Labtech, Germany).

2.10 Statistical analysis
Data denote the mean ± S.E.M. of 3-6 independent HCASMC cultures and were analyzed using Graphpad Prism 8. Significance was assessed using either an unpaired Student's t-test or one- or two-way ANOVA followed by a Bonferroni Post Hoc test where appropriate, with *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 considered significant.

3. Results
3.1 Adaptation to defined pericellular O₂ levels alters HCASMC phenotype and proliferation
Vascular smooth muscle exist in a contractile or a synthetic phenotype, and the degree of differentiation can be detected by the expression of specific markers [40]. Markers of contractile phenotype are divided into early (SMαA, myocardin and SM22-α), mid-term (H-caldesmon and calponin-1) and late (SMMHC-1 and -2 and smoothelin) based on their appearance during embryonic development or differentiation of stem cells toward vascular smooth muscle cells [41-43]. To characterize the effect of pericellular O₂ on the phenotype of HCASMC, contractile markers were examined by immunostaining and immunoblotting. Representative fluorescence images of SM22-α and calponin-1 staining of HCASMC adapted to 18 or 5kPa O₂ are shown in Fig. 1A and C. Although protein expression of SM22-α was affected negligibly under both O₂ levels (Fig. 1B), calponin-1 expression was significantly increased in HCASMC under 5kPa O₂ (Fig. 1D), suggesting that cells cultured long-term under physiological normoxia (5kPa O₂), in the absence of HIF-α stabilization [19], exhibit a more contractile phenotype.

To further characterize HCASMC during long-term culture under 18 or 5kPa O₂, we monitored cell proliferation by determining cell numbers (Fig. 2A) and changes in bioimpedance using a RTCA platform (Fig. 2B). Proliferation of HCASMC was enhanced under 5kPa compared to 18kPa O₂, as evidenced by increased cell numbers after 4 - 5d in culture (Fig. 2A) and a significantly reduced doubling time (Fig. 2B).

3.2 Zn supplementation increases Zn content in HCASMC under 18 but not 5kPa O₂
ICP-MS analysis was employed to determine whether total intracellular Zn content in HCASMC pre-adapted for 5d to 18 or 5kPa O₂ is affected differently by Zn chelation or supplementation. Basal Zn content was affected negligibly in HCASMC under the two O₂ levels (18kPa = 0.52 ± 0.08 ng/µg protein versus 5kPa =
0.66 ± 0.17 ng/μg protein), confirming our previous findings in coronary smooth muscle cells [19]. Treatment of cells with the Zn chelator TPEN (1.25 μM) had no effect on cell viability (Supplementary Fig. 1) and moreover negligible effects on Zn content under either 18 or 5kPa O₂. Pyrithione, an ionophore for Zn [44], facilitates cellular uptake of Zn in cardiomyocytes and other cell types [44, 45]. We initially assessed the viability of HCASMC following co-treatment with different ZnCl₂ (0-20μM) and pyrithione (0-1μM) concentrations and selected concentrations of ZnCl₂ (10μM) + pyrithione (0.5μM) for all subsequent experiments (Supplementary Fig. 1). As shown in Fig. 3A, Zn supplementation significantly increased Zn content in cells adapted to 18kPa O₂ (Veh: 0.52 ± 0.08 ng/μg protein versus ZnCl₂+Py: 1.34 ± 0.26 ng/μg protein) but not in cells adapted to 5kPa O₂ (0.66 ± 0.17 ng/μg protein versus 0.76 ± 0.08 ng/μg protein). Under the same experimental conditions, Zn supplementation significantly increased metallothionein (MT-1) mRNA in cells under 18 or 5kPa O₂ (Fig. 3B).

3.3 Effects of Zn supplementation on NRF2 signaling in HCASMC under 18 or 5kPa O₂

We next examined whether Zn chelation or supplementation influences NRF2 nuclear accumulation and HO-1 and NQO1 mRNA/protein expression in cells pre-adapted for 5d to 18 or 5kPa O₂. Although chelation of Zn had negligible effects on NRF2 nuclear:cytoplasmic ratios, Zn supplementation significantly enhanced NRF2 nuclear accumulation in cells under 18 and 5kPa O₂ (Fig. 4). As shown in Fig. 5, TPEN had negligible effects on HO-1 and NQO1 mRNA/protein expression in cells cultured in complete medium containing low Zn (2.0μM). In contrast, Zn supplementation significantly increased HO-1 and NQO1 mRNA expression in HCASMC pre-adapted to 18kPa O₂, which was markedly attenuated in cells under 5kPa O₂ (Fig. 5 A and B). Furthermore, HO-1 protein expression in response to Zn supplementation was attenuated in HCASMC under 5kPa O₂ (Fig. 5C). Taken together these data suggest that NRF2 signaling is activated by Zn supplementation in cells adapted to 18kPa O₂ and to a lesser extent under 5 kPa O₂, independent of NRF2 nuclear accumulation.

3.4 Effects of hypoxia/reoxygenation on ROS generation, intracellular Zn and glutathione

Ischemia and hypoxia induced damage is exacerbated during reperfusion/reoxygenation and has been attributed to an increased generation of ROS [46]. To mimic reactive oxygen species (ROS) generation during reoxygenation, HCASMC were pre-adapted for 5d to 18 or 5kPa O₂, loaded with the luminescence probe L-012 and exposed to hypoxia (1h) and reoxygenation. As shown in Fig. 6A and B, ROS generation during reoxygenation of HCASMC under 18kPa O₂ was abrogated by PEG-superoxide dismutase, whilst PEG-catalase had a negligible effect, implicating superoxide anions in the reoxygenation-induced free radical burst. In contrast, reoxygenation-induced free radical generation was negligible in cells adapted to 5kPa O₂ (Fig. 6C and D), consistent with our previous findings in brain microvascular endothelial cells [34].

We next determined whether intracellular Zn content and glutathione (GSH) levels were affected differently by hypoxia/reoxygenation in HCASMC pre-adapted to 18 or 5kPa O₂. Total intracellular Zn content was not altered by hypoxia (1h) nor in response to reoxygenation of cells for 1 or 9 h under either 18 or 5kPa O₂, respectively (Supplementary Fig. 2). Hypoxia increased intracellular GSH levels in HCASMC pre-adapted to 18kPa but not 5kPa O₂, with levels returning to basal values over 24h reoxygenation (Supplementary Fig. 3).
Our findings contrast with negligible changes in GSH in mouse peritoneal macrophages cultured under 18kPa O₂ and exposed to hypoxia for 1 h [47].

### 3.5 Effects of hypoxia/reoxygenation on NQO1, GCLM and ZnT1 expression in HCASMC

When we examined the effects of hypoxia/reoxygenation on NRF2 targeted NQO1 and glutamate cysteine ligase modifier subunit (GCLM) expression in HCASMC pre-adapted for 5d to 18 or 5kPa O₂, NQO1 protein expression trended to be lower in cells adapted to 5kPa O₂ and was significantly lower in cells exposed to hypoxia (12h) and reoxygenation (12h) under 5kPa O₂ compared to 18kPa O₂ (Supplementary Fig. 4A). In contrast, negligible changes in GCLM (Supplementary Fig. 4B) and zinc transporter 1 (Zn efflux transporter [48], Supplementary Fig. 4C) expression were detected in cells pre-adapted to 18 or 5kPa O₂ and exposed to hypoxia/reoxygenation.

### 3.6 Effects of Zn chelation or supplementation on ROS generation in HCASMC under hypoxia/reoxygenation

As we established that Zn activates NRF2 signaling in HCASMC under 18 and 5kPa O₂, we next investigated whether Zn chelation or supplementation attenuates reoxygenation-induced free radical generation. HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and then pre-treated for 16h with TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (0.5μM). After incubation with L-012, cells were exposed to hypoxia (1h) and reoxygenation under 18 or 5kPa O₂ respectively in an O₂-controlled plate reader. Under low medium Zn concentration (2μM), TPEN had negligible effects on reoxygenation stimulated L-012 luminescence, whereas Zn supplementation attenuated reoxygenation-induced reactive oxygen species generation in cells under 18 kPa O₂ (Fig. 7A and B). As in our previous study in brain microvascular endothelial cells [34], negligible changes in L-012 luminescence were detected after reoxygenation of HCASMC under 5kPaO₂ (Fig. 7C and D).

### 4. Discussion

The present study, to our knowledge, is the first to investigate the effects of Zn chelation or supplementation on total intracellular Zn content, NRF2 regulated antioxidant gene transcription and hypoxia/reoxygenation induced ROS generation in HCASMC cultured long-term under standard hyperoxia (18kPa O₂) or physiological normoxia (5kPa O₂). Zn supplementation significantly increased total intracellular Zn content in HCASMC under 18 but not 5kPa O₂, whilst metallothionein-1 mRNA expression was upregulated in cells under either pericellular O₂ level. Although Zn supplementation increased NRF2 nuclear accumulation in cells under 18 and 5kPa O₂, NRF2 targeted HO-1 mRNA and protein expression induced by Zn supplementation was attenuated in HCASMC under 5kPa O₂, with a similar trend observed for NQO1. Notably, hypoxia/reoxygenation induced free radical generation was inhibited by Zn supplementation in HCASMC only under 18kPa O₂, consistent with our observation of enhanced ROS generation and antioxidant enzyme expression in cells under hyperoxia.

In view of contractile and synthetic phenotypes in vascular smooth muscle cells, our study is the first to investigate the effects of pericellular O₂ levels on the phenotype of HCASMC, highlighting a more contractile phenotype in cells under 5 compared to 18kPa O₂. Badran et al. recently reviewed the role of ROS as
modulators of vascular smooth muscle phenotype and suggested that excessive ROS generation induces a synthetic phenotype associated with disease, whilst physiological ROS levels are associated with a contractile phenotype [49]. Moreover, activation of NRF2 in response to 7-ketocholesterol stimulated ROS generation has been shown to maintain mouse coronary arterial smooth muscle cells in a differentiated state [50]. Our finding of increased calponin-1 expression in HCASMC under 5kPa O_2 thus further underpins the importance of physiologically relevant O_2 levels for cell culture models in vitro.

Using ICP-MS, we report novel evidence that Zn supplementation (10μM, 16h) induced increases in total Zn content in HCASMC are markedly attenuated in cells under physiological normoxia (5kPa) compared to hyperoxia (18kPa O_2). The Zn ionophore pyrithione enhances Zn uptake in oligodendrocyte progenitor cells [45], cardiac H9c2 cells [44, 51] and isolated papillary muscle [52]. In the present study HCASMC were treated with concentrations of ZnCl_2 + pyrithione and TPEN that had negligible effects on cell viability (see Supplementary Fig. 1). The Zn chelator TPEN (1.25μM) had negligible effects on total intracellular Zn content. It is possible that the low basal Zn concentration in the media for HCASMC may in part explain the lack of a statistical difference between vehicle and TPEN treated cells. Total Zn content may not be altered following treatment of HCASMC with TPEN, since ICP-MS analysis would also detect Zn chelated by TPEN. As reported in many other studies [53], TPEN would be expected to decrease bioavailable Zn levels.

Labile Zn (free Zn^{2+}) levels can increase in cells within seconds, minutes or hours after exogenous Zn supplementation with transcriptional regulation of Zn transporters observed over a longer time scale [54, 55]. Zn ‘muffling’ reactions modulate transient changes in Zn^{2+} after Zn supplementation via zinc transporters (e.g. ZIP1-14 importers and ZnT1-10 exporters) and zinc-binding proteins to maintain a tight control of intracellular Zn^{2+} levels [55, 56]. ZIP2 and ZIP12 have been implicated in endothelial and smooth muscle responses to vascular Zn deficiency [35] and, although ZIP12 expression is low in normal vascular tissue, exposure of human pulmonary vascular smooth muscle cells to hypoxia upregulates ZIP12 mRNA whilst ZIP6, ZIP7 and ZIP10 are unaffected [57]. In rat aortic smooth muscle cells, Zn supplementation (25-50μM) has been shown to decrease ZnT3 and ZnT10 expression and increase senescence [58]. Based on limited information available on the effects of Zn supplementation on ZIP and ZnT transporters in different vascular smooth muscle cell types, further characterization Zn transporters in cells maintained under physiological normoxia and exposed to hypoxia/reoxygenation is warranted.

Oxidative stress and hypoxia can induce release of Zn from metallothioneins (MT), leading to activation of MTF-1 and increased expression of MTs and ZnT1 [27, 59]. In the present study, MT1 mRNA expression was significantly upregulated by Zn supplementation in HCASMC under 18 and 5kPa O_2, consistent with previous studies in human pulmonary vascular smooth muscle cells conducted under ambient air [35]. We previously reported that MT1 and ZnT1 mRNA/protein expression in HCASMC was unaffected by changes in pericellular O_2 levels, and in the present study establish that ZnT1 protein expression is affected negligibly by Zn supplementation in HCASMC under 18 or 5kPa O_2 (see Supplementary Fig. 4C).
NRF2 plays a key role in maintaining cellular redox homeostasis [5-8], and previous studies have established that Zn influences NRF2 activation in different cell types cultured in standard incubators under hyperoxia (18kPa O₂), including endothelial cells [60], human renal tubule cells [25, 61], retinal pigment epithelial cells [26], IMR-32 neuroblastoma cells [62], murine spinal cord neurons [63] and human peripheral blood mononuclear cells [64]. NRF2 protein levels are maintained relatively low under physiological conditions due to rapid ubiquitination and proteasomal degradation mediated by Keap1 [5, 6] and GSK3β phosphorylation of NRF2, leading to β transducin repeats-containing proteins (βTrCP)-mediated degradation and Fyn-mediated nuclear exclusion [7, 65]. Recent evidence in human peripheral blood mononuclear cells suggests that Zn supplementation inhibits HDAC3 activity, using a cell-free assay, and decreases Keap1 mRNA expression without altering NRF2 protein levels [64]. By contrast, Zn supplementation (3d) of human leukemia monocytic THP-1 cells has negligible effects on NRF2 and Keap1 mRNA expression [66].

Our findings establish that upregulation of HO-1 in response to Zn supplementation is attenuated in HCASMC adapted to physiological normoxia (5kPa O₂) (see Fig. 5), consistent with negligible changes in total intracellular Zn content under 5kPa O₂ (Fig. 3B). Kaufman et al. implicated Zn-mediated degradation of Bach1, the mammalian repressor of HO-1, in the regulation of HO-1 expression by Zn in neuroblastoma cells [62]. In this context, specific loss of Bach1 in murine vascular smooth muscle inhibits neointimal hyperplasia and remodelling following vascular injury [67]. The interaction of intracellular Zn³⁺ with His-225, Cys-226 and Cys-613 within Keap1 is associated a conformational change in Keap1 leading to inhibition of NRF2 ubiquitination [23, 24]. Keap1, acting as a Zn sensor and Zn-binding protein, can also release Zn from Cys-273 and Cys-288 under oxidative stress [22, 24]. Zn supplementation has been shown to phosphorylate Akt and/or ERK1/2 and inhibit GSK3β in human renal tubule cells and mouse kidney [25], neonatal ventricular myocytes [17] and H9c2 cardiac cells [51], which would enhance NRF2 nuclear accumulation and downstream antioxidant gene expression [6, 7, 68]. Notably, ZIP6-induced Zn influx in MCF-7 cells has been associated with inhibition of GSK3β [69].

We previously reported that culturing vascular and other cell types under different O₂ levels significantly alters NRF2 regulated redox signaling [19, 31, 34, 70, 71], noting that long-term adaptation of human and murine endothelial cells to physiological normoxia (5kPa O₂) attenuates NRF2 transcriptional activation of HO-1 and NQO1 [19, 31, 34]. Our present findings demonstrate that Zn supplementation enhances NRF2 nuclear accumulation in HCASMC under 18 and 5kPa O₂, noting that Zn induced upregulation of HO-1/NQO1 protein expression is attenuated in cells under 5kPa O₂. Under these experimental conditions, Zn supplementation only protected HCASMC against reoxygenation induced free radical generation during culture under 18kPa O₂ (see Figs. 6 and 7), confirming the protection afforded by the NRF2 inducer sulforaphane against reoxygenation induced O₂⁻ generation in brain microvascular endothelial cells [34] and highlighting the lower redox stress experienced by cells under physiological normoxia [29, 30]. In this context, Kelmanson et al. reported only a slight decrease in H₂O₂ levels in the cytosol and mitochondria of neurons exposed to hypoxia for 30min using genetically encoded Hyper7 biosensors [72]. Notably, pretreatment of rat neonatal cardiomyocytes with

Our finding that Zn supplementation activates NRF2 signaling in HCASMC and can attenuate hypoxia/reoxygenation induced ROS generation provides a basis for design and screening of therapeutic drugs for treatment of coronary heart disease. Given the caveats concerning cell culture under hyperoxia [29, 30, 73-77], the potential influence of pericellular O₂ on chemical fluorescence probes [78] and our present findings that effects of Zn supplementation and the phenotype of HCASMC are altered by physiological normoxia, we encourage researchers to study interactions between Zn and redox signaling in cells under physiological O₂ levels. Moreover, such experiments should also consider zinc concentrations in culture media and the effect that pericellular O₂ has on cellular zinc metabolism and signaling involving or being affected by zinc ions.

Authors contributions
F.Y., M.J.S. and G.E.M. conceptualized the study; F.Y., M.J.S., A.G., A.M. developed the methodology; F.Y. performed and analysed all experiments and A.G. conducted the ICP-MS analyses; F.Y. and G.E.M. wrote the manuscript, which all authors reviewed. G.E.M. is the guarantor of this study, with responsibility for the integrity of the data and data analysis.

Declaration of competing interest
The authors declare that they have no known competing interests that could have influenced the study.

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Supplementary data to this article can be found online at
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Figures and Legends

A  Early marker: SM22-α  C  Mid-term marker: Calponin-1

B  D

Fig.1 Long-term culture of HCASMC under physiological normoxia (5kPa O₂) upregulates calponin-1 expression

A and C, Representative immunofluorescence images of SM22-α or calponin-1 and DAPI stained nuclei in HCASMC pre-adapted for 5d to 18 or 5kPa O₂. B and D, Representative immunoblots and densitometric analysis of SM22-α and calponin-1 expression relative to β-actin. Data denote mean ± S.E.M., n=3-6 independent cell cultures (color-coded), unpaired Student's t-test, **P<0.01.
Fig. 2 Proliferation of HCASMC during long-term culture under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to either 18 or 5kPa O₂. A-B, Cells seeded at 3500 cells/well into 96-well plates and cell number counted and total protein content measured over 1 – 5 days in culture. C, Cells seeded at 7000 cells/well in E-Plates® and doubling time monitored continuously over 6 days using an iCELLigence platform. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded), two-way ANOVA followed by a Bonferroni Post Hoc test analysis, *P<0.05, **P<0.01, ***P < 0.001.
Fig. 3 Total intracellular Zn content and metallothionein mRNA expression in HCASMC treated with TPEN or supplemented with Zn under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and then treated with vehicle (Veh, 0.01% DMSO), TPEN (Zn²⁺ chelator, 1.25μM) or ZnCl₂ (10μM) and 2-mercaptopyridine N-oxide sodium salt (Zn ionophore pyrithione, 0.5μM) (ZnCl₂+Py). A, ICP-MS analysis of total Zn content in HCASMC following treatment for 16h with Veh, TPEN or ZnCl₂+Py. Data denote mean ± S.E.M., n=4 independent cultures (color-coded), two-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001.
Fig. 4  Zn supplementation induces NRF2 nuclear accumulation in HCASMC under 18 or 5kPa O₂

A, Representative NRF2 positive immunofluorescence and DAPI stained nuclei in HCASMC pre-adapted to 18 or 5kPa O₂ and then treated for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM), respectively. B, Quantification of NRF2 nuclear:cytoplasmic ratio in HCASMC treated with Veh, TPEN or ZnCl₂+Py. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded) with 20-30 cells analyzed in each culture, two-way ANOVA followed by a Bonferroni Post Hoc test analysis, **P<0.01, ***P<0.001. Scale bar = 20μm.
Fig. 5 Effect of Zn supplementation on HO-1 and NQO1 mRNA and protein expression in HCASMC under 18 or 5kPa O₂

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂. **A and B**, mRNA expression of HO-1 and NQO1 in HCASMC treated for 6h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM), respectively. Values normalized to three housekeeping (TBP, RPLPO and SDHA). **C and D**, Representative immunobLOTS and densitometric analysis of HO-1 and NQO1 expression relative to β-actin following treatment for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM). Data denote mean ± S.E.M., n=4-6 independent cultures (color-coded), two-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, ***P<0.001.
Fig. 6 Reoxygenation induced reactive oxygen species generation in HCASMC under 18 or 5kPa O2  
A and C. Representative L-012 luminescence traces in HCASMC pre-adapted for 5d to 18 or 5kPa O2. Cells were treated with Veh (0.01% DMSO), PEG-superoxide dismutase (pSOD, 20U/ml) or PEG-catalase (pCAT, 200U/ml). Cells were incubated with L-012 and transferred to an O2-regulated plate reader gassed with 18 or 5kPa O2, respectively. L-012 luminescence was detected in the plate reader, with O2 reduced to 1kPa and reoxygenation under 18 or 5kPa O2, respectively. The red line indicates the pericellular O2 levels within the plate reader. B and D. Area under curve summary of reoxygenation-induced L-012 luminescence changes. L-012 signal in panel B (18kPa O2) denotes area under the curve x10^6 and in panel D (5kPa O2) area under the curve x10^4. Data denote mean ± S.E.M., n=6 independent cultures, one-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, ***P<0.001.
Fig. 7 Zn supplementation attenuates reoxygenation-induced reactive oxygen species generation in HCASMC under 18kPa O₂

A and C, Representative L-012 luminescence traces in HCASMC pre-adapted for 5d to 18 or 5kPa O₂. Cells were treated for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM). Cells were incubated with L-012 and transferred to an O₂-regulated plate reader. L-012 luminescence was detected in the plate reader gassed with 18 or 5kPa O₂, with O₂ then reduced to 1kPa and reoxygenation under either 18 or 5kPa O₂, respectively. The red line indicates the pericellular O₂ levels within the plate reader. B and D, Area under curve summary of reoxygenation-induced L-012 luminescence changes. L-012 signal in panel B (18kPa O₂) denotes area under the curve x10⁶ and in panel D (5kPa O₂) area under the curve x10⁴. Data denote mean ± S.E.M., n=4 independent cultures, one-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05.
Redox Biology - Supplementary Data

Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation

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Supplementary Table 1 - qPCR primers

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Abbreviations: RPLP0, ribosomal protein lateral stalk subunit P0; TBP, TATA-binding protein; SDHA, succinate dehydrogenase complex, subunit A; HO-1, heme oxygenase-1, NQO1, NAD(P)H quinone dehydrogenase 1

Supplementary Fig.1 Viability of HCASMC following treatment with ZnCl2/pyrithione or TPEN

HCASMC pre-adapted for 5d to 18kPa O2 were seeded in triplicate at a concentration of 10^4 cells/well in 96-well plates and cultured for 48h. Cells were then treated for 16h in the absence (control medium) or presence of different concentrations of ZnCl2/pyrithione or TPEN and an MTT assay used to assess cell viability. A, Pyrithione (0.5µM) + ZnCl2 (10, 12 or 14µM). B, ZnCl2 (10µM) + pyrithione (0.25, 0.5, 0.75 or 1µM). C, TPEN (0.5, 0.75, 1.0, 1.25 or 1.5µM). Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded), one-way or two-way ANOVA with Tukey’s or Bonferroni’s multiple comparisons test, *P<0.05, **P<0.01.

Abbreviations: TPEN, N,N,N’,N’-tetrakis (2-pyridylmethyl) ethylenediamine
Supplementary Fig. 2 Effects of hypoxia/reoxygenation on total intracellular Zn content in HCASMC under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and cells exposed to hypoxia and reoxygenation. A. ICP-MS analysis of total intracellular Zn content in HCASMC under basal conditions, hypoxia (1kPa, 1h) and reoxygenation (for 1h and 9h) under 18 or 5kPa O₂, respectively. Changes between 18kPa or 5kPa and 1kPa O₂ required 30 min with cells maintained in the O₂-controlled workstation. B. Summary of total Zn content for each condition and time point. Data denote mean ± S.E.M., n=4-10 independent cultures, two-way ANOVA with Tukey’s multiple comparisons test.

Supplementary Fig. 3 Effects of hypoxia/reoxygenation on intracellular glutathione levels in HCASMC under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and intracellular glutathione (GSH) then measured under basal conditions (control), hypoxia (1h) only or followed by reoxygenation (HR) for 1h or 24h under 18 or 5kPa O₂, respectively. Data denote mean ± S.E.M., n=4 independent cultures (color-coded), two-way ANOVA with Tukey’s or Bonferroni’s multiple comparisons test, *P<0.05.
Supplementary Fig. 4 NQO1, GCLM and ZnT1 expression in HCASMC under hypoxia-reoxygenation

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ (control) and then subjected to hypoxia (H) for 12h or hypoxia for 12 h followed by reoxygenation for 12h (HR) under 18 or 5kPa O₂, respectively. Representative immunoblots and densitometric analyses of NQO1 (A), GCLM (B) and ZnT1 (C) expression relative to β-actin. Data denote mean ± S.E.M., n=3 independent paired cultures (color-coded), two-way ANOVA with Tukey’s multiple comparisons test, *P<0.05.
Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation

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Short Title: Zinc supplementation protects against hypoxia/reoxygenation

Keywords
Coronary artery smooth muscle cells; Metals; Metallomics; Metallothionein; Zinc; NRF2; Redox status; Physiological normoxia; Hyperoxia, Hypoxia, Oxygen, Hypoxia-reoxygenation

Abbreviations
GCLM, glutamate cysteine ligase modifier subunit; GSK3β, glycogen synthase kinase 3β; HCASMC, human coronary artery smooth muscle cells; HO-1, heme oxygenase; ICP-MS, inductively coupled plasma mass spectrometry; Keap1, Kelch-like ECH-associated protein 1; MT, metallothionein; NQO1, NAD(P)H:quinone oxidoreductase-1, NRF2, nuclear factor-erythroid 2 p45-related factor 2; ROS, reactive oxygen species; TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine; ZnT1, zinc transporter 1

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Highlights

- Physiological normoxia upregulates calponin-1 expression in coronary artery smooth muscle cells
- Physiological normoxia attenuates zinc accumulation following zinc supplementation
- Zinc supplementation activates NRF2 signaling and HO-1, NQO1 and MT-1 mRNA expression
- Zinc supplementation attenuates hypoxia/reoxygenation induced superoxide generation

Graphical Abstract
Abstract
Zinc (Zn) has antioxidant, anti-inflammatory and anti-proliferative actions, with Zn dysregulation associated with coronary ischemia/reperfusion injury and smooth muscle cell dysfunction. As the majority of studies concerning Zn have been conducted under non-physiological hyperoxic conditions, we compare the effects of Zn chelation or supplementation on total intracellular Zn content, antioxidant NRF2 targeted gene transcription and hypoxia/reoxygenation-induced reactive oxygen species generation in human coronary artery smooth muscle cells (HCASMC) pre-adapted to hyperoxia (18kPa O$_2$) or normoxia (5kPa O$_2$). Expression of the smooth muscle marker SM22-α was unaffected by lowering pericellular O$_2$, whereas calponin-1 was significantly upregulated in cells under 5kPa O$_2$, indicating a more physiological contractile phenotype under 5kPa O$_2$. Inductively coupled plasma mass spectrometry established that Zn supplementation (10μM ZnCl$_2$ + 0.5μM pyrithione) significantly increased total Zn content in HCASMC under 18 but not 5kPa O$_2$. Zn supplementation increased metallothionein mRNA expression and NRF2 nuclear accumulation in cells under 18 or 5kPa O$_2$. Notably, NRF2 regulated HO-1 and NQO1 mRNA expression in response to Zn supplementation was only upregulated in cells under 18 but not 5kPa. Furthermore, whilst hypoxia increased intracellular glutathione (GSH) in cells pre-adapted to 18 but not 5kPa O$_2$, reoxygenation had negligible effects on GSH or total Zn content. Reoxygenation-induced superoxide generation in cells under 18kPa O$_2$ was abrogated by PEG-superoxide dismutase but not by PEG-catalase, and Zn supplementation, but not Zn chelation, attenuated reoxygenation-induced superoxide generation in cells under 18 but not 5kPaO$_2$, consistent with a lower redox stress under physiological normoxia. Our findings highlight that culture of HCASMC under normoxia recapitulates an in vivo contractile phenotype and that effects of Zn on NRF2 signaling are altered by oxygen tension.
1. Introduction

Zinc (Zn) has emerged as a valuable biomarker in diagnosis and therapy in coronary artery and heart disease associated with oxidative stress and redox dysregulation [1-3]. Nuclear factor E2-related factor 2 (NRF2) is a well-known antioxidant transcription factor [4-8] and plays a critical role in the maintenance of cellular redox homeostasis in oxidative stress and ischemia-reperfusion injury [9, 10]. Although Zn supplementation has been reported to reduce superoxide (O2−) generation, lower apoptotic indices, restore ATP levels and attenuate NADPH oxidase mediated oxidative stress [2, 11-14], there is evidence that Zn supplementation at the onset of reperfusion enhances the severity of myocardial infarction (MI) [15] and increases reactive oxygen species (ROS) generation in cardiomyocytes and aortic smooth muscle cells [16-18]. We recently established that NRF2 activation regulates total Zn content in coronary vascular cells in a cell-type specific manner under physiological oxygen levels [19]. Based on these findings and conflicting reports on Zn supplementation, further investigation of the relationship between Zn and NRF2 targeted cellular antioxidant defenses in IR and hypoxia/reoxygenation is warranted.

As a redox-inactive metal, Zn plays an important role in oxidative stress by affording some protection of thiol groups against reactive oxygen species (ROS) damage [20, 21]. NRF2 transcriptional activity is increased by ROS interacting zinc coordination site in Keap1 [22], leading to inhibition of NRF2 ubiquitination and proteasomal degradation [23, 24]. In human renal tubular cells, Zn supplementation induced inhibition of GSK3β has been linked to an inhibition of NRF2 nuclear export and enhanced transcriptional activity [25]. Zn has also been shown to regulate expression of the NRF2 target glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis [26]. Moreover, Zn can activate the metal regulatory element binding transcription factor 1 (MTF-1), which plays an important role in regulating antioxidant responses and maintaining metal homeostasis [27]. Activation of MTF-1 upregulates expression of metallothioneins (MT) and the selenoprotein1 gene, which encodes an antioxidant glutathione-binding protein known to scavenge free radicals [27]. MTF-1 and NRF2 transcription factors are thus linked through a pool of free Zn2+ modulated by both MT and transcriptional machinery [28].

We recently highlighted cell-type differences in redox signaling and total intracellular Zn content in human coronary artery endothelial and smooth muscle cells (HCASMC) upon lowering pericellular O2 levels from standard cell culture hyperoxia (18kPa) to physiological normoxia (5kPa) and hypoxia (1kPa) [19]. In the present study, we investigate whether Zn chelation or supplementation affect intracellular Zn content and NRF2 redox signaling differentially in HCASMC cultured long-term (5 days) under hyperoxia or physiological normoxia in an O2-controlled workstation. In the context of hypoxia/reoxygenation, we report the first evidence that Zn supplementation significantly attenuates reoxygenation induced ROS generation in HCASMC under 18 but not 5kPa O2. In support of our recent recommendations [29, 30], the present study further highlights the importance of assessing redox signaling, Zn metabolism and the effects of Zn supplementation in cells cultured under pericellular O2 levels encountered in vivo.
2. Methods and Materials

2.1 HCASMC culture under defined pericellular O₂ levels
As previously described [19], primary human coronary artery smooth muscle cells (HCASMC, PromoCell, Germany) were cultured in Smooth Muscle Cell Basal Medium 2 (PromoCell), supplemented with growth medium 2 supplement pack (PromoCell) and 1% penicillin (100U/ml)/streptomycin (100µg/ml). Cells were treated with medium containing 2µM Zn or with medium supplemented with a Zn chelator (TPEN, 1.25µM) or ZnCl₂ (10µM) + pyrithione (Zn ionophore, 0.5µM). HCASMC were pre-adapted for 5d in a dual Scitive O₂-controlled workstation (Baker, USA) under 18kPa O₂ (hyperoxia) or 5kPa O₂ (physiological normoxia) and 5% CO₂ at 37°C. All protocols and experiments were conducted within the O₂-controlled workstation and/or plate reader (CLARIOstar, BMG Labtech, Germany) [31-34].

2.2 Measurement of cell proliferation using real time cell analysis (RTCA) platform
Cell proliferation was assessed using an RTCA (iCELLigence™, Acea Biosciences) platform [31], which uses non-invasive electrical impedance to quantify cell proliferation label-free in real-time. HCASMC adapted to 18 or 5kPa O₂ were seeded into E-Plates® in triplicate at a concentration of 7000 cells/well. Adherent cells at the electrode-solution interface impede electron flow, and the magnitude of impedance (Cell Index, CI) serves as an index of cell proliferation. Cell adhesion was measured over the first 2h to achieve a baseline CI and proliferation then recorded over 6 days with media changed every 2d.

2.3 Inductively coupled plasma mass spectrometry analysis of total intracellular Zn content in HCASMC
We previously described ICP-MS protocols for measuring total intracellular Zn content in HCASMC lysates collected in purified trace metal free water with a resistivity ≥18.2 MΩcm obtained from a Milli-Q system (Merck Millipore, USA) [19]. Cell lysates were introduced to an ICP-QMS via a Cetac ASX-520 autosampler (Teledyne, USA) coupled to a SeaSpray glass nebulizer fitted to a quartz cyclonic spray chamber. Zn concentrations were normalised to cell protein measured using the bicinchoninic acid (BCA) assay.

2.4 NRF2 nuclear accumulation assessed by immunofluorescence
To assess nuclear translocation of NRF2 after treatment with a Zn chelator or Zn supplementation, HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and then seeded into 8-chambered coverslips (Ibidi, Germany) for 48h. Cells were then treated for 16h with (i) vehicle (Veh, 0.01% dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK), (ii) N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (Zn chelator, TPEN, 1.25µM, Sigma-Aldrich, UK) [35] or (iii) ZnCl₂ (10µM, Sigma-Aldrich, UK) and 2-mercaptopypyridine N-oxide sodium salt (Zn²⁺ ionophore pyrithione, 0.5µM, Sigma-Aldrich, UK). Immunofluorescence analysis was performed using a primary anti-NRF2 antibody (Santa Cruz, USA) and donkey anti-Rabbit DyLight® 488 conjugated secondary antibody (Bethyl Laboratories, USA). Fluorescence was visualized at ×40 magnification using a fluorescence microscope (Etaluma LS720, USA), with images quantified as the ratio of nuclear:cytoplasmic NRF2 immunofluorescence, with cell nuclei stained with DAPI [36].
2.5 *Immunoblotting*

Whole cell lysates were collected using SDS lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, UK), separated by gel electrophoresis, electro-transferred onto polyvinylidene difluoride membranes (Millipore, Sigma, USA) and probed with primary and HRP-conjugated secondary antibodies (Millipore, Sigma, USA): NQO1 (Santa Cruz, USA), HO-1 (BD Biosciences, USA), ZnT1 (Abcam, UK) and β-actin (Sigma-Aldrich, UK) and analyzed by enhanced chemiluminescence (Millipore, Sigma, USA). Images were captured using a G:Box system (Syngene, UK) and densitometric analysis conducted using ImageJ software (National Institutes of Health, USA), as previously described [19].

2.6 *Quantitative RT-PCR*

HCASMC RNA was isolated using a RNeasy® Mini Kit (Qiagen, Germany) and RNA content and purity assessed using a spectrophotometer (NanoDrop Technologies, USA) [31]. Total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). HO-1, NQO1 and MT-1 mRNA were assessed by real-time qPCR (Applied Biosystems, USA) and normalized to the geometric mean of ribosomal protein lateral stalk subunit P0 (RPLP0), TATA-binding protein (TBP) and succinate dehydrogenase complex, subunit A (SDHA) (primer sequences in Supplementary Table 1).

2.7 *L-012 chemiluminescence measurements of ROS generation in HCASMC under hypoxia/reoxygenation*

HCASMC were seeded into white clear bottomed 96-well plates in quadruplicate and adapted for 5d under 18 or 5kPa O₂. As previously described [34], cell monolayers were incubated in the absence or presence of polyethylene glycol superoxide dismutase (pSOD, 20U/ml, Sigma-Aldrich, UK) or polyethylene glycol catalase (pCAT, 200U/ml, Sigma-Aldrich, UK) to scavenge superoxide or H₂O₂ respectively. To determine the effects of Zn chelation or supplementation on ROS generation, cells were pre-adapted for 5d to 18 or 5kPa O₂ and then treated for 16h with (i) vehicle (Veh, 0.01% DMSO), (ii) TPEN (1.25μM) or (iii) ZnCl₂ (10μM ZnCl₂ + pyrithione 0.5μM). After incubation with the chemiluminescent luminol analogue L-012 (8-amino-5-chloro-7-phenylpyridol[3,4-d] pyridazine-1,4-(2H,3H)dione sodium salt, 10μM, Tocris Bioscience, UK), cells were rapidly transferred from the O₂-controlled workstation to an O₂-controlled plate reader (CLARIOstar, BMG Labtech, UK) at 37°C [34]. Cells were then exposed to hypoxia (1kPa O₂) for 1h and reoxygenation under either 18 or 5kPa O₂, respectively, and chemiluminescence measured at 60s intervals over 3h and expressed as mean light units×10⁶ or 10⁴/mg protein.

L-012 is widely used to measure superoxide (O₂⁻) and other reactive oxygen species (ROS) in biological systems [37]. Although O₂⁻ alone does not react with L-012 to emit luminescence, oxidation of the probe to its radical and reaction of the luminol radical with self-generated O₂⁻ during oxidation of L-012 leads to the emission of blue light, which can be inhibited by superoxide dismutase (SOD) [37].

2.8 *Effects of hypoxia/reoxygenation on intracellular glutathione levels*

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ in an O₂-regulated workstation and then exposed to hypoxia for 1h by lowering O₂ in the workstation to 1kPa and reoxygenation for 1h or 24h under 18 or 5kPa O₂, respectively (see Supplementary Fig. 3). GSH levels were determined using a fluorometric assay [38].
Luminescence and fluorescence were measured in a plate reader (CLARIOstar, BMG Labtech, Germany) and expressed as nmol/mg protein, as previously described [31].

2.9 Effects of ZnCl₂ and pyrithione co-treatment on cell viability
Cell viability was assessed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, U.K.), as previously described [39]. HCASMC were co-treated for 16h with ZnCl₂ (10, 12 or 14µM) and the Zn ionophore pyrithione (0.25, 0.5, 0.75 or 1µM) and then incubated with 5µg/ml MTT for 3h at 37°C. Insoluble formazan salts were dissolved in DMSO and absorbance at 570nm measured in a plate reader (CLARIOstar, BMG Labtech, Germany).

2.10 Statistical analysis
Data denote the mean ± S.E.M. of 3-6 independent HCASMC cultures and were analyzed using Graphpad Prism 8. Significance was assessed using either an unpaired Student's t-test or one- or two-way ANOVA followed by a Bonferroni Post Hoc test where appropriate, with *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 considered significant.

3. Results
3.1 Adaptation to defined pericellular O₂ levels alters HCASMC phenotype and proliferation
Vascular smooth muscle exist in a contractile or a synthetic phenotype, and the degree of differentiation can be detected by the expression of specific markers [40]. Markers of contractile phenotype are divided into early (SMαA, myocardin and SM22-α), mid-term (H-caldesmon and calponin-1) and late (SMMHC-1 and -2 and smoothelin) based on their appearance during embryonic development or differentiation of stem cells toward vascular smooth muscle cells [41-43]. To characterize the effect of pericellular O₂ on the phenotype of HCASMC, contractile markers were examined by immunostaining and immunoblotting. Representative fluorescence images of SM22-α and calponin-1 staining of HCASMC adapted to 18 or 5kPa O₂ are shown in Fig. 1A and C. Although protein expression of SM22-α was affected negligibly under both O₂ levels (Fig. 1B), calponin-1 expression was significantly increased in HCASMC under 5kPa O₂ (Fig. 1D), suggesting that cells cultured long-term under physiological normoxia (5kPa O₂), in the absence of HIF-α stabilization [19], exhibit a more contractile phenotype.

To further characterize HCASMC during long-term culture under 18 or 5kPa O₂, we monitored cell proliferation by determining cell numbers (Fig. 2A) and changes in bioimpedance using a RTCA platform (Fig. 2B). Proliferation of HCASMC was enhanced under 5kPa compared to 18kPa O₂, as evidenced by increased cell numbers after 4 - 5d in culture (Fig. 2A) and a significantly reduced doubling time (Fig. 2B).

3.2 Zn supplementation increases Zn content in HCASMC under 18 but not 5kPa O₂
ICP-MS analysis was employed to determine whether total intracellular Zn content in HCASMC pre-adapted for 5d to 18 or 5kPa O₂ is affected differently by Zn chelation or supplementation. Basal Zn content was affected negligibly in HCASMC under the two O₂ levels (18kPa = 0.52 ± 0.08 ng/µg protein versus 5kPa =
0.66 ± 0.17 ng/μg protein), confirming our previous findings in coronary smooth muscle cells [19]. Treatment of cells with the Zn chelator TPEN (1.25 μM) had no effect on cell viability (Supplementary Fig. 1) and moreover negligible effects on Zn content under either 18 or 5kPa O₂. Pyrithione, an ionophore for Zn [44], facilitates cellular uptake of Zn in cardiomyocytes and other cell types [44, 45]. We initially assessed the viability of HCASMC following co-treatment with different ZnCl₂ (0-20μM) and pyrithione (0-1μM) concentrations and selected concentrations of ZnCl₂ (10μM) + pyrithione (0.5μM) for all subsequent experiments (Supplementary Fig. 1). As shown in Fig. 3A, Zn supplementation significantly increased Zn content in cells adapted to 18kPa O₂ (Veh: 0.52 ± 0.08 ng/μg protein versus ZnCl₂-Py: 1.34 ± 0.26 ng/μg protein) but not in cells adapted to 5kPa O₂ (0.66 ± 0.17 ng/μg protein versus 0.76 ± 0.08 ng/μg protein). Under the same experimental conditions, Zn supplementation significantly increased metallothionein (MT-1) mRNA in cells under 18 or 5kPa O₂ (Fig. 3B).

3.3 Effects of Zn supplementation on NRF2 signaling in HCASMC under 18 or 5kPa O₂

We next examined whether Zn chelation or supplementation influences NRF2 nuclear accumulation and HO-1 and NQO1 mRNA/protein expression in cells pre-adapted for 5d to 18 or 5kPa O₂. Although chelation of Zn had negligible effects on NRF2 nuclear:cytoplasmic ratios, Zn supplementation significantly enhanced NRF2 nuclear accumulation in cells under 18 and 5kPa O₂ (Fig. 4). As shown in Fig. 5, TPEN had negligible effects on HO-1 and NQO1 mRNA/protein expression in cells cultured in complete medium containing low Zn (2.0μM). In contrast, Zn supplementation significantly increased HO-1 and NQO1 mRNA expression in HCASMC pre-adapted to 18kPa O₂, which was markedly attenuated in cells under 5kPa O₂ (Fig. 5 A and B). Furthermore, HO-1 protein expression in response to Zn supplementation was attenuated in HCASMC under 5kPa O₂ (Fig. 5C). Taken together these data suggest that NRF2 signaling is activated by Zn supplementation in cells adapted to 18kPa O₂ and to a lesser extent under 5 kPa O₂, independent of NRF2 nuclear accumulation.

3.4 Effects of hypoxia/reoxygenation on ROS generation, intracellular Zn and glutathione

Ischemia and hypoxia induced damage is exacerbated during reperfusion/reoxygenation and has been attributed to an increased generation of ROS [46]. To mimic reactive oxygen species (ROS) generation during reoxygenation, HCASMC were pre-adapted for 5d to 18 or 5kPa O₂, loaded with the luminescence probe L-012 and exposed to hypoxia (1h) and reoxygenation. As shown in Fig. 6A and B, ROS generation during reoxygenation of HCASMC under 18kPa O₂ was abrogated by PEG-superoxide dismutase, whilst PEG-catalase had a negligible effect, implicating superoxide anions in the reoxygenation-induced free radical burst. In contrast, reoxygenation-induced free radical generation was negligible in cells adapted to 5kPa O₂ (Fig. 6C and D), consistent with our previous findings in brain microvascular endothelial cells [34].

We next determined whether intracellular Zn content and glutathione (GSH) levels were affected differently by hypoxia/reoxygenation in HCASMC pre-adapted to 18 or 5kPa O₂. Total intracellular Zn content was not altered by hypoxia (1h) nor in response to reoxygenation of cells for 1 or 9 h under either 18 or 5kPa O₂, respectively (Supplementary Fig. 2). Hypoxia increased intracellular GSH levels in HCASMC pre-adapted to 18kPa but not 5kPa O₂, with levels returning to basal values over 24h reoxygenation (Supplementary Fig. 3).
Our findings contrast with negligible changes in GSH in mouse peritoneal macrophages cultured under 18kPa O₂ and exposed to hypoxia for 1 h [47].

3.5 Effects of hypoxia/reoxygenation on NQO1, GCLM and ZnT1 expression in HCASMC

When we examined the effects of hypoxia/reoxygenation on NRF2 targeted NQO1 and glutamate cysteine ligase modifier subunit (GCLM) expression in HCASMC pre-adapted for 5d to 18 or 5kPa O₂, NQO1 protein expression trended to be lower in cells adapted to 5kPa O₂ and was significantly lower in cells exposed to hypoxia (12h) and reoxygenation (12h) under 5kPa O₂ compared to 18kPa O₂ (Supplementary Fig. 4A). In contrast, negligible changes in GCLM (Supplementary Fig. 4B) and zinc transporter 1 (Zn efflux transporter [48], Supplementary Fig. 4C) expression were detected in cells pre-adapted to 18 or 5kPa O₂ and exposed to hypoxia/reoxygenation.

3.6 Effects of Zn chelation or supplementation on ROS generation in HCASMC under hypoxia/reoxygenation

As we established that Zn activates NRF2 signaling in HCASMC under 18 and 5kPa O₂, we next investigated whether Zn chelation or supplementation attenuates reoxygenation-induced free radical generation. HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and then pre-treated for 16h with TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (0.5μM). After incubation with L-012, cells were exposed to hypoxia (1h) and reoxygenation under 18 or 5kPa O₂ respectively in an O₂-controlled plate reader. Under low medium Zn concentration (2μM), TPEN had negligible effects on reoxygenation stimulated L-012 luminescence, whereas Zn supplementation attenuated reoxygenation-induced reactive oxygen species generation in cells under 18 kPa O₂ (Fig. 7A and B). As in our previous study in brain microvascular endothelial cells [34], negligible changes in L-012 luminescence were detected after reoxygenation of HCASMC under 5kPaO₂ (Fig. 7C and D).

4. Discussion

The present study, to our knowledge, is the first to investigate the effects of Zn chelation or supplementation on total intracellular Zn content, NRF2 regulated antioxidant gene transcription and hypoxia/reoxygenation induced ROS generation in HCASMC cultured long-term under standard hyperoxia (18kPa O₂) or physiological normoxia (5kPa O₂). Zn supplementation significantly increased total intracellular Zn content in HCASMC under 18 but not 5kPa O₂, whilst metallothionein-1 mRNA expression was upregulated in cells under either pericellular O₂ level. Although Zn supplementation increased NRF2 nuclear accumulation in cells under 18 and 5kPa O₂, NRF2 targeted HO-1 mRNA and protein expression induced by Zn supplementation was attenuated in HCASMC under 5kPa O₂, with a similar trend observed for NQO1. Notably, hypoxia/reoxygenation induced free radical generation was inhibited by Zn supplementation in HCASMC only under 18kPa O₂, consistent with our observation of enhanced ROS generation and antioxidant enzyme expression in cells under hyperoxia.

In view of contractile and synthetic phenotypes in vascular smooth muscle cells, our study is the first to investigate the effects of pericellular O₂ levels on the phenotype of HCASMC, highlighting a more contractile phenotype in cells under 5 compared to 18kPa O₂. Badran et al. recently reviewed the role of ROS as
modulators of vascular smooth muscle phenotype and suggested that excessive ROS generation induces a synthetic phenotype associated with disease, whilst physiological ROS levels are associated with a contractile phenotype [49]. Moreover, activation of NRF2 in response to 7-ketocholesterol stimulated ROS generation has been shown to maintain mouse coronary arterial smooth muscle cells in a differentiated state [50]. Our finding of increased calponin-1 expression in HCASMC under 5kPa O$_2$ thus further underpins the importance of physiologically relevant O$_2$ levels for cell culture models in vitro.

Using ICP-MS, we report novel evidence that Zn supplementation (10μM, 16h) induced increases in total Zn content in HCASMC are markedly attenuated in cells under physiological normoxia (5kPa) compared to hyperoxia (18kPa O$_2$). The Zn ionophore pyrithione enhances Zn uptake in oligodendrocyte progenitor cells [45], cardiac H9c2 cells [44, 51] and isolated papillary muscle [52]. In the present study HCASMC were treated with concentrations of ZnCl$_2$ + pyrithione and TPEN that had negligible effects on cell viability (see Supplementary Fig. 1). The Zn chelator TPEN (1.25μM) had negligible effects on total intracellular Zn content. It is possible that the low basal Zn concentration in the media for HCASMC may in part explain the lack of a statistical difference between vehicle and TPEN treated cells. Total Zn content may not be altered following treatment of HCASMC with TPEN, since ICP-MS analysis would also detect Zn chelated by TPEN. As reported in many other studies [53], TPEN would be expected to decrease bioavailable Zn levels.

Labile Zn (free Zn$^{2+}$) levels can increase in cells within seconds, minutes or hours after exogenous Zn supplementation with transcriptional regulation of Zn transporters observed over a longer time scale [54, 55]. Zn 'muffling' reactions modulate transient changes in Zn$^{2+}$ after Zn supplementation via zinc transporters (e.g. ZIP1-14 importers and ZnT1-10 exporters) and zinc-binding proteins to maintain a tight control of intracellular Zn$^{2+}$ levels [55, 56]. ZIP2 and ZIP12 have been implicated in endothelial and smooth muscle responses to vascular Zn deficiency [35] and, although ZIP12 expression is low in normal vascular tissue, exposure of human pulmonary vascular smooth muscle cells to hypoxia upregulates ZIP12 mRNA whilst ZIP6, ZIP7 and ZIP10 are unaffected [57]. In rat aortic smooth muscle cells, Zn supplementation (25-50μM) has been shown to decrease ZnT3 and ZnT10 expression and increase senescence [58]. Based on limited information available on the effects of Zn supplementation on ZIP and ZnT transporters in different vascular smooth muscle cell types, further characterization Zn transporters in cells maintained under physiological normoxia and exposed to hypoxia/reoxygenation is warranted.

Oxidative stress and hypoxia can induce release of Zn from metallothioneins (MT), leading to activation of MTF-1 and increased expression of MTs and ZnT1 [27, 59]. In the present study, MT1 mRNA expression was significantly upregulated by Zn supplementation in HCASMC under 18 and 5kPa O$_2$, consistent with previous studies in human pulmonary vascular smooth muscle cells conducted under ambient air [35]. We previously reported that MT1 and ZnT1 mRNA/protein expression in HCASMC was unaffected by changes in pericellular O$_2$ levels, and in the present study establish that ZnT1 protein expression is affected negligibly by Zn supplementation in HCASMC under 18 or 5kPa O$_2$ (see Supplementary Fig. 4C).
NRF2 plays a key role in maintaining cellular redox homeostasis [5-8], and previous studies have established that Zn influences NRF2 activation in different cell types cultured in standard incubators under hyperoxia (18kPa O_2), including endothelial cells [60], human renal tubule cells [25, 61], retinal pigment epithelial cells [26], IMR-32 neuroblastoma cells [62], murine spinal cord neurons [63] and human peripheral blood mononuclear cells [64]. NRF2 protein levels are maintained relatively low under physiological conditions due to rapid ubiquitination and proteosomal degradation mediated by Keap1 [5, 6] and GSK3β phosphorylation of NRF2, leading to β transducin repeats-containing proteins (βTrCP)-mediated degradation and Fyn-mediated nuclear exclusion [7, 65]. Recent evidence in human peripheral blood mononuclear cells suggests that Zn supplementation inhibits HDAC3 activity, using a cell-free assay, and decreases Keap1 mRNA expression without altering NRF2 protein levels [64]. By contrast, Zn supplementation (3d) of human leukemia monocytic THP-1 cells has negligible effects on NRF2 and Keap1 mRNA expression [66].

Our findings establish that upregulation of HO-1 in response to Zn supplementation is attenuated in HCASMC adapted to physiological normoxia (5kPa O_2) (see Fig. 5), consistent with negligible changes in total intracellular Zn content under 5kPa O_2 (Fig. 3B). Kaufman et al. implicated Zn-mediated degradation of Bach1, the mammalian repressor of HO-1, in the regulation of HO-1 expression by Zn in neuroblastoma cells [62]. In this context, specific loss of Bach1 in murine vascular smooth muscle inhibits neointimal hyperplasia and remodelling following vascular injury [67]. The interaction of intracellular Zn^{2+} with His-225, Cys-226 and Cys-613 within Keap1 is associated a conformational change in Keap1 leading to inhibition of NRF2 ubiquitination [23, 24]. Keap1, acting as a Zn sensor and Zn-binding protein, can also release Zn from Cys-273 and Cys-288 under oxidative stress [22, 24]. Zn supplementation has been shown to phosphorylate Akt and/or ERK1/2 and inhibit GSK3β in human renal tubule cells and mouse kidney [25], neonatal ventricular myocytes [17] and H9c2 cardiac cells [51], which would enhance NRF2 nuclear accumulation and downstream antioxidant gene expression [6, 7, 68]. Notably, ZIP6-induced Zn influx in MCF-7 cells has been associated with inhibition of GSK3β [69].

We previously reported that culturing vascular and other cell types under different O_2 levels significantly alters NRF2 regulated redox signaling [19, 31, 34, 70, 71], noting that long-term adaptation of human and murine endothelial cells to physiological normoxia (5kPa O_2) attenuates NRF2 transcrip-tional activation of HO-1 and NQO1 [19, 31, 34]. Our present findings demonstrate that Zn supplementation enhances NRF2 nuclear accumulation in HCASMC under 18 and 5kPa O_2, noting that Zn induced upregulation of HO-1/NQO1 protein expression is attenuated in cells under 5kPa O_2. Under these experimental conditions, Zn supplementation only protected HCASMC against reoxygenation induced free radical generation during culture under 18kPa O_2 (see Figs. 6 and 7), confirming the protection afforded by the NRF2 inducer sulforaphane against reoxygenation induced O_2^- generation in brain microvascular endothelial cells [34] and highlighting the lower redox stress experienced by cells under physiological normoxia [29, 30]. In this context, Kelmanson et al. reported only a slight decrease in H_2O_2 levels in the cytosol and mitochondria of neurons exposed to hypoxia for 30min using genetically encoded Hyper7 biosensors [72]. Notably, pretreatment of rat neonatal cardiomyocytes with

Our finding that Zn supplementation activates NRF2 signaling in HCASMC and can attenuate hypoxia/reoxygenation induced ROS generation provides a basis for design and screening of therapeutic drugs for treatment of coronary heart disease. Given the caveats concerning cell culture under hyperoxia [29, 30, 73-77], the potential influence of pericellular O₂ on chemical fluorescence probes [78] and our present findings that effects of Zn supplementation and the phenotype of HCASMC are altered by physiological normoxia, we encourage researchers to study interactions between Zn and redox signaling in cells under physiological O₂ levels. Moreover, such experiments should also consider zinc concentrations in culture media and the effect that pericellular O₂ has on cellular zinc metabolism and signaling involving or being affected by zinc ions.

Authors contributions
F.Y., M.J.S. and G.E.M. conceptualized the study; F.Y., M.J.S., A.G., A.M. developed the methodology; F.Y. performed and analysed all experiments and A.G. conducted the ICP-MS analyses; F.Y. and G.E.M. wrote the manuscript, which all authors reviewed. G.E.M. is the guarantor of this study, with responsibility for the integrity of the data and data analysis.

Declaration of competing interest
The authors declare that they have no known competing interests that could have influenced the study.

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Supplementary data to this article can be found online at
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Fig. 1 Long-term culture of HCASMC under physiological normoxia (5kPa O₂) upregulates calponin-1 expression

A and C, Representative immunofluorescence images of SM22-α or calponin-1 and DAPI stained nuclei in HCASMC pre-adapted for 5d to 18 or 5kPa O₂. B and D, Representative immunoblots and densitometric analysis of SM22-α and calponin-1 expression relative to β-actin. Data denote mean ± S.E.M., n=3-6 independent cell cultures (color-coded), unpaired Student's t-test, **P<0.01.
Fig. 2 Proliferation of HCASMC during long-term culture under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to either 18 or 5kPa O₂. **A-B**, Cells seeded at 3500 cells/well into 96-well plates and cell number counted and total protein content measured over 1 – 5 days in culture. **C**, Cells seeded at 7000 cells/well in E-Plates® and doubling time monitored continuously over 6 days using an iCELLigence platform. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded), two-way ANOVA followed by a Bonferroni Post Hoc test analysis, *P<0.05, **P<0.01, ***P < 0.001.
Fig. 3 Total intracellular Zn content and metallothionein mRNA expression in HCASMC treated with TPEN or supplemented with Zn under 18 or 5kPa O₂

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and then treated with vehicle (Veh, 0.01% DMSO), TPEN (Zn⁺⁺ chelator, 1.25μM) or ZnCl₂ (10μM) and 2-mercaptopyridine N-oxide sodium salt (Zn ionophore pyrithione, 0.5μM) (ZnCl₂+Py). A. ICP-MS analysis of total Zn content in HCASMC following treatment for 16h with Veh, TPEN or ZnCl₂+Py. Data denote mean ± S.E.M., n=4 independent cultures (color-coded), two-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001.
Fig. 4 Zn supplementation induces NRF2 nuclear accumulation in HCASMC under 18 or 5kPa O$_2$

A. Representative NRF2 positive immunofluorescence and DAPI stained nuclei in HCASMC pre-adapted to 18 or 5kPa O$_2$ and then treated for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl$_2$ (10μM) + pyrithione (Py, 0.5μM), respectively. B. Quantification of NRF2 nuclear:cytoplasmic ratio in HCASMC treated with Veh, TPEN or ZnCl$_2$+Py. Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded) with 20-30 cells analyzed in each culture, two-way ANOVA followed by a Bonferroni Post Hoc test analysis, **P<0.01, ***P<0.001. Scale bar = 20μm.
Fig. 5 Effect of Zn supplementation on HO-1 and NQO1 mRNA and protein expression in HCASMC under 18 or 5kPa O₂

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂. A and B, mRNA expression of HO-1 and NQO1 in HCASMC treated for 6h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM), respectively. Values normalized to three housekeeping (TBP, RPLPO and SDHA). C and D, Representative immunoblots and densitometric analysis of HO-1 and NQO1 expression relative to β-actin following treatment for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM). Data denote mean ± S.E.M., n=4-6 independent cultures (color-coded), two-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, ***P<0.001.
Fig. 6 Reoxygenation induced reactive oxygen species generation in HCASMC under 18 or 5kPa O₂

**A and C.** Representative L-012 luminescence traces in HCASMC pre-adapted for 5d to 18 or 5kPa O₂. Cells were treated with Veh (0.01% DMSO), PEG-superoxide dismutase (pSOD, 20U/ml) or PEG-catalase (pCAT, 200U/ml). Cells were incubated with L-012 and transferred to an O₂-regulated plate reader gassed with 18 or 5kPa O₂, respectively. L-012 luminescence was detected in the plate reader, with O₂ reduced to 1kPa and reoxygenation under 18 or 5kPa O₂, respectively. The red line indicates the pericellular O₂ levels within the plate reader.

**B and D.** Area under curve summary of reoxygenation-induced L-012 luminescence changes. L-012 signal in panel B (18kPa O₂) denotes area under the curve x10⁶ and in panel D (5kPa O₂) area under the curve x10⁴. Data denote mean ± S.E.M., n=6 independent cultures, one-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05, ***P<0.001.
Fig. 7 Zn supplementation attenuates reoxygenation-induced reactive oxygen species generation in HCASMC under 18kPa O₂

A and C. Representative L-012 luminescence traces in HCASMC pre-adapted for 5d to 18 or 5kPa O₂. Cells were treated for 16h with Veh (0.01% DMSO), TPEN (1.25μM) or ZnCl₂ (10μM) + pyrithione (Py, 0.5μM). Cells were incubated with L-012 and transferred to an O₂-regulated plate reader. L-012 luminescence was detected in the plate reader gassed with 18 or 5kPa O₂, with O₂ then reduced to 1kPa and reoxygenation under either 18 or 5kPa O₂, respectively. The red line indicates the pericellular O₂ levels within the plate reader. B and D. Area under curve summary of reoxygenation-induced L-012 luminescence changes. L-012 signal in panel B (18kPa O₂) denotes area under the curve x10⁶ and in panel D (5kPa O₂) area under the curve x10⁴. Data denote mean ± S.E.M., n=4 independent cultures, one-way ANOVA followed by Bonferroni’s multiple comparisons test, *P<0.05.
Redox Biology - Supplementary Data

Vascular protection afforded by zinc supplementation in human coronary artery smooth muscle cells mediated by NRF2 signaling under hypoxia/reoxygenation

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Supplementary Table 1 - qPCR primers

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Abbreviations: RPLP0, ribosomal protein lateral stalk subunit P0; TBP, TATA-binding protein; SDHA, succinate dehydrogenase complex, subunit A; HO-1, heme oxygenase-1, NQO1, NAD(P)H quinone dehydrogenase 1

Supplementary Fig.1 Viability of HCASMC following treatment with ZnCl\(_2\)/pyrithione or TPEN

HCASMC pre-adapted for 5d to 18kPa \(\text{O}_2\) were seeded in triplicate at a concentration of 10\(^4\) cells/well in 96-well plates and cultured for 48h. Cells were then treated for 16h in the absence (control medium) or presence of different concentrations of ZnCl\(_2\)/pyrithione or TPEN and an MTT assay used to assess cell viability. A, Pyrithione (0.5µM) + ZnCl\(_2\) (10, 12 or 14µM). B, ZnCl\(_2\) (10µM) + pyrithione (0.25, 0.5, 0.75 or 1µM). C, TPEN (0.5, 0.75, 1.0, 1.25 or 1.5µM). Data denote mean ± S.E.M., n=3-4 independent cultures (color-coded), one-way or two-way ANOVA with Tukey’s or Bonferroni’s multiple comparisons test, *\(P<0.05\), **\(P<0.01\).

Abbreviations: TPEN, N,N,N’,N’-tetrakis (2-pyridylmethyl) ethylenediamine
Supplementary Fig. 2 Effects of hypoxia/reoxygenation on total intracellular Zn content in HCASMC under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and cells exposed to hypoxia and reoxygenation. A, ICP-MS analysis of total intracellular Zn content in HCASMC under basal conditions, hypoxia (1kPa, 1h) and reoxygenation (for 1h and 9h) under 18 or 5kPa O₂, respectively. Changes between 18kPa or 5kPa and 1kPa O₂ required 30 min with cells maintained in the O₂-controlled workstation. B, Summary of total Zn content for each condition and time point. Data denote mean ± S.E.M., n=4-10 independent cultures, two-way ANOVA with Tukey’s multiple comparisons test.

Supplementary Fig. 3 Effects of hypoxia/reoxygenation on intracellular glutathione levels in HCASMC under 18 or 5kPa O₂
HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ and intracellular glutathione (GSH) then measured under basal conditions (control), hypoxia (1h) only or followed by reoxygenation (HR) for 1h or 24h under 18 or 5kPa O₂, respectively. Data denote mean ± S.E.M., n=4 independent cultures (color-coded), two-way ANOVA with Tukey’s or Bonferroni’s multiple comparisons test, *P<0.05.
Supplementary Fig. 4 NQO1, GCLM and ZnT1 expression in HCASMC under hypoxia-reoxygenation

HCASMC were pre-adapted for 5d to 18 or 5kPa O₂ (control) and then subjected to hypoxia (H) for 12h or hypoxia for 12 h followed by reoxygenation for 12h (HR) under 18 or 5kPa O₂, respectively. Representative immunoblots and densitometric analyses of NQO1 (A), GCLM (B) and ZnT1 (C) expression relative to β-actin. Data denote mean ± S.E.M., n=3 independent paired cultures (color-coded), two-way ANOVA with Tukey’s multiple comparisons test, *P<0.05.
Authors declare no conflicts of interest in this study.