

# Hydrogen peroxide depolarizes mitochondria and inhibits IP<sub>3</sub>-evoked Ca<sup>2+</sup> release in the endothelium of intact arteries

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## ABSTRACT

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a mitochondrial-derived reactive oxygen species (ROS) that regulates vascular signalling transduction, vasoconstriction and vasodilation. Although the physiological role of ROS in endothelial cells is acknowledged, the mechanisms underlying H<sub>2</sub>O<sub>2</sub> regulation of signalling in native, fully-differentiated endothelial cells is unresolved. In the present study, the effects of H<sub>2</sub>O<sub>2</sub> on Ca<sup>2+</sup> signalling were investigated in the endothelium of intact rat mesenteric arteries. Spontaneous local Ca<sup>2+</sup> signals and acetylcholine evoked Ca<sup>2+</sup> increases were inhibited by H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> inhibition of acetylcholine-evoked Ca<sup>2+</sup> signals was reversed by catalase. H<sub>2</sub>O<sub>2</sub> exerts its inhibition on the IP<sub>3</sub> receptor as Ca<sup>2+</sup> release evoked by photolysis of caged IP<sub>3</sub> was suppressed by H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> suppression of IP<sub>3</sub>-evoked Ca<sup>2+</sup> signalling may be mediated by mitochondria. H<sub>2</sub>O<sub>2</sub> depolarized mitochondria membrane potential. Acetylcholine-evoked Ca<sup>2+</sup> release was inhibited by depolarisation of the mitochondrial membrane potential by the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or complex 1 inhibitor, rotenone. We propose that the suppression of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release by H<sub>2</sub>O<sub>2</sub> arises from the decrease in mitochondrial membrane potential. These results suggest that mitochondria may protect themselves against Ca<sup>2+</sup> overload during IP<sub>3</sub>-linked Ca<sup>2+</sup> signals by a H<sub>2</sub>O<sub>2</sub> mediated negative feedback depolarization of the organelle and inhibition of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release.

## 1. Introduction

The endothelium is the single layer of cells that lines the entire cardiovascular system and it is exposed constantly to a wide range of mechanical and chemical stimuli. The endothelium responds to these stimuli by releasing Ca<sup>2+</sup>-dependent vasoactive factors that include nitric oxide, prostacyclin, endothelium-derived contracting factors, von Willebrand factor, tissue plasminogen activator and endothelial derived hyperpolarising factor (1, 2). These vasoactive factors allow the endothelium to regulate almost all cardiovascular activities including vascular tone, immune responses, angiogenesis and vascular remodelling [1].

There is accumulating evidence that reactive oxygen species (ROS) also regulates endothelial function. ROS modulates endothelial cell growth, proliferation, endothelium-dependent relaxation, cytoskeletal reorganization, inflammatory responses and endothelium-regulated vascular remodelling. Among various ROS, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) fulfils the prerequisites for serving as an intracellular messenger and acting as a cell-cell signalling molecule. H<sub>2</sub>O<sub>2</sub> is a small and non-polar molecule produced by several cell processes that include mitochondria and NADPH oxidase [17,29,76]. During mitochondrial ATP production,

the electron transport chain leaks electrons from complexes I and III resulting in the formation of superoxide anion radical (O<sub>2</sub><sup>-</sup>) [11]. O<sub>2</sub><sup>-</sup> generates H<sub>2</sub>O<sub>2</sub> spontaneously, or by the activity of superoxide dismutases. Although O<sub>2</sub><sup>-</sup> is not membrane permeable, H<sub>2</sub>O<sub>2</sub> can diffuse across biological membranes or may cross membrane boundaries via channels like aquaporins [8] to regulate physiological and pathological cellular processes [3,62,75,85].

Many of the systems that produce H<sub>2</sub>O<sub>2</sub>, such as mitochondria, are modulated by the cytoplasmic Ca<sup>2+</sup> concentration [6,24,36]. Ca<sup>2+</sup> released from IP<sub>3</sub>Rs may result in Ca<sup>2+</sup> signals that propagate into the mitochondrial matrix [35,59]. Changes in mitochondrial Ca<sup>2+</sup> may lead to enhanced ATP synthesis [74] and, as a result, increased ROS production [68]. Conversely, increased H<sub>2</sub>O<sub>2</sub> generated by mitochondrial activity may modulate Ca<sup>2+</sup> signalling to exert control on endothelial function. For example, in various cultured cell lines, H<sub>2</sub>O<sub>2</sub> evokes Ca<sup>2+</sup> release from the internal Ca<sup>2+</sup> store [27,37,77]. These observations raise the possibility that there may be feedback regulation of mitochondrial ATP production by changes in the cell activity mediated via the cytoplasmic Ca<sup>2+</sup> concentration. To explore this possibility we measured the effects of H<sub>2</sub>O<sub>2</sub> on Ca<sup>2+</sup> signalling in the endothelium in large numbers of endothelial cells in intact blood vessels. We show

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that H<sub>2</sub>O<sub>2</sub> depolarises mitochondria and suppresses IP<sub>3</sub> evoked Ca<sup>2+</sup> signalling.

## 2. Methods

### 2.1. Animals

All animal husbandry and euthanasia were carried out in accordance with the prior approval of the University of Strathclyde Animal Welfare and Ethical Review Body and under relevant UK Home Office Regulations, [Schedule 1 of the Animals (Scientific Procedures) Act 1986, UK]. Strathclyde BPU is a conventional unit which undertakes FELASA quarterly health monitoring. Male Sprague-Dawley rats (10–12 weeks old), from an in-house colony, were used in the study. Animals were housed 3 per cage (RC2F cages, North Kent Plastics Company, UK), provided with enrichment (aspen wood chew sticks and hanging huts), nesting material (Sizzle nest, LBS Technology, UK), and fresh water and chow (RM1, Special Diet Services, UK) were available *ad libitum*. Room temperature was 19–23 °C (set point 21 °C), humidity was 45–65 %, and a 12 h light cycle was used. Rats were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg, Pentaject, Merial Animal Health Ltd, UK).

### 2.2. Endothelial Ca<sup>2+</sup> imaging

First order mesenteric arteries were isolated, placed into a physiological saline solution (PSS), cleaned of adherent fat and then used immediately. Each artery was then cut open and pinned flat on a Sylgard block, with endothelial cells facing upward (*en face* preparation). The endothelium was then loaded with acetoxymethyl ester form of the Ca<sup>2+</sup> indicator, Cal-520 (5 μM) and 0.02% pluronic F-127 in DMSO, for 30 min at 37 °C [55,79–81]. Following incubation, arteries were gently washed before the Sylgard block was inverted and placed in a custom-made bath chamber. The bottom of the chamber was a 0-thickness glass coverslip and two (0.2 μm diameter) steel pins were set between the coverslip and the block to prevent endothelial cells from contacting the coverslip, and to allow solutions to flow across the endothelium. Ca<sup>2+</sup> images were acquired at 10 Hz on an inverted fluorescence microscope (TE300, Nikon, Japan) using a 40×, 1.4 NA oil immersion lens and a back-illuminated electron-multiplying charge-coupled device (EMCCD) camera (1024 × 1024 13 μm pixels; iXon 888; Andor, UK). Fluorescence excitation (488 nm wavelength) illumination was provided by a monochromator (Horiba, UK).

### 2.3. Localized flash photolysis

In some experiments, the endothelial Ca<sup>2+</sup> response to local photolysis of caged IP<sub>3</sub> was examined. In these experiments, the endothelium was loaded membrane permeant, caged IP<sub>3</sub> (5 μM) for 30 min at 37 °C. A xenon flash lamp (Rapp Optoelektronik, Germany) was used to uncage IP<sub>3</sub> [13,47,80]. The output light was filtered using a UG-5 filter to select ultraviolet light. The light was focused and merged into the excitation light path via a fibre optic bundle and long pass dichroic mirror attached to the lens part of the microscope's epi-illumination attachment [13,53,58]. The area of the photolysis site (~80 μm diameter) resulted from the fiber optic diameter and the objective lens magnification (40x).

### 2.4. Imaging endothelial mitochondria

To assess mitochondrial membrane potential, arteries were pinned out in a Sylgard coated chamber designed for use on an upright microscope. Mitotracker Green FM (100 nM) was added to the PSS and the endothelium was incubated for 20 min followed by 20 min washing. Tetramethylrhodamine ethyl ester (TMRE) (60 nM) was added to the PSS and the endothelium was incubated 10 min [20,21,80]. TMRE

(60 nm) was subsequently present in all perfusion solutions. Minimal photobleaching of TMRE was observed over the 5 min recording periods used. TMRE and Mitotracker Green images (10 Hz) were acquired on an upright microscope (Eclipse FN1; Nikon, Japan) equipped with a 60× water immersion objective (1.0 numerical aperture) and an EMCCD camera (iXon 888; Andor, UK).

### 2.5. Experimental protocols

The effect of H<sub>2</sub>O<sub>2</sub> on basal endothelial Ca<sup>2+</sup> activity was studied using a non-cumulative concentration response in the same preparation. In these experiments, a Ca<sup>2+</sup>-free PSS was used and H<sub>2</sub>O<sub>2</sub> added to the Ca<sup>2+</sup>-free perfusate. To prevent depletion of internal Ca<sup>2+</sup> stores, arteries were incubated in Ca<sup>2+</sup>-containing PSS between each exposure to H<sub>2</sub>O<sub>2</sub>.

The effect of H<sub>2</sub>O<sub>2</sub> (with or without catalase, 1000 U ml<sup>-1</sup>) on evoked (acetylcholine, ACh; 100 nM) endothelial Ca<sup>2+</sup> activity was studied in paired experiments. In these experiments, a control response (5-minute recording) to ACh (flowed rate: 1.5 ml min<sup>-1</sup>) was obtained before the tissue was washed for 5 min, and allowed to equilibrate for 10 min. ACh was then applied a second time, together with H<sub>2</sub>O<sub>2</sub> (with or without catalase), and the responses compared. ACh and H<sub>2</sub>O<sub>2</sub> (with or without catalase) were applied via separate syringe pumps each at 0.75 ml min<sup>-1</sup>. The effects of various pharmacological interventions on ACh-evoked Ca<sup>2+</sup> signalling were also studied in paired experiments in which control responses were first obtained and then the endothelium was incubated with each antagonist for 20 min. Following the incubation period, ACh was applied a second time and responses compared to control. Each pharmacological agent was present throughout the second exposure to ACh.

Experiments utilising caged-IP<sub>3</sub> also used a paired experimental design. An initial response to photolysis was recorded, and the tissue was then rested for 10 min. The endothelium was then incubated with H<sub>2</sub>O<sub>2</sub> for 20 min before a second response, using the same photolysis location, was obtained.

### 2.6. Data analysis

Automated analysis of endothelial Ca<sup>2+</sup> imaging recordings was carried out using custom-written Python routines [44,80,81]. In brief, average intensity projections were used to generate regions-of-interest (ROI) around each cell. The Ca<sup>2+</sup> response of each endothelial cell was then extracted by averaging the fluorescence intensity within each ROI, for each cell and image in the dataset. Each ROI/cell was assigned an identification number so that the response of each cell could be compared within experimental series. Fluorescence signals are expressed as ratios (F/F<sub>0</sub>) of fluorescence counts (F) relative to baseline (control) values before stimulation (F<sub>0</sub>). The baseline (F<sub>0</sub>) was identified automatically as the 100 frame (10 s) period exhibiting the lowest noise prior to the introduction of any agonist. The total number of oscillations, and the amplitude of each oscillation were then extracted for each cell using a zero-crossing peak-detection algorithm [82] for signals exceeding 3 times the standard deviation of baseline noise.

### 2.7. Statistics

All data are presented as mean ± SEM of n biological replicates. Data were analysed using repeated measures one-way ANOVA with Geisser-Greenhouse correction and Dunnett's multiple comparisons test, or paired *t*-test as appropriate. A *p* value less than 0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, USA) was used to run the statistical analysis.

## 2.8. Reagents and chemicals

The PSS consisted of (in mM):145 NaCl, 2.0 MOPS, 4.7 KCl, 1.3  $\text{NaH}_2\text{PO}_4$ , 5.0 Glucose, 1.17, MgCl, 2.0 CaCl, 0.02 EDTA (pH adjusted to 7.4 with NaOH). In experiments using  $\text{Ca}^{2+}$  free PSS,  $\text{CaCl}_2$  was replaced with  $\text{MgCl}_2$  on an equimolar basis and EGTA (1 mM) was included. Caged- $\text{IP}_3$  (caged- $\text{IP}_3$  4,5-dimethoxy-2-nitrobenzyl) was obtained from Slichem (Germany). Cal-520 was obtained from Abcam (UK). Pluronic F-127 was obtained from Invitrogen (UK). Mitotracker Green FM was obtained from Invitrogen (UK). All other drugs and chemicals were obtained from Sigma (UK). Stock solutions of ACh, catalase-polyethylene glycol and  $\text{H}_2\text{O}_2$  were prepared by dissolving each chemical in double-distilled, dionized water. 2-aminoethoxydiphenyl borate (2-APB), caged- $\text{IP}_3$ , Cal-520, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), oligomycin, TMRE and Mitotracker Green FM were dissolved in DMSO.

## 2.9. Data availability

All data underpinning this study is available from the authors upon reasonable request.

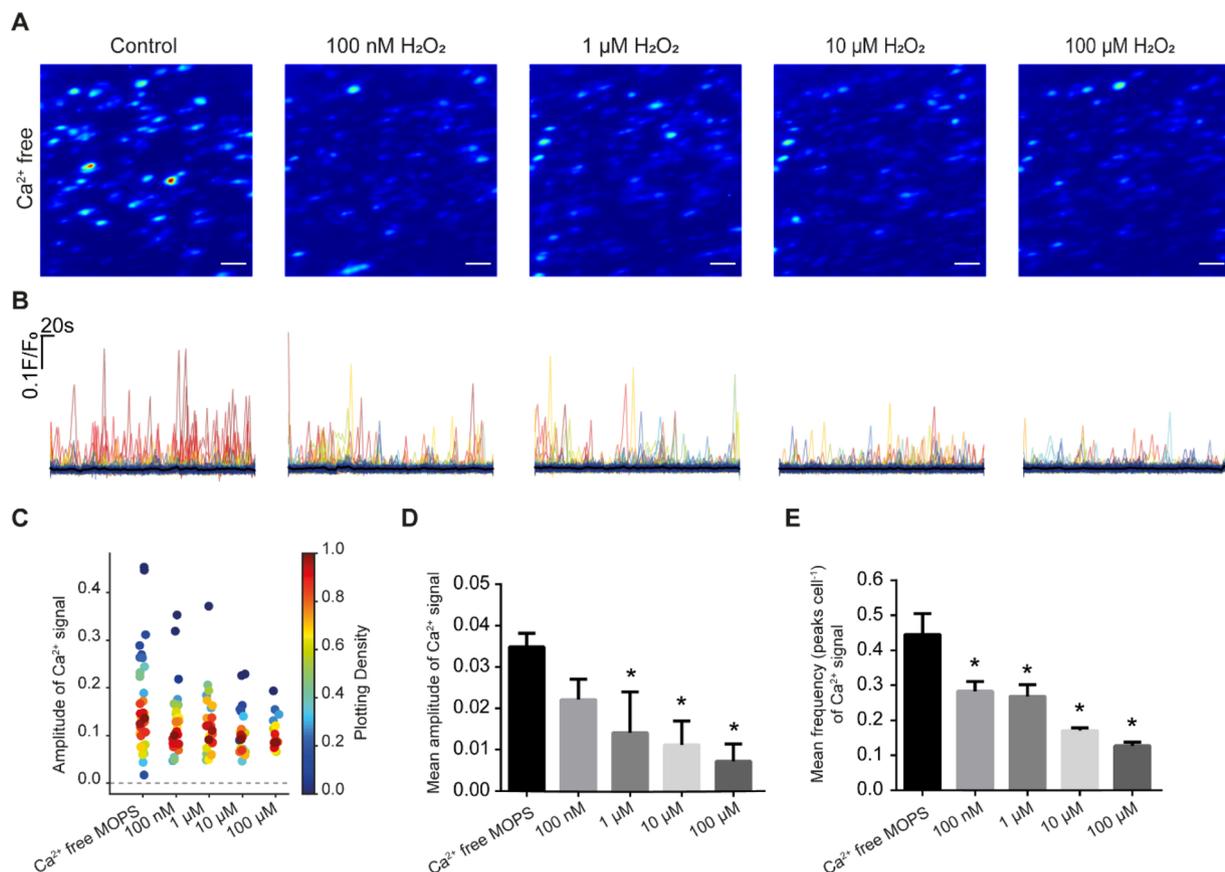
## 3. Results

To determine if  $\text{H}_2\text{O}_2$  alters spontaneous  $\text{Ca}^{2+}$  release from the internal store in intact mesenteric arteries,  $\text{H}_2\text{O}_2$  (100 nM, 1  $\mu\text{M}$ , 10  $\mu\text{M}$  and 100  $\mu\text{M}$ ) was applied in a  $\text{Ca}^{2+}$  free PSS (Fig. 1). Between  $\text{H}_2\text{O}_2$

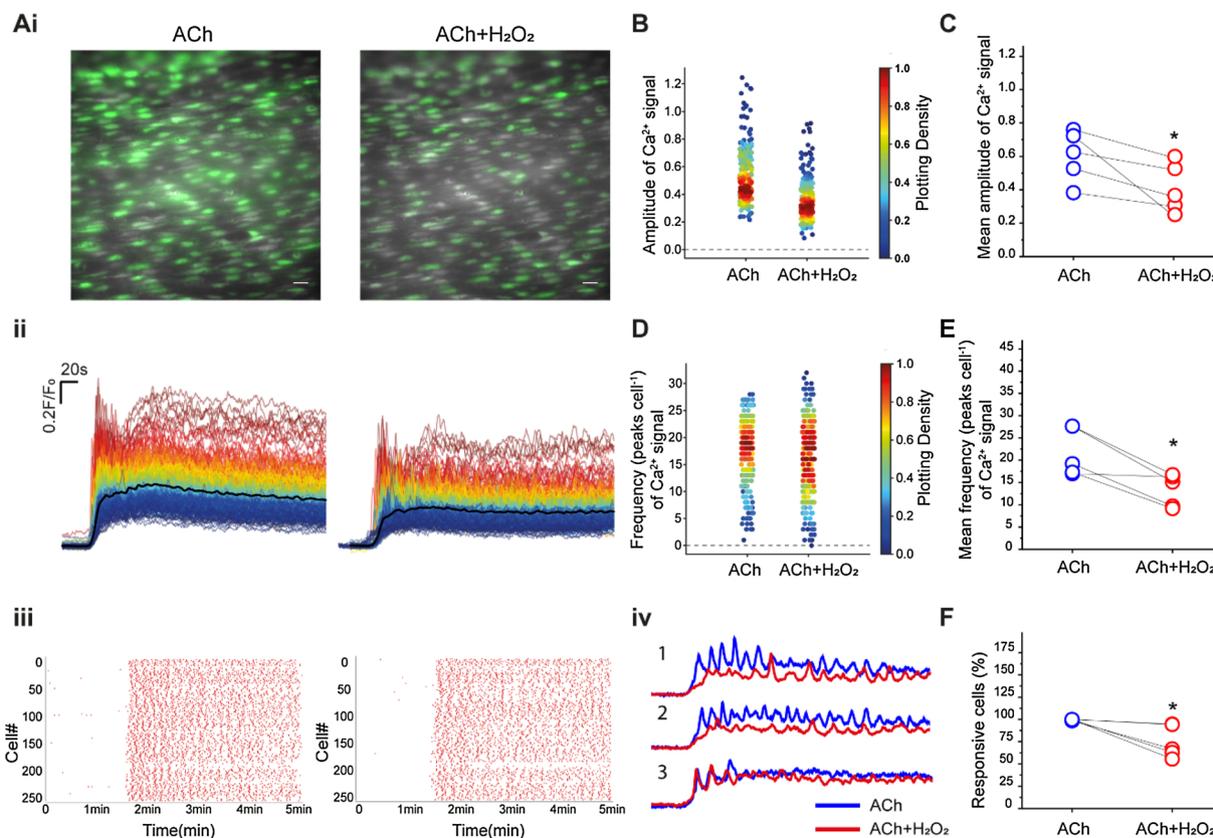
applications arteries were washed in PSS (containing  $\text{Ca}^{2+}$ ) to allow the internal  $\text{Ca}^{2+}$  stores to refill. As the concentration of  $\text{H}_2\text{O}_2$  increased, spontaneous  $\text{Ca}^{2+}$  release events decreased (Fig. 1). As spontaneous  $\text{Ca}^{2+}$  release arises from  $\text{IP}_3$ -receptor activity [44,80,81], these results suggest that  $\text{H}_2\text{O}_2$  may suppress  $\text{Ca}^{2+}$  release from the internal  $\text{Ca}^{2+}$  store.

To further examine the effect of  $\text{H}_2\text{O}_2$  on  $\text{Ca}^{2+}$  release from the store, the effects of the free radical were examined on ACh-evoked  $\text{Ca}^{2+}$  release. ACh (100 nM) evoked substantial  $\text{Ca}^{2+}$  signals that were heterogeneous across the endothelium and the amplitude and frequency of  $\text{Ca}^{2+}$  oscillations varied across cells. (Fig. 2A) (see also [2,38,44,49,52,55,81]). After washing out ACh, the endothelium was allowed to rest for 10 min and then challenged again with ACh (100 nM) and  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) applied simultaneously.  $\text{H}_2\text{O}_2$  suppressed several aspects of ACh-evoked  $\text{Ca}^{2+}$  signalling. There was a reduction in the percentage of cells responding to ACh, a decrease in the amplitude, and a reduction frequency of oscillations in the presence of  $\text{H}_2\text{O}_2$  when compared to controls (ACh alone; Fig. 2B-F). The effect of  $\text{H}_2\text{O}_2$  on endothelial cells was also heterogeneous, and the free radical affected the  $\text{Ca}^{2+}$  response of some cells more than others' (see Fig. 2Aiv). In the absence of  $\text{H}_2\text{O}_2$ , ACh (100 nM; 10 min. apart) evoked reproducible  $\text{Ca}^{2+}$  signals (Figure S1).

To determine if the internal  $\text{Ca}^{2+}$  store content was altered by  $\text{H}_2\text{O}_2$ , ionomycin (2  $\mu\text{M}$ , in  $\text{Ca}^{2+}$  free PSS) was applied in the absence or presence of  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ). Ionomycin-evoked  $\text{Ca}^{2+}$  signals were not significantly altered by  $\text{H}_2\text{O}_2$  (Fig. 3A). Two measurements were used in this analysis; the amplitude of ionomycin-induced  $\text{Ca}^{2+}$  release and the



**Fig. 1.** Effect of  $\text{H}_2\text{O}_2$  on spontaneous local  $\text{Ca}^{2+}$  signals arising from the internal  $\text{Ca}^{2+}$  stores. (A) Pseudo-colour images of spontaneous  $\text{Ca}^{2+}$  signals activity (red high, blue low amplitude) over a 5 min period in control ( $\text{Ca}^{2+}$ -free PSS) and with  $\text{H}_2\text{O}_2$  (100 nM to 100  $\mu\text{M}$ ). Between each recording,  $\text{H}_2\text{O}_2$  was washed out and  $\text{Ca}^{2+}$  was restored to the bathing medium (10 min) to permit the store to refill. Scale bar: 20  $\mu\text{M}$ . (B)  $\text{Ca}^{2+}$  signals measured in ~200 cells shown in A. (C) Density plot of mean peak value of  $\text{Ca}^{2+}$  signalling in  $\text{Ca}^{2+}$  free MOPS and increasing concentrations of  $\text{H}_2\text{O}_2$ . Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values. (D) Summary of mean peak value of  $\text{Ca}^{2+}$  signalling in all cells. (E) Summary of number of  $\text{Ca}^{2+}$  signalling peaks in all cells  $n = 5$ , \* $p < 0.05$  vs  $\text{Ca}^{2+}$ -free MOPS.



**Fig. 2.** ACh-evoked Ca<sup>2+</sup> release was suppressed by H<sub>2</sub>O<sub>2</sub>. (Ai) Pseudo-colour images (green) of Ca<sup>2+</sup> signalling evoked by ACh (100 nM), and ACh (100 nM) in the presence of H<sub>2</sub>O<sub>2</sub> (100 μM). Scale bar: 20 μM. (Aii) Overlaid Ca<sup>2+</sup> signalling traces from ~200 cells (shown in A) with the average shown as the black line in response to ACh and ACh+H<sub>2</sub>O<sub>2</sub>. Individual Ca<sup>2+</sup> traces are coloured according to the magnitude of the control (ACh) response. (Aiii) Rastergram plot of Ca<sup>2+</sup> signals. Each red dot represents a Ca<sup>2+</sup> peak in each cell (shown on the left axis) ACh (100 nM) left-side and ACh (100 nM) + H<sub>2</sub>O<sub>2</sub> (100 μM) right-side. (Aiv) The effect of H<sub>2</sub>O<sub>2</sub> varied on Ca<sup>2+</sup> signals across cells. The panel shows traces of Ca<sup>2+</sup> signalling from three typical cells from (Ai). The blue lines are the Ca<sup>2+</sup> signals evoked by ACh (100 nM) and the red lines ACh (100 nM) + H<sub>2</sub>O<sub>2</sub> (100 μM). Cell 3 was largely unaffected by H<sub>2</sub>O<sub>2</sub>. (B) Density plot of mean peak value of Ca<sup>2+</sup> signalling from cells treated with ACh (100 nM) and ACh (100 nM) + H<sub>2</sub>O<sub>2</sub> (100 μM). Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values (C) Summary of mean peak value of Ca<sup>2+</sup> signalling in all cells. (D) Density plot of the frequency of Ca<sup>2+</sup> signals. Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values (E) Summary of frequency of Ca<sup>2+</sup> oscillations in all cells. (F) Summary of percentage of ACh-responsive cells. For all summary data (D–F) n = 6; \*p < 0.05.

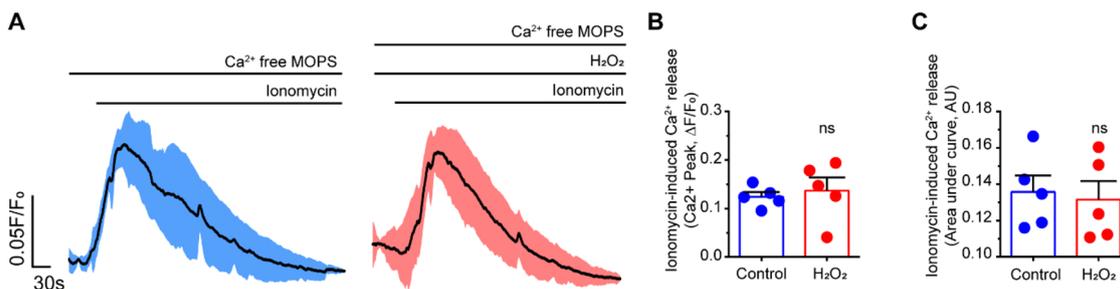
area under the curve (Fig. 3B, C). Each measure was unchanged suggesting that H<sub>2</sub>O<sub>2</sub> did not deplete the internal Ca<sup>2+</sup> store.

To confirm that the suppression of ACh-evoked Ca<sup>2+</sup> signalling in endothelial cells arose from H<sub>2</sub>O<sub>2</sub>, catalase-peg (1000 U/ml) was used to breakdown H<sub>2</sub>O<sub>2</sub>. Catalase by itself did not alter the Ca<sup>2+</sup> signal evoked by ACh (100 nM) when compared to controls (Fig. 4A–F). Furthermore, in the presence of catalase, H<sub>2</sub>O<sub>2</sub> (100 μM) did not alter the amplitude (Fig. 4B, D), or the frequency (Fig. 4C, F) of the ACh-evoked Ca<sup>2+</sup> signals, nor did it alter the percentage of cells activated by ACh

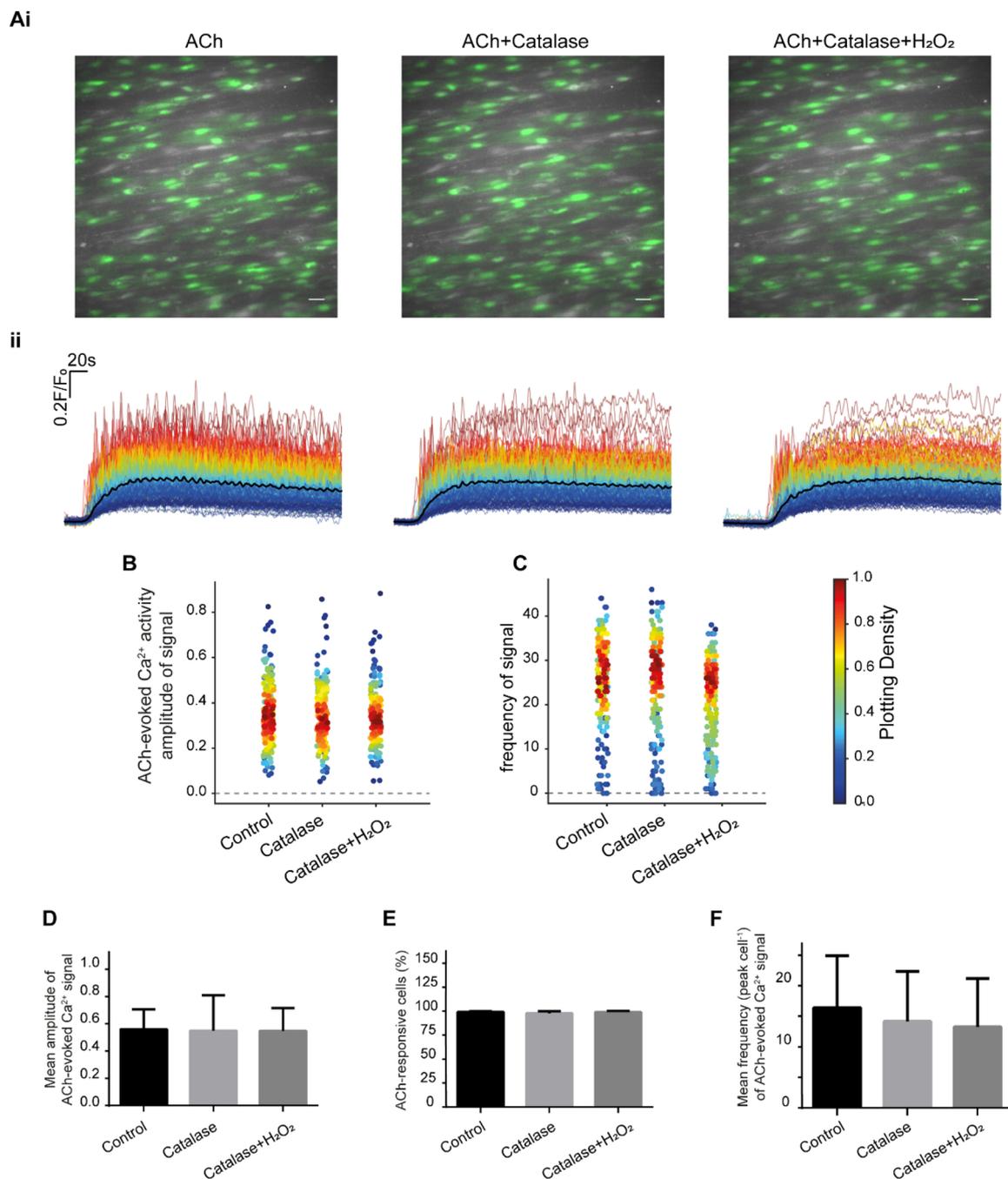
(Fig. 4E). These data suggest that H<sub>2</sub>O<sub>2</sub> suppresses ACh-evoked Ca<sup>2+</sup> signals in native endothelial cells.

In native endothelial cells, ACh-evoked Ca<sup>2+</sup> release requires activation of IP<sub>3</sub>Rs [1]. In support, ACh-evoked Ca<sup>2+</sup> release was rapidly blocked by 2-APB (Fig. 5A–F). 2-APB significantly attenuated the amplitude (97% reduction; Fig. 5A, B, C) and frequency (99% reduction; Fig. 5E) of ACh-evoked Ca<sup>2+</sup> signals, and the percentage of active cells activated by ACh (97% reduction; Fig. 5D).

To determine which part of the IP<sub>3</sub> pathway Ca<sup>2+</sup> release was



**Fig. 3.** Internal Ca<sup>2+</sup> store content was unchanged in H<sub>2</sub>O<sub>2</sub>. (A) The Ca<sup>2+</sup> store content was assessed using ionomycin (2 μM) applied in a Ca<sup>2+</sup>-free PSS. The left panel shows the averaged ionomycin-induced Ca<sup>2+</sup> transient in the absence of H<sub>2</sub>O<sub>2</sub> (100 μM) while the right panel is in the presence of H<sub>2</sub>O<sub>2</sub>. The black lines is the mean of five independent preparations blue and red lines show the standard error of the mean (SEM). (B–C) Summary data of mean peak response (B) and area under the curve (C) of ionomycin-induced Ca<sup>2+</sup> increase (n = 5).

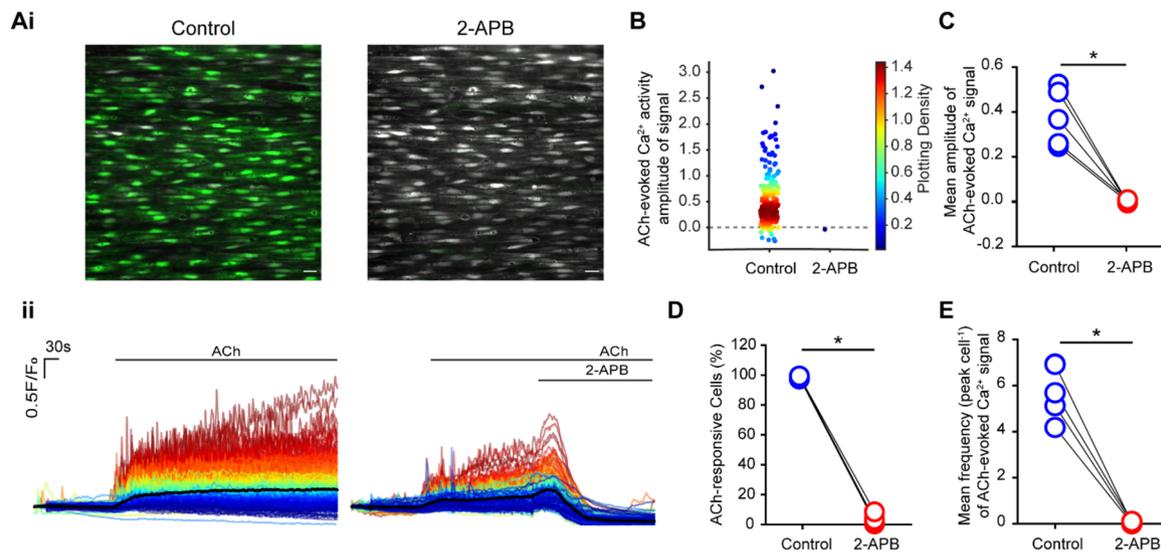


**Fig. 4.** Catalase eliminated the effect of H<sub>2</sub>O<sub>2</sub> on ACh-evoked Ca<sup>2+</sup> signalling. (Ai) Pseudo colour (green) images of Ca<sup>2+</sup> signalling evoked by ACh (100 nM), ACh (100 nM) + Catalase (1000 U/ml) and ACh (100 nM) + Catalase (1000 U/ml) + H<sub>2</sub>O<sub>2</sub> (100 μM). Scale bar: 20 μM. (Aii) Overlaid Ca<sup>2+</sup> signalling traces from each endothelial cell with treatment of ACh (100 nM), ACh (100 nM) + Catalase (1000 U/ml) and ACh (100 nM) + Catalase (1000 U/ml) + H<sub>2</sub>O<sub>2</sub> (100 μM). Individual Ca<sup>2+</sup> traces are coloured according to the magnitude of the control (ACh) response. (B) Density plot of mean peak value of Ca<sup>2+</sup> signalling. Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values. (C) Density plot of number of the frequency Ca<sup>2+</sup> signalling. (D) Summary data showing mean peak value of Ca<sup>2+</sup> signalling in all cells. (E) Summary data showing the percentage of active cells. (F) Summary data showing the frequency of Ca<sup>2+</sup> signals in all cells ( $n = 6$  for all summary data).

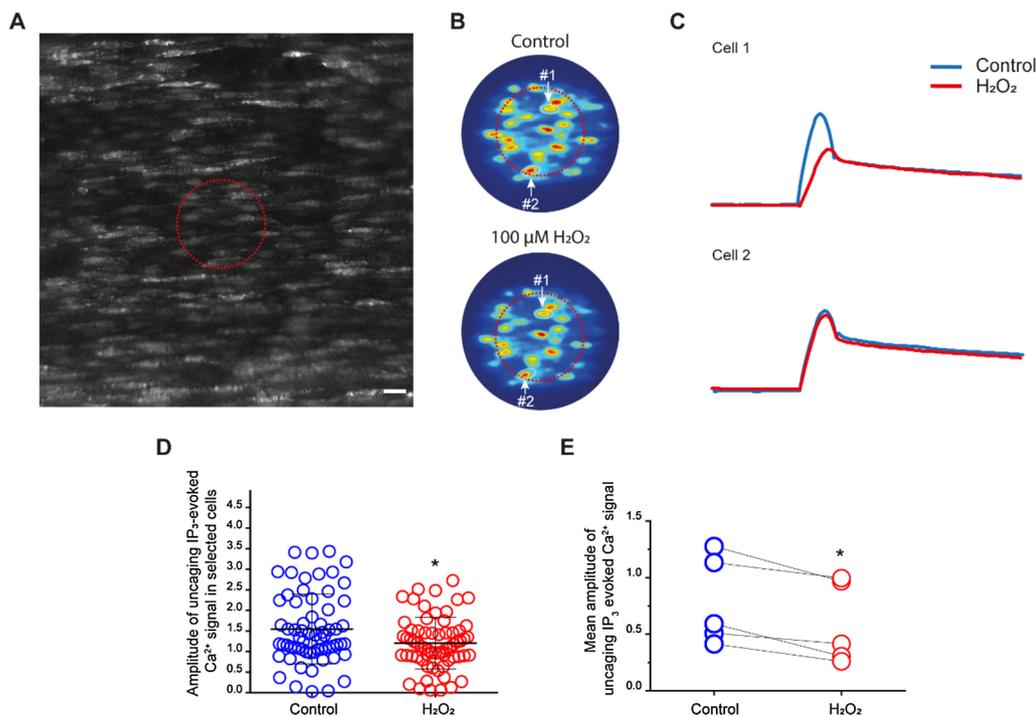
modified by H<sub>2</sub>O<sub>2</sub>, we performed experiments using the membrane-permeant, photoactivatable form of IP<sub>3</sub> (caged-IP<sub>3</sub> 5 μM). IP<sub>3</sub>, released via the photolysis of caged-IP<sub>3</sub>, directly activates IP<sub>3</sub>Rs [13,51] and evoked a Ca<sup>2+</sup> response (Fig. 6A-E). H<sub>2</sub>O<sub>2</sub> (100 μM) significantly attenuated the Ca<sup>2+</sup> response to photolysis of caged-IP<sub>3</sub> (22% reduction; Fig. 6C-E). Again, there was heterogeneity in the sensitivity to H<sub>2</sub>O<sub>2</sub> and some cells were less affected than others (Fig. 6C). These results demonstrate that H<sub>2</sub>O<sub>2</sub> reduces ACh-evoked Ca<sup>2+</sup> release by altering either the activity of IP<sub>3</sub> receptors or the interaction between IP<sub>3</sub> and

IP<sub>3</sub>Rs.

Since H<sub>2</sub>O<sub>2</sub> is reported to increase the activity of IP<sub>3</sub>Rs [11], the question arises as to how H<sub>2</sub>O<sub>2</sub> is able to decrease IP<sub>3</sub>-evoked Ca<sup>2+</sup> release. Mitochondria exert profound control of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release [58,80] and H<sub>2</sub>O<sub>2</sub> has been shown to alter mitochondrial function [56]. These observations raise the possibility that H<sub>2</sub>O<sub>2</sub> may exert effects on IP<sub>3</sub>R indirectly. To determine if mitochondria mediate the effects of H<sub>2</sub>O<sub>2</sub>, we investigated the effect of uncoupling mitochondria on Ca<sup>2+</sup> release from the internal Ca<sup>2+</sup> store. To do this, the uncoupler,



**Fig. 5.** 2-APB inhibits ACh-evoked  $\text{Ca}^{2+}$  signalling. (Ai) Pseudo colour (green) images of  $\text{Ca}^{2+}$  signals evoked by ACh (100 nM) and ACh (100 nM) with 2-APB (100  $\mu\text{M}$ ). Scale bar: 20  $\mu\text{M}$ . (Aii) Overlaid  $\text{Ca}^{2+}$  signalling traces of ACh (100 nM; left) and ACh (100 nM) with 2-APB (100  $\mu\text{M}$ ; right). Individual  $\text{Ca}^{2+}$  traces are coloured according to the magnitude of the control (ACh) response. (B) Density plot of mean peak value of  $\text{Ca}^{2+}$  signalling. Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values. (C) Summary data of the mean peak value of  $\text{Ca}^{2+}$  signalling (E) percentage of active cells and (D) number of peaks of  $\text{Ca}^{2+}$  signalling. For all summary data (D–E),  $n = 5$ ,  $* p < 0.05$ .

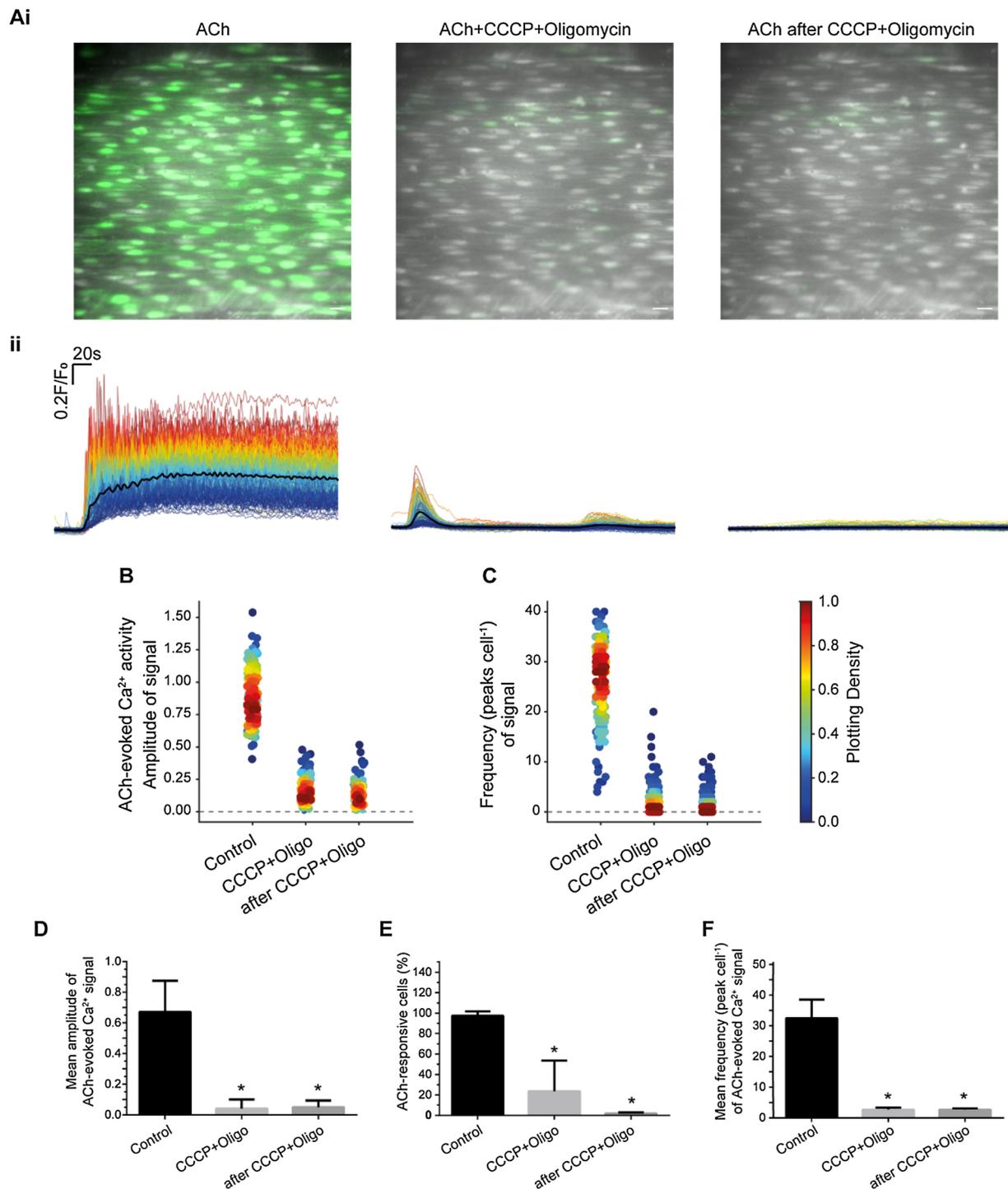


**Fig. 6.**  $\text{H}_2\text{O}_2$  suppresses  $\text{IP}_3$  receptor activity. (A) Representative image showing endothelial cells of an intact artery. The red circle marks the area that was selected for photolysis of caged  $\text{IP}_3$ . Scale bar: 20  $\mu\text{M}$ . (B) Heat map of  $\text{Ca}^{2+}$  signalling stimulated by photolysis of caged  $\text{IP}_3$  in the pre-selected area. (C) Uncaging  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  signalling traces of two representative single cells (cell 1 and cell 2 from B) before and after 20 min incubation of  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ). As with ACh there was heterogeneity in the responses of cells to  $\text{H}_2\text{O}_2$ . The peak value of the  $\text{Ca}^{2+}$  signal was either unaltered (cell 2) or suppressed (cell 1) by  $\text{H}_2\text{O}_2$ . (D) Summary data showing the  $\text{Ca}^{2+}$  signal peak after uncaging. Control: blue;  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ): red ( $n = 5$ ). (E) Averaged peak value of  $\text{Ca}^{2+}$  signals from five different experiments. Control: blue;  $\text{H}_2\text{O}_2$  treated: red, ( $n = 5$ ;  $*p < 0.05$ ).

CCCP, and the complex I inhibitor, rotenone, were used in separate experiments. Each drug was used in combination with the ATP synthase inhibitor oligomycin, to prevent reversal of the ATP synthase. CCCP (5  $\mu\text{M}$ ) and oligomycin (6  $\mu\text{M}$ ) inhibited ACh-evoked  $\text{Ca}^{2+}$  signalling (Fig. 7A, B & Figure S2); the inhibition remained even after CCCP and oligomycin wash out (Fig. 7A, B). CCCP and oligomycin significantly reduced the amplitude and frequency of ACh-evoked  $\text{Ca}^{2+}$  signals, and the percentage of cells activated by ACh (Fig. 7C–F). Similarly, rotenone (2  $\mu\text{M}$ ) and oligomycin (6  $\mu\text{M}$ ) also inhibited ACh-evoked  $\text{Ca}^{2+}$  signalling (Fig. 8A–F). These results demonstrate that mitochondrial regulate  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release and that mitochondrial membrane potential depolarization inhibits  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release.

To explore the role  $\text{H}_2\text{O}_2$  plays in mitochondria-regulated  $\text{Ca}^{2+}$

signaling, mitochondrial membrane potential was assessed using TMRE. TMRE is a lipophilic cation that is rapidly sequestered by the negatively-charged ( $\sim -180$  mV) mitochondrial membrane potential [21]. TMRE was imaged for 30 min to ensure the stability of the indicator. After 30 min,  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) was introduced and TMRE imaged for a further 30 min.  $\text{H}_2\text{O}_2$  caused a significant decrease in TMRE fluorescence intensity (Fig. 9A, B). These findings suggest that  $\text{H}_2\text{O}_2$  may suppress  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release by depolarizing mitochondria. In a control experiments, to confirm mitochondrial localization, TMRE (60 nM) was loaded together with mitotracker green (100 nM). The two mitochondrial indicators largely overlapped in their localization (Figure S3). As expected from a mitochondrial localization of the dyes, mitochondrial membrane potential depolarization with CCCP (5  $\mu\text{M}$ ;



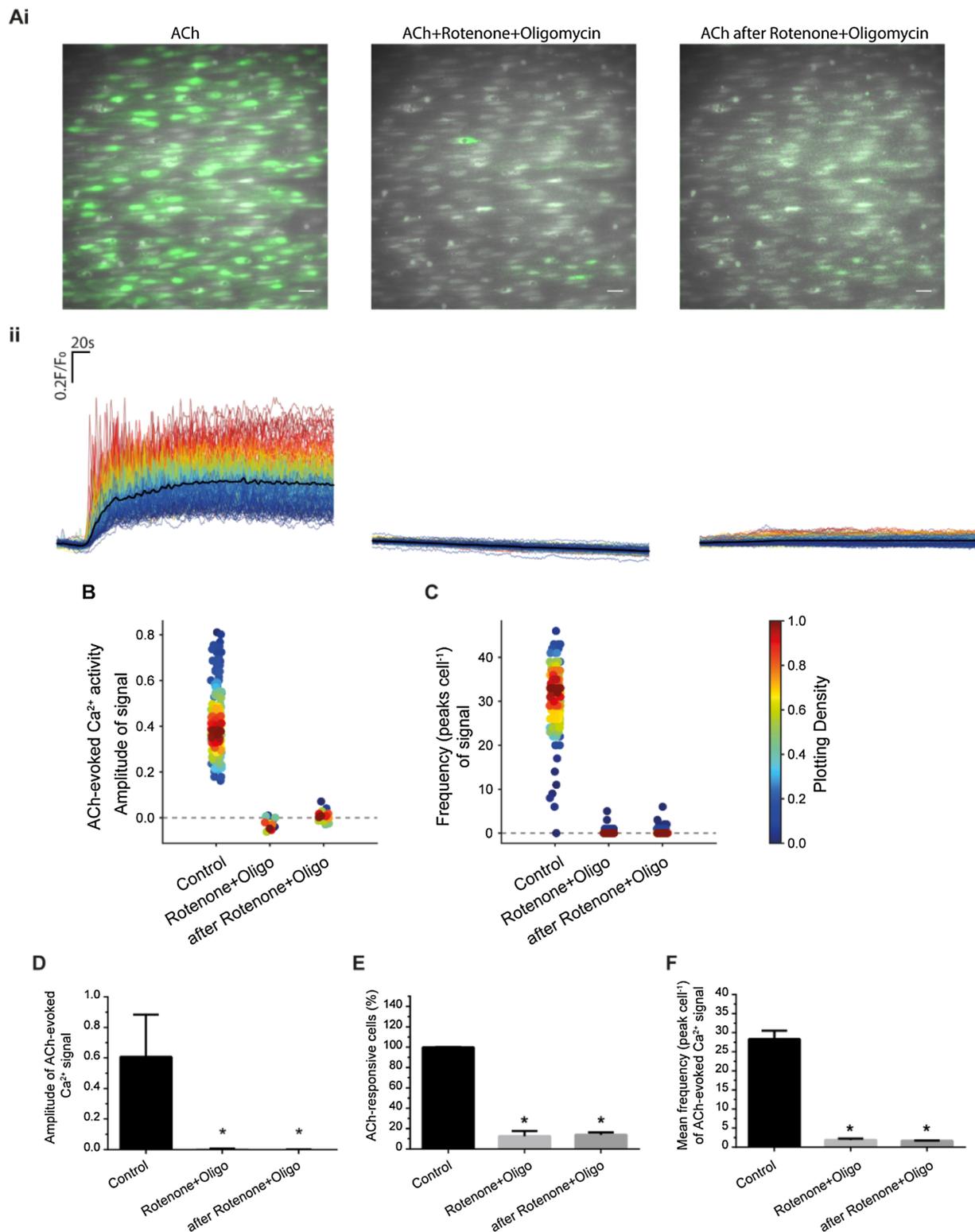
**Fig. 7.** The uncoupler, CCCP, and ATP synthase blocker, oligomycin, inhibited ACh-evoked Ca<sup>2+</sup> signals. (Ai) Pseudo colour (green) images of Ca<sup>2+</sup> activity evoked by ACh (100 nM; left), ACh (100 nM) + CCCP (5  $\mu$ M) + oligomycin (6  $\mu$ M; middle), and after washout of the CCCP and oligomycin (right). Scale bar: 20  $\mu$ M. (Aii) Overlaid single cell Ca<sup>2+</sup> traces from the endothelial cells shown in Aii. Individual Ca<sup>2+</sup> traces are coloured according to the magnitude of the control (ACh) response. (B) Density plot of mean peak value of the Ca<sup>2+</sup> signal. Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values (C) Density plot of the frequency of Ca<sup>2+</sup> signals. (D) Summary of the mean peak value of Ca<sup>2+</sup> signals in all cells, (E) Percentage of active cells (F) and the frequency of signals in all cells. For all summary data (D–F),  $n = 3$ ,  $*p < 0.05$ .

applied with oligomycin (6  $\mu$ M)) dispersed punctuate TMRE staining and reduced mitotracker green labelling (Figure S3).

#### 4. Discussion

Interaction between the internal Ca<sup>2+</sup> store and mitochondrial are critical in regulating cell signalling and cell performance. Several

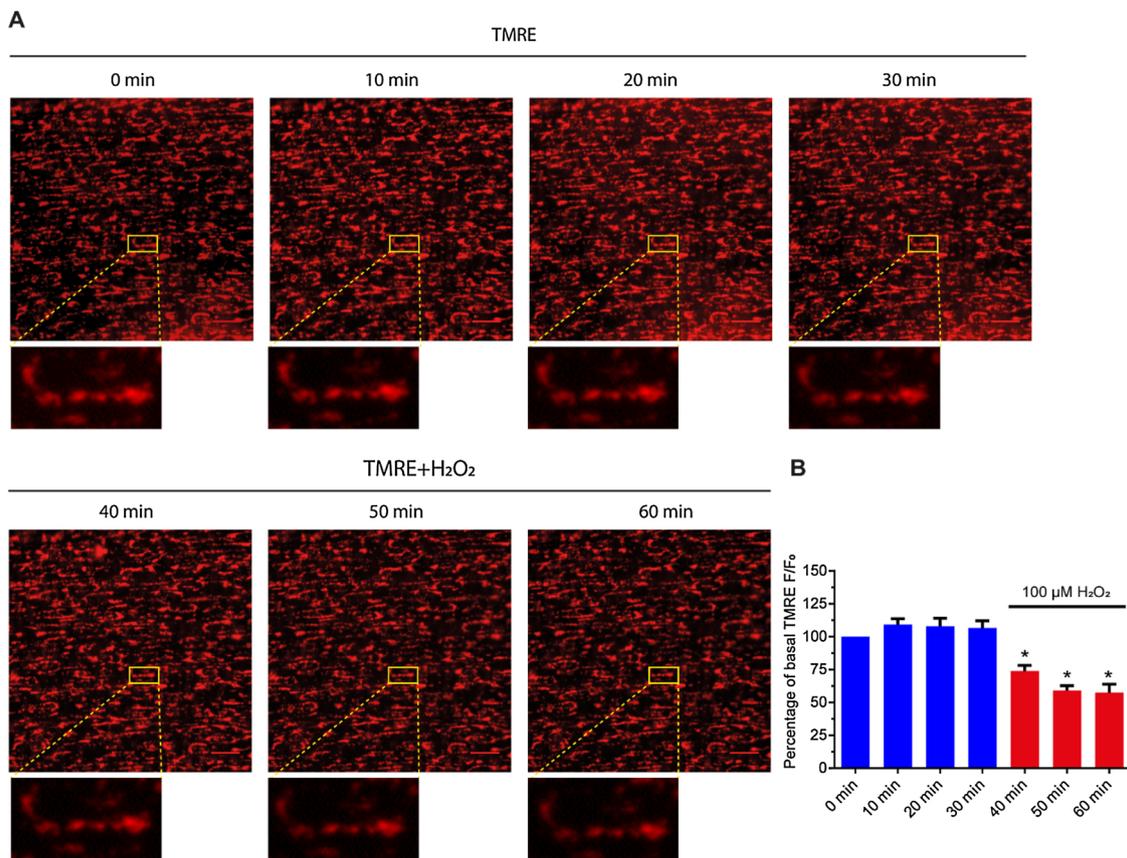
diffusible mediators communicate between the two organelles to control cell and tissue function. Of these, Ca<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> are of particular significance. Mitochondria are a major source, and the internal Ca<sup>2+</sup> store a target, for H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> modulates Ca<sup>2+</sup> transport mechanisms on the internal Ca<sup>2+</sup> store [5,12,24,60] and in turn, Ca<sup>2+</sup> release from the store modulates mitochondrial function by regulating the enzymes of the Krebs cycle and oxidative phosphorylation [41]. Ca<sup>2+</sup>-induced



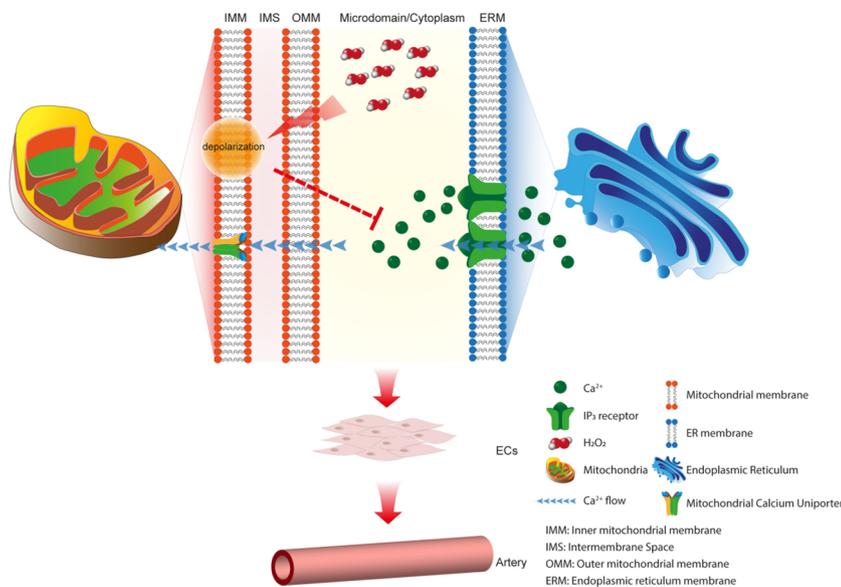
**Fig. 8.** Inhibition of complex I blocked ACh-evoked Ca<sup>2+</sup> signalling. (Ai) Pseudo colour (green) images of Ca<sup>2+</sup> signals evoked by ACh (100 nM), ACh (100 nM) + Rotenone (2 μM) + oligomycin (6 μM) and after washout of rotenone and oligomycin. Scale bar: 20 μM. (Aii) Overlaid Ca<sup>2+</sup> signalling traces, from the cells shown in Ai, evoked by ACh (100 nM), ACh (100 nM) + Rotenone (2 μM) + oligomycin (6 μM) and after washout of rotenone and oligomycin. (B) Density plot of mean peak value of Ca<sup>2+</sup> signalling. Individual data points have been coloured (from blue, low to red, high) according to the density (i.e. occurrence) of particular values (C) Density plot of number of peaks of Ca<sup>2+</sup> signalling. (D) Summary of the mean peak value of Ca<sup>2+</sup> signals in all cells, (E) Percentage of active cells (F) and the frequency of signals in all cells. For all summary data (D–F), n = 3, \*p < 0.05.

changes in metabolic rate result in altered oxygen consumption, respiratory chain electron leakage and H<sub>2</sub>O<sub>2</sub> levels [14]. Here, we have demonstrated H<sub>2</sub>O<sub>2</sub> depolarises mitochondria and inhibits spontaneous

and agonist-evoked IP<sub>3</sub>-induced Ca<sup>2+</sup> signals. We suggest that suppression of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release arises from a H<sub>2</sub>O<sub>2</sub>-induced decrease in mitochondrial membrane potential (Fig. 10).



**Fig. 9.** Depolarisation of mitochondria by H<sub>2</sub>O<sub>2</sub>. (A) Pseudo colour images (red) of the mitochondrial membrane potential (reported by the membrane potential sensitive dye TMRE). In control (0 min ~ 30 min) and after H<sub>2</sub>O<sub>2</sub> (100 μM; 30 min–60 min). H<sub>2</sub>O<sub>2</sub> decrease the mitochondrial membrane potential as revealed by the decrease in mitochondrial TMRE fluorescence intensity. Scale bar: 20 μm. (B) Quantification of the normalized intensity of TMRE (n = 6). \*p < 0.05.



**Fig. 10.** Proposed mechanism for mitochondria regulation Ca<sup>2+</sup> signalling in endothelial cells. The mitochondrial membrane potential is depolarized by H<sub>2</sub>O<sub>2</sub>. The depolarized membrane potential limits the driving force for Ca<sup>2+</sup> uptake by mitochondria. As result, the concentration of Ca<sup>2+</sup> in microdomain area between mitochondria and endoplasmic reticulum is increased and the ion suppresses Ca<sup>2+</sup> release through IP<sub>3</sub> receptor from the endoplasmic reticulum. Limited Ca<sup>2+</sup> endothelial and artery function. On the other hand, reduced uptake of Ca<sup>2+</sup> into mitochondria will lead to less production of ATP, therefore, reduced the metabolic production of H<sub>2</sub>O<sub>2</sub>. The negative feedback loop may help endothelial cells maintain normal metabolism and cell survival.

Mitochondria are potent modulators of IP<sub>3</sub>-evoked Ca<sup>2+</sup> release. Ca<sup>2+</sup> uptake by mitochondria may promote Ca<sup>2+</sup> release from IP<sub>3</sub>Rs [18,19,23,43,54,65,72,73,78], limit IP<sub>3</sub>-evoked Ca<sup>2+</sup> signals [4,34] or slow IP<sub>3</sub>-evoked Ca<sup>2+</sup> wave progression [10,15,32,61,69,84]. At least two mechanisms have been proposed to account for mitochondrial control of IP<sub>3</sub>R activity. First, at sites of close contact between the internal Ca<sup>2+</sup> store and mitochondria [26,48], channels on the internal Ca<sup>2+</sup> store and mitochondrial channels (e.g. the uniporter and voltage-

dependent anion-selective channel) may cluster, and Ca<sup>2+</sup> uptake into mitochondria occurs at these sites [25,33,63,64]. Ca<sup>2+</sup> uptake depends critically on the mitochondrial membrane potential. As the membrane potential decreases, so does mitochondrial Ca<sup>2+</sup> uptake. Mitochondrial Ca<sup>2+</sup> uptake limits a negative feedback process that operates at IP<sub>3</sub> receptors to maintain Ca<sup>2+</sup> release [19,54]. In smooth muscle, mitochondrial Ca<sup>2+</sup> uptake is fast enough to regulate local spontaneous Ca<sup>2+</sup> signals arising from IP<sub>3</sub>Rs (Ca<sup>2+</sup> puffs) [57] and regulates store-

operated  $\text{Ca}^{2+}$  entry [59], demonstrating tight functional coupling between  $\text{IP}_3\text{Rs}$  and mitochondria. However, in other studies, close coupling between the internal store and mitochondria was not required for mitochondrial control of  $\text{Ca}^{2+}$  release to occur [80]. In the second mechanism proposed to account for mitochondrial control of the internal store, mitochondrial ATP production modulates  $\text{Ca}^{2+}$  release. When ATP production was restricted  $\text{Ca}^{2+}$  release was inhibited. This mechanism permits mitochondria to control  $\text{Ca}^{2+}$  release while being positioned far from the internal  $\text{Ca}^{2+}$  store [80]. Several studies show that ATP maintains  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release. ATP potentiates  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release in permeabilized cells and from native endoplasmic reticulum vesicles, and it enhances activation of  $\text{IP}_3$ -gated channels and purified, reconstituted  $\text{IP}_3\text{Rs}$  [28,39,50,66] by increasing the open time of the channel s[7]. Thus factors provided by mitochondria may diffuse to  $\text{IP}_3\text{R}$  to maintain  $\text{IP}_3\text{R}$  activity.

The present results highlight another control mechanism which may operate between mitochondria and the internal  $\text{Ca}^{2+}$  store i.e. diffusion of  $\text{H}_2\text{O}_2$ . Our results suggest that the control of  $\text{IP}_3$  release by  $\text{H}_2\text{O}_2$  is indirect and mediated by depolarization of mitochondria so combines aspects from both proposals (diffusible substance and mitochondrial depolarization).  $\text{H}_2\text{O}_2$ -mediated depolarization of the mitochondrial membrane potential will decrease the driving force for  $\text{Ca}^{2+}$  uptake by mitochondria and so limit negative feedback control of  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release. Several proposals exist for the mechanisms by which  $\text{H}_2\text{O}_2$  may depolarize mitochondria [30,31] and include inhibition of alpha-ketoglutarate dehydrogenase [22,56] or succinate dehydrogenase [56], or activation of the permeability transition pore [83]. Interestingly, the effect of  $\text{H}_2\text{O}_2$  was not homogeneous across all cells and some were affected more than others. The reason for the heterogeneity is not clear, but perhaps differences in basal levels of  $\text{H}_2\text{O}_2$ , or metabolic state of the cells may contribute. Alternatively, antioxidant enzymes whose activities are directed at reducing hydrogen peroxide, such as catalase, glutathione peroxidase, and thioredoxin peroxidase, may vary across cells.

While the present results suggest that  $\text{H}_2\text{O}_2$  inhibition of  $\text{IP}_3\text{R}$  is indirect and mediated via mitochondria, ROS may also directly alter  $\text{IP}_3$  receptor activity. However,  $\text{H}_2\text{O}_2$  is often reported to promote rather than inhibit  $\text{IP}_3\text{R}$  activity. When  $\text{H}_2\text{O}_2$  transients were prevented,  $\text{Ca}^{2+}$  oscillations were inhibited in some cells, implying that  $\text{H}_2\text{O}_2$  makes subsequent  $\text{Ca}^{2+}$  release more likely to occur [11]. In support of these observations, in various cultured endothelial cell lines (HAECs, LECs, HUVECs and CVECs),  $\text{H}_2\text{O}_2$  induces  $\text{Ca}^{2+}$  release from the internal store [27,37,77]. Various exogenously added oxidants stimulate rather than inhibit  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  release. Thimerosal [12,16,42], t-butylhydroperoxide [9], and diamide [45,46] each increase  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release. In the case of thimerosal, the proposed mechanism involves an increased sensitivity of the receptor to  $[\text{IP}_3]$  that results in  $\text{Ca}^{2+}$  oscillations occurring at basal  $[\text{IP}_3]$  in unstimulated cells [5,40]. Although sensitization to  $\text{IP}_3$  may be a general mechanism responsible for the action of other oxidants, it has also been suggested that they (oxidants) may alter the sensitivity of  $\text{IP}_3\text{R}$  to  $\text{Ca}^{2+}$  [9,45]. Our results suggest that the indirect effect of  $\text{H}_2\text{O}_2$  on endothelial mitochondria may dominate control of  $\text{IP}_3\text{R}$ . Since  $\text{H}_2\text{O}_2$  may increase  $\text{IP}_3\text{R}$  activity, these findings buttress the proposal that inhibition of  $\text{Ca}^{2+}$  release by  $\text{H}_2\text{O}_2$  is indirect.

$\text{H}_2\text{O}_2$  regulates several key modulators of cell activity including cell proliferation, migration, and differentiation, and because  $\text{H}_2\text{O}_2$  is membrane permeable, the ROS may exert widespread control across many endothelial cells to regulate cardiovascular function.  $\text{H}_2\text{O}_2$  mediates at least part of its effects through changes in  $\text{Ca}^{2+}$  signalling.  $\text{Ca}^{2+}$  influx in native and in cultured endothelial cell lines is evoked by  $\text{H}_2\text{O}_2$ . In the native endothelium of superior epigastric arteries,  $\text{H}_2\text{O}_2$  evoked  $\text{Ca}^{2+}$  increases result from  $\text{Ca}^{2+}$  influx via TRPV4 channels [67]. In mouse and human lung microvascular endothelial cell lines (MLMVEC, HPAE, H5V and HLMVEC)  $\text{H}_2\text{O}_2$  evokes  $\text{Ca}^{2+}$  influx via TRPV4 or TRPM2 [70,71]. Our results reveal an additional complexity in the

effects of  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  may inhibit  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release in native endothelial cells. The inhibition of  $\text{IP}_3$  evoked  $\text{Ca}^{2+}$  by  $\text{H}_2\text{O}_2$  may be an indirect and mediated via depolarization of the mitochondrial membrane potential. This process may serve as a negative feedback modulation of mitochondrial function.  $\text{Ca}^{2+}$  increases associated with cell activity may initially stimulate mitochondrial ATP production. However, an increase in electron transport activity will result in increased ROS production, with a decrease in mitochondrial membrane potential and inhibition of  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  release as a result.  $\text{H}_2\text{O}_2$  mediated mitochondrial depolarization may be a mechanism by which the organelles inhibit  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  signalling to protect themselves against  $\text{Ca}^{2+}$  overload.

#### Author contributions

XZ, MDL, CW & JGM developed the concept. XZ performed the experiments. JGM & XZ drafted the manuscript. JGM, ZX, CW & MDL edited and revised the manuscript. CW & JGM sourced funding. All authors approved the final version of the manuscript.

#### Declaration of Competing Interest

None.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ceca.2019.102108>.

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