Effect of Hydrogen Peroxide and Superoxide Anions on Cytosolic Ca\(^{2+}\): Comparison of Endothelial Cells from Large-Sized and Small-Sized Arteries

Lei Sun, Ho-Yan Yau, On-Chai Lau, Yu Huang, Xiaoqiang Yao

School of Biomedical Sciences and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, China

Abstract

We compared the Ca\(^{2+}\) responses to reactive oxygen species (ROS) between mouse endothelial cells derived from large-sized arteries, aortas (aortic ECs), and small-sized arteries, mesenteric arteries (MAECs). Application of hydrogen peroxide (H\(_2\)O\(_2\)) caused an increase in cytosolic Ca\(^{2+}\) levels ([Ca\(^{2+}\)]) in both cell types. The [Ca\(^{2+}\)], rises diminished in the presence of U73122, a phospholipase C inhibitor, or Xestospongin C (XeC), an inhibitor for inositol-1,4,5-trisphosphate (IP\(_3\)) receptors. Removal of Ca\(^{2+}\) from the bath also decreased the [Ca\(^{2+}\)], rises in response to H\(_2\)O\(_2\). In addition, treatment of endothelial cells with H\(_2\)O\(_2\) reduced the [Ca\(^{2+}\)], responses to subsequent challenge of ATP. The decreased [Ca\(^{2+}\)], responses to ATP were resulted from a pre-depletion of intracellular Ca\(^{2+}\) stores by H\(_2\)O\(_2\). Interestingly, we also found that Ca\(^{2+}\) store depletion was more sensitive to H\(_2\)O\(_2\) treatment in endothelial cells of mesenteric arteries than those of aortas. Hypoxanthine-xanthine oxidase (HX-XO) was also found to induce [Ca\(^{2+}\)], rises in both types of endothelial cells, the effect of which was mediated by superoxide anions and H\(_2\)O\(_2\) but not by hydroxyl radical. H\(_2\)O\(_2\) contribution in HX-XO-induced [Ca\(^{2+}\)], rises were more significant in endothelial cells from mesenteric arteries than those from aortas. In summary, H\(_2\)O\(_2\) could induce store Ca\(^{2+}\) release via phospholipase C-IP\(_3\) pathway in endothelial cells. Resultant emptying of intracellular Ca\(^{2+}\) stores contributed to the reduced [Ca\(^{2+}\)], responses to subsequent ATP challenge. The [Ca\(^{2+}\)], responses were more sensitive to H\(_2\)O\(_2\) in endothelial cells of small-sized arteries than those of large-sized arteries.

Introduction

Vascular endothelial cells in vivo are constantly exposed to ROS that are released from neutrophils, macrophages, and vascular smooth muscle cells [1,2]. Moreover, endothelial cells themselves are generators of ROS [1,2]. The main ROS that are produced include superoxide anions, H\(_2\)O\(_2\), hydroxyl radicals and peroxynitrite. Functionally, ROS play a key role in physiological and pathological processes in endothelial cells. For example, H\(_2\)O\(_2\) at physiological concentration serves as an endothelium-derived hyperpolarizing factor (EDHF), mediating vascular relaxation [3]. However, excessive production of ROS causes extensive damage to the structure and function of endothelial cells, leading to endothelial dysfunction [1]. Evidence indicates that ROS-induced endothelial function and dysfunction are often preceded by an alteration in endothelial Ca\(^{2+}\) responses, which serves as an important second messenger to induce diverse responses.

Reports showed that superoxide anions [5], H\(_2\)O\(_2\) [6–9], and hydroxyl radical [5,10] are all capable of inducing [Ca\(^{2+}\)], rises in vascular endothelial cells. The [Ca\(^{2+}\)], rises could result from ROS actions on the plasma membrane ion channels [11], IP\(_3\) production [7,12], IP\(_3\) receptors [13,14], and/or endoplasmic reticulum Ca\(^{2+}\)-ATPase [6]. In addition to their direct action on endothelial Ca\(^{2+}\), ROS treatment may alter the [Ca\(^{2+}\)], responses of endothelial cells to a variety of physiological agonists including ATP and bradykinin [7,9,12]. However, the results of these studies are often controversial. In some studies, ROS treatment was found to enhance the agonist-induced [Ca\(^{2+}\)], rises [12], whereas in other studies ROS were found to attenuate [9,15] or have no effect [7] on the agonist-induced [Ca\(^{2+}\)], responses.

Although there have been a great number of studies investigating the ROS effect on [Ca\(^{2+}\)], in endothelial cells, most of these reports only investigated the endothelial cells derived from large-sized arteries [5–10,12,15]. The role of ROS on [Ca\(^{2+}\)], in endothelial cells of small-sized arteries has received little attention [but see 8]. It is unclear whether there is any difference in ROS-induced [Ca\(^{2+}\)], responses in endothelial cells from different-sized arteries. Large-sized arteries and small-sized arteries differ in their function. Small-sized arteries such as mesenteric arteries are resistance arteries that play a key role in blood pressure control. Vasoactive factors in small-sized arteries are often different from that in large-sized arteries. For example, while nitric oxide is the major vasodilator in large arteries, EDHFs often play a more important role as vasodilators in small-sized arteries [16].

In the present study, we compared the effect of H\(_2\)O\(_2\) on [Ca\(^{2+}\)], in endothelial cells from large-sized arteries, aortas (aortic ECs), and small-sized arteries, mesenteric arteries (MAECs). We found that H\(_2\)O\(_2\) stimulated IP\(_3\) production to induce store Ca\(^{2+}\) release
in both cell types. H\textsubscript{2}O\textsubscript{2} treatment depleted intracellular [Ca\textsuperscript{2+}]i stores, resulted in a decreased [Ca\textsuperscript{2+}], response to subsequent ATP challenge. The Ca\textsuperscript{2+} store depletion was more sensitive to H\textsubscript{2}O\textsubscript{2} in endothelial cells of small-sized arteries than those of large-sized arteries.

**Results**

**Both Ca\textsuperscript{2+} entry and store Ca\textsuperscript{2+} release contributed to H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], rises**

The effect of H\textsubscript{2}O\textsubscript{2} on [Ca\textsuperscript{2+}], was investigated in aortic ECs and MAECs. H\textsubscript{2}O\textsubscript{2} at 5 mM caused marked [Ca\textsuperscript{2+}], rises in both types of cells that were bathed in normal physiological saline solution (N-PSS) containing 1 mM Ca\textsuperscript{2+} (Figure 1A–1D). The amplitude of [Ca\textsuperscript{2+}], rises to H\textsubscript{2}O\textsubscript{2} was reduced to 0.5 mM or to nominal Ca\textsuperscript{2+}-free (0Ca\textsuperscript{2+}-PSS), suggesting a contribution of Ca\textsuperscript{2+} entry to the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], rises. Significant [Ca\textsuperscript{2+}]i rises to H\textsubscript{2}O\textsubscript{2} could still be observed even when bath was Ca\textsuperscript{2+}-free, suggesting that store Ca\textsuperscript{2+} release also contributed to the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], rises.

H\textsubscript{2}O\textsubscript{2} enhanced IP\textsubscript{3} production and store Ca\textsuperscript{2+} release

It is well documented that IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores are the major intracellular Ca\textsuperscript{2+} stores, and that the Ca\textsuperscript{2+} release from the stores hinges on the production on IP\textsubscript{3}, which is generated through activity of phospholipase C (PLC) [17]. Figure 2A–2D show that treatment of the cells with XeC, an IP\textsubscript{3} receptor inhibitor, at 10 \textmu M for 20 min almost abolished the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], rises in both aortic ECs and MAECs. Furthermore, a PLC inhibitor U73122 (10 \textmu M) markedly reduced the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], rises, whereas its inactive analog U73343 (10 \textmu M) had no effect (Figure 3A–3D). These results suggest that the action of H\textsubscript{2}O\textsubscript{2} mediated through IP\textsubscript{3}, which binds to IP\textsubscript{3} receptors to release Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores. This was confirmed by experiments that measures IP\textsubscript{3} production (Figure 4). Treatment of cells with H\textsubscript{2}O\textsubscript{2} caused a H\textsubscript{2}O\textsubscript{2} concentration-dependent increase in IP\textsubscript{3} levels in both types of endothelial cells (Figure 4).

H\textsubscript{2}O\textsubscript{2} reduced the [Ca\textsuperscript{2+}], responses to ATP in a H\textsubscript{2}O\textsubscript{2} concentration and incubation time dependent manner

We next examined the effect of H\textsubscript{2}O\textsubscript{2} treatment on agonist (ATP)-induced [Ca\textsuperscript{2+}], rises. The cells were first pre-incubated with H\textsubscript{2}O\textsubscript{2} (500 \textmu M or 1 mM) for 30 min, followed by 30 \textmu M ATP application to evoke [Ca\textsuperscript{2+}], responses. Figure 5A and 5B show the representative traces of [Ca\textsuperscript{2+}], responses to ATP in aortic ECs and MAECs that were pre-incubated with different concentrations of H\textsubscript{2}O\textsubscript{2}. A marked difference was found between aortic ECs and MAECs. While both cells lost the [Ca\textsuperscript{2+}], responses to ATP after 1 mM H\textsubscript{2}O\textsubscript{2} treatment, a relatively low concentration of 500 \textmu M H\textsubscript{2}O\textsubscript{2} could abolish the ATP responses in MAECs but had no effect in aortic ECs (Figure 5A–5D). To further confirm the difference between aortic ECs and MAECs, time series experiments were carried out. 500 \textmu M H\textsubscript{2}O\textsubscript{2} caused a time dependent decrease in the [Ca\textsuperscript{2+}], responses to ATP in MAECs (Figure 5F) but not in aortic ECs (Figure 5E).

![Figure 1. Effect of extracellular Ca\textsuperscript{2+} on H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}]i rises in aortic ECs and MAECs.](image-url)
H$_2$O$_2$ induced Ca$^{2+}$ store depletion

The reduced [Ca$^{2+}$]$_i$ responses to ATP could result from a decreased Ca$^{2+}$ entry or a reduced Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. To focus on the store Ca$^{2+}$ release alone, we next studied the ATP (30 μM)-induced [Ca$^{2+}$]$_i$ rises in cells bathed in a nominal Ca$^{2+}$-free solution (Figure 6). Under this condition, [Ca$^{2+}$]$_i$ rises could only be attributed to the store Ca$^{2+}$ release. The results show that ATP still triggered large [Ca$^{2+}$]$_i$ responses, which could be abolished by pre-treating aortic ECs for 25–30 min with 1 mM H$_2$O$_2$ but not 500 μM H$_2$O$_2$ (Figure 6A and 6C). For MAECs, treatment with a lower concentration (500 μM, 26–30 min) was enough to abolish the [Ca$^{2+}$]$_i$ responses to ATP (Figure 6B and 6D).

To further confirm the findings, Mag-fluo4/AM, a dye that stains Ca$^{2+}$ in intracellular Ca$^{2+}$ stores, was used to directly measure the store Ca$^{2+}$ content. As shown in Figure 6E–6F, treatment with 500 μM H$_2$O$_2$ for 26–30 min caused a marked reduction of store Ca$^{2+}$ content by 33±6% (n = 3) in MAECs but had no significant effect in aortic ECs. These data suggest that MAECs were more sensitive to H$_2$O$_2$ treatment than aortic ECs with regard to their responses in Ca$^{2+}$ store depletion and ATP-induced [Ca$^{2+}$]$_i$ rises. The controls in Figure 6E and 6F were time controls, in which the cells went through 30 min incubation in the absence of H$_2$O$_2$. In time control, Mag-fluo4 fluorescence only decreased by 8±6% (n = 3) in MAECs and by 3±7% (n = 3) in aortic ECs. The small reduction in Mag-fluo4 fluorescence in the control experiments could be due to light-sensitive quenching of Mag-fluo4 as described elsewhere [18].

[Ca$^{2+}$]$_i$ responses to ATP in the absence of H$_2$O$_2$

We also compared ATP-induced Ca$^{2+}$ store release in aortic ECs and MAECs in the absence of H$_2$O$_2$ pretreatment. Cells bathed in a nominal Ca$^{2+}$-free solution were challenged with different concentrations of ATP. In both cell types, ATP evoked [Ca$^{2+}$]$_i$ rises in a concentration dependent manner (Figure 7). Furthermore, the [Ca$^{2+}$]$_i$ response in MAECs was more sensitive to ATP than that in aortic ECs (Figure 7).

Non-involvement of hydroxyl radical

The effect of H$_2$O$_2$ on [Ca$^{2+}$]$_i$ could result from the action of H$_2$O$_2$ itself or from its metabolic product hydroxyl radical. Catalase was used to remove H$_2$O$_2$ and DMSO was used to scavenge hydroxyl radical. Pretreatment of cells with 2000 U/ml catalase for 30 min abolished the H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ rises in both types of endothelial cells, whereas 2% DMSO had no effect (Figure 8A–8D). Our data suggest hydroxyl radical was not involved in the H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ rises in both types of endothelial cells.

HX-XO-induced [Ca$^{2+}$]$_i$ rises were caused by superoxide anion and hydrogen peroxide

Effect of HX-XO on [Ca$^{2+}$]$_i$ was also studied. HX-XO reacts to yield superoxide anions, which may spontaneously or enzymatically dismutate into H$_2$O$_2$ [4]. Application of HX-XO (200 μM and 20 nU/ml, respectively) evoked rapid [Ca$^{2+}$]$_i$ rises in both types of endothelial cells. Pre-incubation of the cells for 20 min

Figure 2. Effect of XeC on H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ rises in aortic ECs and MAECs. A and B. Representative traces showing the [Ca$^{2+}$]$_i$ rises in response to 5 mM H$_2$O$_2$, with or without XeC. The cells were pre-treated with or without 10 μM XeC for 20 min in N-PSS before H$_2$O$_2$ challenge. Fluorescence intensity before H$_2$O$_2$ application was normalized to 1 as F0. C and D. Summary of data showing the effect of XeC (C and D) on H$_2$O$_2$-induced maximal [Ca$^{2+}$]$_i$ rises in aortic ECs (C) and MAECs (D) as expressed in F1/F0. Mean ± SEM of 3 to 9 independent experiments (10 to 15 cells per experiment). *, P<0.05 as compared to control. doi:10.1371/journal.pone.0025432.g002
with 250 U/ml superoxide dismutase (SOD), an enzyme that causes superoxide dismutation, reduced the \([\text{Ca}^{2+}]_i\) rises (Figure 9A–9D). Pretreatment with catalase (2000 U/ml, 30 min) also reduced the HX-XO-induced \([\text{Ca}^{2+}]_i\) rises (Figure 9A–9D). Catalase had a larger effect on the HX-XO-induced \([\text{Ca}^{2+}]_i\) responses in MAECs (reduction by 71 ± 60%, \(n = 13\)) than in aortic ECs (reduction by 47 ± 60%, \(n = 10\)) (Figure 9C and 9D). Combined treatment of SOD and catalase almost completely abolished the HX-XO-induced \([\text{Ca}^{2+}]_i\) rises in both types of endothelial cells (Figure 9A–9D).

**Discussion**

\([\text{Ca}^{2+}]_i\) change is an important early signal for ROS-induced endothelial function and dysfunction. However, only a few studies have investigated ROS-induced \([\text{Ca}^{2+}]_i\) signaling in the endothelial cells derived from small-sized arteries \([8,19]\) and it is unclear whether there is any difference in ROS-induced \([\text{Ca}^{2+}]_i\) responses in endothelial cells from different-sized arteries. In the present study, we compared the effect of \(\text{H}_2\text{O}_2\) on \([\text{Ca}^{2+}]_i\) in endothelial cells from large-sized arteries and small-sized arteries. The results show that \(\text{H}_2\text{O}_2\) stimulated \([\text{Ca}^{2+}]_i\) rises in both cell types. The \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) responses in MAECs (reduction by 71 ± 0%, \(n = 13\)) than in aortic ECs (reduction by 47 ± 0%, \(n = 10\)) (Figure 9C and 9D). Combined treatment of SOD and catalase almost completely abolished the HX-XO-induced \([\text{Ca}^{2+}]_i\) rises in both types of endothelial cells (Figure 9A–9D).
induced [Ca^{2+}]_{i} rises in response to 30 μM ATP. The cells were pre-treated with or without H_{2}O_{2} (500 μM or 1 mM as indicated) in N-PSS for 30 min, followed by ATP challenge. Control had no H_{2}O_{2} treatment. Fluorescence intensity before ATP application was normalized to 1 as F0. Figure 5. Effect of H_{2}O_{2} pre-treatment on ATP-induced [Ca^{2+}]_{i} rises in aortic ECs and MAECs. A and B. Representative traces showing the [Ca^{2+}]_{i} rises in response to 30 μM ATP. The cells were pre-treated with or without H_{2}O_{2} (500 μM or 1 mM as indicated) in N-PSS for 30 min, followed by ATP challenge. Control had no H_{2}O_{2} treatment. Fluorescence intensity before ATP application was normalized to 1 as F0. C and D. Summary of data showing the ATP-induced maximal [Ca^{2+}]_{i} rises as in A and B, expressed in F1/F0. E and F. Summary of data showing the ATP-induced maximal [Ca^{2+}]_{i} rises after the cells were treated with 500 μM H_{2}O_{2} for different period of time in N-PSS. Mean±SEM of 3 to 7 independent experiments (10 to 15 cells per experiment). *, P<0.05 as compared to control. doi:10.1371/journal.pone.0025432.g005

Figure 5. Effect of H_{2}O_{2} on Aortic ECs and MAECs

Effect of H_{2}O_{2} on Aortic ECs and MAECs

induced [Ca^{2+}]_{i} rises [9,15]. In other studies, ROS treatment was found to enhance [9,12] or have no effect on the agonist-induced [Ca^{2+}]_{i} responses [7]. In the present study, we found that H_{2}O_{2} treatment reduced the [Ca^{2+}]_{i} responses to ATP in H_{2}O_{2} concentration-dependent and H_{2}O_{2} incubation time-dependent manners in mouse aortic ECs and MAECs. The reduced [Ca^{2+}]_{i} responses to ATP were due to a pre-depletion of intracellular Ca^{2+} stores during H_{2}O_{2} treatment. Two lines of evidence support this: 1) After H_{2}O_{2} treatment, the store Ca^{2+} release in response to ATP became much smaller. 2) Direct measurement of store Ca^{2+} content by Mag-fluo4 demonstrated a reduction in store Ca^{2+} content after H_{2}O_{2} treatment. Interestingly, our data clearly indicate that endothelial cells from small-sized arteries (MAECs) were more sensitive to H_{2}O_{2} treatment than those of large-sized arteries (aortic ECs) with regard to their store Ca^{2+} release and subsequent [Ca^{2+}]_{i} responses to ATP. This type of differential sensitivity/response of store Ca^{2+} release to ROS treatment could explain some data conflicts in the literature. For example, Volk et al., reported that, in rat liver artery endothelial cells, ROS treatment had no effect on the [Ca^{2+}]_{i} responses to subsequent ATP or histamine challenge [7]. But they used a relatively low concentration of ROS [7]. It is possible that such a low concentration of ROS might not be sufficient to cause marked store Ca^{2+} depletion. As a result, no change in [Ca^{2+}]_{i} responses to agonists would be expected.
What could be the underlying cellular mechanism for the higher sensitivity of \([\text{Ca}^{2+}]_i\) responses to H2O2 in MAECs than in aortic ECs? H2O2-induced IP3 production was similar in MAECs and aortic ECs, therefore IP3 production was not the reason. Alternatively, this could be due to more abundant IP3 receptor expression and/or a higher IP3 receptor sensitivity to IP3 in MAECs than in aortic ECs. If this is true, \([\text{Ca}^{2+}]_i\) responses to other agonists is also expected to be higher in MAECs than in aortic ECs. Indeed, we found that similar high sensitivity of intracellular store Ca2+ release to ATP in MAECs than in aortic ECs (Figure 7). Therefore, we speculate that MAECs may express more IP3 receptors and/or the sensitivity of IP3 receptors to IP3 may be higher in MAECs than in aortic ECs.

The higher sensitivity of \([\text{Ca}^{2+}]_i\) responses to H2O2 in the endothelial cell of small-sized arteries could have physiological and/or pathological implication. At physiological concentration, H2O2 is a vasodilator and it causes endothelium-dependent and endothelium-independent vascular dilation [3,23,24]. The effect of H2O2 as a vascular dilator is often found in small-sized arteries and arterioles [3,25]. In contrast, in large-sized arteries nitric acid is a more important vascular dilator [26]. Because \([\text{Ca}^{2+}]_i\) rises endothelial cells often trigger vascular dilation, a more sensitive \([\text{Ca}^{2+}]_i\) response to H2O2 in endothelial cells would allow H2O2 to serve as a more effective vascular dilator in small-sized arteries and arterioles. On the other hand, a high \([\text{Ca}^{2+}]_i\) sensitivity to H2O2 could also have pathological consequence. Excessive Ca2+
accumulation may lead to endothelial cell apoptosis and cell death [4]. Therefore, it is possible that endothelial cells in small-sized arteries or arterioles might be more vulnerable to ROS-induced cell damage.

H$_2$O$_2$ can be converted to hydroxyl radical in the presence of Fe$^{2+}$ [4]. However, in the present study the effect of H$_2$O$_2$ on [Ca$^{2+}$]$_i$ rises in endothelial cells could not be attributed to hydroxyl radical, because the H$_2$O$_2$ effect was not affected by DMSO, which is an efficient hydroxyl radical scavenger [21]. In contrast, H$_2$O$_2$ effect was abolished by catalase, which converts H$_2$O$_2$ to O$_2$ and H$_2$O, suggesting a direct action of H$_2$O$_2$. We also investigated the effect of HX-XO on [Ca$^{2+}$]$_i$ in mouse aortic ECs and MAECs. HX-XO is one of the most widely used methods to generate superoxide anions, which may in turn dismutate into H$_2$O$_2$ spontaneously or enzymatically [4]. We found that the HX-XO-induced [Ca$^{2+}$]$_i$ rises could be attributed to involvement of superoxide anions and H$_2$O$_2$ but not hydroxyl radicals in both types of endothelial cells, because the response was reduced by SOD and catalase but not by DMSO. There were relatively more H$_2$O$_2$ contribution in HX-XO-induced [Ca$^{2+}$]$_i$ rises in endothelial cells of small-sized arteries (MAECs) than in those of large-sized arteries (aortic ECs). Previously, different reports have claimed different ROS, including H$_2$O$_2$ [5,7,10], hydroxyl radical [10], and/or superoxide anions [5,10], to be the contributing factors that were involved in HX-XO provoked-[Ca$^{2+}$]$_i$ rises in endothelial cells. The discrepancy in results could be due to a variety of factors including endothelial cell sources and/or culture conditions.

Figure 7. ATP-induced store Ca$^{2+}$ release in endothelial cells in the absence of H$_2$O$_2$ pretreatment. Shown were the maximal [Ca$^{2+}$] changes to different concentration of ATP (1 μM, 10 μM, 30 μM, 100 μM) in aortic ECs and MAECs as expressed in F1/F0. Cells were bathed in 0Ca$^{2+}$-PSS in the absence of H$_2$O$_2$ pretreatment. Mean ± SEM of 3 to 7 independent experiments (10 to 15 cells per experiment). *, P<0.05 as compared to aortic ECs. doi:10.1371/journal.pone.0025432.g007

Effect of H$_2$O$_2$ on Aortic ECs and MAECs

Figure 8. Effect of catalase and DMSO on H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ rises in aortic ECs and MAECs. A and B. Representative traces of H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ rises in the presence or absence of catalase or DMSO in N-PSS. 2000 U/ml catalase or 2% DMSO was added 30 min prior to the addition of H$_2$O$_2$ (5 mM). Fluorescence intensity before application of H$_2$O$_2$ was normalized to 1 as F0. C and D. Summary of data showing the effect of 2000 U/ml catalase and 2% DMSO treatment on H$_2$O$_2$-induced maximal [Ca$^{2+}$]$_i$ rises in aortic ECs (C) and MAECs (D) as expressed in F1/F0. Mean±SEM of 3 to 4 independent experiments (10 to 15 cells per experiment), *, P<0.05 as compared to control. doi:10.1371/journal.pone.0025432.g008
In conclusion, we found both Ca\textsuperscript{2+} entry and store Ca\textsuperscript{2+} release contributed to the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}]i rises in endothelial cells. H\textsubscript{2}O\textsubscript{2} treatment depleted the intracellular Ca\textsuperscript{2+} stores, resulting in reduced [Ca\textsuperscript{2+}]i responses to subsequent agonist challenge. The store Ca\textsuperscript{2+} release and subsequent [Ca\textsuperscript{2+}]i responses to ATP were more sensitive to H\textsubscript{2}O\textsubscript{2} treatment in endothelial cells of small-sized arteries than those of large-sized arteries. This study highlights the similarity and difference of ROS-induced [Ca\textsuperscript{2+}]i responses in endothelial cells from large-sized arteries and small-sized arteries.

**Methods**

**Ethics statement**

We followed Guide for Animal Care and Use of Laboratory Animals published by the US National Institute of Health. The protocols for animal experiments were approved by Animal Experimentation Ethics Committee, The Chinese University of Hong Kong (approval number # 09/060/MIS).

**Primary Cell Culture**

Animals were supplied by the Laboratory Animal Service Center of the Chinese University of Hong Kong (Hong Kong, China). We followed Guide for Animal Care and Use of Laboratory Animals published by the US National Institute of Health. The protocols for animal experiments were approved by Animal Experimentation Ethics Committee, The Chinese University of Hong Kong (approval number # 09/060/MIS). Male C57 mice (8–12 weeks) were sacrificed by inhalation of CO\textsubscript{2}. Primary cultured aortic endothelial cells (aortic ECs) and mesenteric artery endothelial cells (MAECs) were dissociated from mouse aorta and mesenteric arteries of the first to tertiary branches (internal diameter = 60–200 μm), respectively, using the methods described elsewhere [27]. Aortic ECs and MAECs were cultured in endothelial cell growth medium supplemented with 1% bovine brain extract.

**[Ca\textsuperscript{2+}]i Measurement**

Cells were prepared and loaded with a membrane permeant fluorescence dye Fluo4/AM (Molecular Probes, Inc., NJ) for observing their [Ca\textsuperscript{2+}]i, responses to H\textsubscript{2}O\textsubscript{2} or HX-XO or ATP. Briefly, the cells were seeded on circular glass discs at 37°C for 1 hr in dark at room temperature with 10 μM Fluo4/AM and 0.02% Pluronic acid F-127 in normal physiological saline solution (N-PSS), which contained in mM: 1 CaCl\textsubscript{2}, 140 NaCl, 1 KCl, 1 MgCl\textsubscript{2}, 10 glucose, and 5 Hepes at pH 7.4. The circular discs containing the endothelial cells were then pinned in a specially designed chamber. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot 200). During experiments, cells were bathed in N-PSS or 0.5Ca\textsuperscript{2+}-PSS or 0Ca\textsuperscript{2+}-PSS. The composition of 0.5Ca\textsuperscript{2+}-PSS and 0Ca\textsuperscript{2+}-PSS was similar to N-PSS except for Ca\textsuperscript{2+} concentration (0.5 mM CaCl\textsubscript{2} for 0.5Ca\textsuperscript{2+}-PSS, and nominal Ca\textsuperscript{2+}-free for 0Ca\textsuperscript{2+}-PSS). All agents were applied directly to the bath along the side of the chamber. Solutions were then mixed by pipetting gently up and down for a few times. Experiments were
performed at room temperature. Fluorescence signals were recorded by MRC-1000 Laser Scanning Confocal Imaging System with MRC-1000 software (Bio-Rad) with the excitation wavelength of 488 nm and a 515 nm-long pass emission filter. The $Ca^{2+}$ responses displayed as the ratio of fluorescence relative to the intensity before $H_2O_2$ or ATP or HX-XO (F1/F0). Due to variation in $[Ca^{2+}]$, responses between different batches of cells, each series of experiments had its own control.

Measuring $Ca^{2+}$ Content in Intracellular $Ca^{2+}$ Stores

Cells were loaded with fluorescence dye Mag-fluo4/AM (Molecular Probes, Inc., USA) for observing the $Ca^{2+}$ level in intracellular $Ca^{2+}$ stores. Briefly, cells were seeded on circular glass plates at 37°C overnight supplemented with the culture medium. As for the fluorescence dye loading, cells were incubated with 5 $\mu$M Mag-fluo4/AM in dark at 37°C for 45 min, and 0.02% Pluronic acid F-127 in N-PSS. Cells were then washed with the indicator-free N-PSS and incubated at 37°C for 45 min to unload the Mag-fluo4 from cytoplasm. The circular discs containing the endothelial cells were then pinned down in a specially designed chamber. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot 200). Mag-fluo4 fluorescence was recorded by MRC-1000 Laser Scanning Confocal Imaging System with MRC-1000 software (Bio-Rad) with the excitation wavelength of 488 nm and a 515 nm-long pass emission filter. The fluorescent signals were then collected beginning from the end of 30-minute $H_2O_2$ treatment. The change in store $Ca^{2+}$ was cut off during the period of $H_2O_2$ treatment. The $Ca^{2+}$ fluorescence was displayed as Mag-fluo4 intensity change in percentage. Each series of experiments had its own control.

$IP_3$ measurement

The amount of $IP_3$ was measured using HitHunter™ $IP_3$ Assay Fluorescence Polarization Detection-Green Kits (DiscoverRx Tech, Fremont, CA, USA), a reliable and convenient methodology based on competitive binding between an $IP_3$ fluorescence tracer and unlabeled $IP_3$ from the cell lysates or standards. Free $IP_3$ competes at the $IP_3$ binding protein and allows the $IP_3$ tracer to rotate freely upon excitation with plane polarized light. The polarized signal is inversely proportional to the amount of the free unlabeled $IP_3$. Thus, polarization signal is decreased when the concentration of free $IP_3$ is increased [22]. Briefly, aortic ECs and MAECs were treated with different concentrations of $H_2O_2$ (500 $\mu$M, 2 $mM$, 5 $mM$) for 5 min in black 96-well plates. The cellular reactions were terminated by placing cells on ice followed by addition of 0.2 N perchloric acid to lyse the cells. The plate was then shaken at 650 rpm for 5 min. The $IP_3$ tracer was subsequently added to each well, followed by $IP_3$ binding protein. The polarized fluorescence from the $IP_3$ tracer (fluorescein) was read using a Wallac EnVision™ Microplate Reader (Perkin Elmer, Wallac, EnVision, Finland) with a polarization mirror, a 485 nm excitation filter and a 530 nm emission filter. $IP_3$ concentration was calculated from the $IP_3$ standard curve and expressed as pmole/1 × 10^6 cells.

Data Analysis

Data Analysis was performed with Software Confocal Assistant and Metafluor. All representative traces were plotted by using Prism 3.0 (GraphPad, San Diego, CA, USA). Summarized data were expressed as the mean±SEM and analyzed with two-tailed Student’s t test at a p<0.05 level of significance.

Author Contributions

Conceived and designed the experiments: LS H-YY O-CL XY. Performed the experiments: LS O-CL XY. Analyzed the data: LS O-CL XY. Contributed reagents/materials/analysis tools: LS O-CL XY. Wrote the paper: LS XY O-CL YH.
23. Yang Z, Zhang A, Altura BT, Altura BM (1999) Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta involvement of Ca\(^{2+}\) and other cellular metabolites. Gen Pharmacol 33: 325–336.

24. Thengchaisri N, Kuo L (2003) Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. Am J Physiol Heart Circ Physiol 285: H2255–H2263.

25. Liu Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC, et al. (2003) Mitochondrial sources of H\(_2\)O\(_2\) generation play a key role in flow-mediated dilation in human coronary resistance arteries. Circ Res 93: 573–580.

26. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakajo R, et al. (1996) The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol 28: 703–711.

27. Ashley RA, Dubaque SH, Dvorak B, Woodward SS, Williams SK, et al. (2002) Erythropoietin stimulates vasculogenesis in neonatal rat mesenteric microvascular endothelial cells. Pediatr Res 51: 472–478.

Effect of H\(_2\)O\(_2\) on Aortic ECs and MAECs