Mitochondria depletion abolishes agonist-induced Ca\(^{2+}\) plateau in airway smooth muscle cells: potential role of H\(_2\)O\(_2\)

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Mitochondria depletion abolishes agonist-induced Ca\(^{2+}\) plateau in airway smooth muscle cells: potential role of H\(_2\)O\(_2\). Am J Physiol Lung Cell Mol Physiol 298: L178–L188, 2010. doi:10.1152/ajplung.00134.2009.—The mechanisms by which mitochondria regulate agonist-induced Ca\(^{2+}\) oscillations in airway smooth muscle cells remain unclear. By exposing to ethidium bromide and supplying pyruvate as an energy source, we established mitochondrial DNA (mtDNA)-depleted rat airway smooth muscle cells (RASMCs) with maintained cellular energy status upon an exposure to 2 \(\mu\)M histamine. [Ca\(^{2+}\)]\(_i\) in control RASMCs increased to a peak followed by a plateau above baseline, whereas [Ca\(^{2+}\)]\(_i\) in mtDNA-depleted RASMCs jumped to a peak and then declined to baseline without any plateau. mtDNA depletion appeared attenuated intracellular reactive oxygen species generation induced by histamine. By coexposure to 2 \(\mu\)M histamine and 0.1 \(\mu\)M exogenous H\(_2\)O\(_2\), which did not affect [Ca\(^{2+}\)]\(_i\), by itself, the above difference in [Ca\(^{2+}\)]\(_i\), kinetics in mtDNA-depleted RASMCs was reversed. Intracellular H\(_2\)O\(_2\) decomposition abolishes histamine-induced sustained elevation in [Ca\(^{2+}\)]\(_i\), in RASMCs. Thus, mitochondria regulate agonist-induced sustained [Ca\(^{2+}\)]\(_i\) elevation by a H\(_2\)O\(_2\)-dependent mechanism.

AN IMPORTANT ROLE OF MITOCHONDRIA in shaping agonist-stimulated cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\)) kinetics has been studied in some types of cells (1, 2, 3, 4, 36). In almost all previous studies, pharmacological inhibitors have been predominantly employed to elucidate mitochondrial dysfunction in altering [Ca\(^{2+}\)]\(_i\); signaling kinetics (1, 2, 3, 4, 36). As the only organelle containing DNA except nuclei in eukaryotic cells, mitochondrial dysfunction can result from mitochondrial DNA (mtDNA) mutation or depletion. A previous study showed that a low concentration of ethidium bromide (EB) only inhibited the replication and transcription of mtDNA without substantially affecting nuclear DNA (nDNA) (21), and the mtDNA-depleted cells can maintain their energy status, survive, and proliferate by supplementing exogenous uridine and pyruvate (21).

A very recent study reported that mtDNA depletion eliminated receptor-mediated [Ca\(^{2+}\)]\(_i\) oscillations (38). The artificial mtDNA depletion can be an alternative strategy for the investigation of mitochondria in [Ca\(^{2+}\)]\(_i\) signaling. In addition to [Ca\(^{2+}\)]\(_i\) oscillations, a biphasic [Ca\(^{2+}\)]\(_i\) elevation consisting of a peak response followed by a sustained elevation represents another major pattern of [Ca\(^{2+}\)]\(_i\) signaling at the global cellular level (26). The mechanism underlying how mitochondria affect receptor-mediated biphasic [Ca\(^{2+}\)]\(_i\) elevations has not been documented. Mitochondria are both the major site of cellular energy production and that of hydrogen peroxide (H\(_2\)O\(_2\)) production. Previous studies (9, 12, 17, 40) including ours (17, 40) have shown the importance of intracellular H\(_2\)O\(_2\) in Ca\(^{2+}\) signaling. mtDNA-depleted cells with maintained basal level of energy status may provide a unique way to specifically reveal the mechanistic role of mitochondria-derived H\(_2\)O\(_2\) in biphasic [Ca\(^{2+}\)]\(_i\) elevations.

Airway smooth muscle cell (SMC) is a kind of parenchyma cell with multifunction associated with mitochondrial status including mtDNA mutation and/or deletion. It has been shown recently that mitochondria regulate ion channel activity in SMC membrane by the control of [Ca\(^{2+}\)]\(_i\), or redox status of the cell (5). Another recent study in tracheal SMC suggested a close spatial association between the mitochondria and the sarcoplasmic reticulum (SR) as the structural basis for Ca\(^{2+}\) trafficking between the two organelles (7).

Using mtDNA depletion strategy, we show in rat airway SMC that mitochondria depletion abolishes agonist-induced sustained [Ca\(^{2+}\)]\(_i\) elevation, which is probably due to diminished intracellular H\(_2\)O\(_2\) generation.

MATERIALS AND METHODS

Animal welfare. All experiments involving Sprague-Dawley rats for tracheal smooth muscle cell culture were approved by the Institutional Animal Care and Use Committee.

Cell culture. Rat airway smooth muscle cells (RASMCs) were cultured from rat tracheal tissue explants using the procedures modified from Hirst (13). The adherent connective tissue in tracheal explants was carefully removed from the adventitial surface, and the airway epithelium was disrupted by firm scraping across the luminal surface with a blade. RASMCs at ~80% confluence in five to eight passages in culture were either maintained in DMEM as control or treated in DMEM supplemented with 110 \(\mu\)g/ml sodium pyruvate, 50 \(\mu\)g/ml uridine, and three different concentrations of EB (50, 100, or 200 ng/ml), respectively (21, 27). Unless specifically stated, all other chemicals and reagents were purchased from Sigma.

To identify the expression of smooth muscle-specific \(\alpha\)-actin, RASMCs planted and fixed in 24-well plates were incubated overnight at 4°C with rat anti-smooth muscle-specific \(\alpha\)-actin monoclonal antibody (Santa Cruz, 1:100) or with 0.5% BSA as negative control and then visualized by FITC-labeled goat anti-rat IgG (Santa Cruz). Hoechst-33258 was used to visualize the nucleus.

mtDNA extraction and quantification, Southern blot. Total DNA was extracted from RASMCs using a QIAamp Mini Kit (Qiagen).
digested with the restriction enzyme PvuII to linearize the circular mtDNA. The resulting fragments were separated by electrophoresis through a 0.7% agarose gel, denatured, and transferred to a positively charged nylon filter and hybridized simultaneously with the mtDNA probe (2574-4122 portion of a human mtDNA fragment encompassing the gene for NADH dehydrogenase subunit 1, 92% homology with rat mtDNA; American Type Culture Collection) and a nuclear probe (partial sequence of rat 18s ribosomal DNA) tagged with digoxin dUTP (Roche) by random-primed labeling.

Ultrastuctural assessment by electron microscopy. RASMCs for electron microscopy assessment were prepared according to the method of Kislev et al. (23). Briefly, glutaraldehyde-fixed RASMC pellets were postfixed with 1% osmium tetroxide for 1 h at room temperature, washed with 0.1 M phosphate buffer and dehydrated in a graded series of ethyl alcohol and acetone, transferred into a mixture of Epon with acetone (1:1), and presoaked for 2 h embedded in pure Epon. After polymerization in an oven at constant 80°C for 10 h, the ultrathin sections were cut on a UCT ultramicrotome (Leica Ultracut, Germany) and stained with uranyl acetate solution followed by lead citrate. The ultrastructure of RASMCs was examined using a transmission electron microscope (JEI Tecnai G2 12, Netherlands), and the intact mitochondria were quantified in 10 random fields of cytoplasm (2.5 × 2.0 μM).

Assay of cellular ATP. The luminometric ATP assay for quantitative ATP measurement was performed using the ATP assay kit (Sigma) and a luminometer (Berthold LB 9507). The relative light unit (RLU) was converted to ATP content using an ATP standard curve, which is generated by linear regression between the logarithm values of a series of known ATP concentrations (10 nM-100 μM) and emitted RLU. The cellular ATP level was normalized by protein concentration for each sample.

Cytosolic Ca2+ measurement. [Ca2+], measurement using the fluorescent Ca2+ indicator of fura 2 has been fully described before (17, 40).

Measurements of intracellular reactive oxygen species. The reactive oxygen species (ROS)-sensitive fluorescent indicator 2,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to detect the generation of intracellular ROS in RASMCs as previously described in detail (17).

Transfection of shRNAi against STIM1. Effective shRNAi specifically against stromal interaction molecule-1 (STIM1) and its nonspecific control were purchased from Origene (Rockville, MD). The shRNAi constructs simultaneously encode GFP for identification of transfected RASMCs individually. The transfection of shRNAi/GFP specifically targeted to STIM1 or to a nonspecific control into RASMCs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, RASMCs grown on coverslips were used for transfection with 1 μg of shRNAi/GFP against STIM1 or a nonspecific control in 100 μl of DMEM containing 20% CO2 for 4.5–5 h. After transfection, the RASMCs were loaded with fura 2 and then transferred to the fluorescent microscope for [Ca2+]i measurement. The transfected RASMC was first identified by GFP expression under a fluorescent microscope with a wavelength of 485 nm for excitation and 520 nm for emission. Then, the cell was excited alternatively by 340/380 nm, and the emitted fluorescence of 510 nm was continuously monitored before and after the challenge of 2 μM histamine.

Statistical analysis. Data are reported as means ± SD (11). Comparisons between two groups were made with the Student’s t-test, and comparisons among multiple groups were made with one-way ANOVA analysis. A difference was considered significant at P < 0.05.

RESULTS

Morphological and immunocytochemical analysis. RASMCs in culture were long fusiform or triangle, and nuclei shapes were mainly orbicular-ovate. Typical “hills and valleys” structures were observed when RASMCs were completely confluent (Fig. 1A). SMCs were confirmed by indirect immunocytochemical staining of smooth muscle-specific α-actin (Fig. 1B).

mtDNA depletion. Southern blot imaging and semiquantitative analysis of hybridized mtDNA bands found that 200 ng/ml of EB pretreatment induced significant depletion of mtDNA in RASMCs (P < 0.05 vs. control, 50 and 100 ng/ml EB pretreatment, respectively, n = 3 for each, Fig. 2, A and B).

Electronic microscopy identified more than 80% decline in the quantity of mitochondia in EB-pretreated RASMCs compared with the control cells (Fig. 2C). Swollen and/or disrupted mitochondrial structures were found in EB-pretreated RASMCs, whereas cell membrane, nucleus, and endoplasmic reticulum remained intact in both EB-pretreated and control cells (not shown). These results are consistent with previous studies showing that EB treatment at an appropriate concentration can selectively destroy mitochondrial structure (21, 22).

As shown in Fig. 2D, cellular ATP contents of mtDNA-depleted RASMCs are similar to those of control RASMCs, indicating that mitochondria depletion does not affect cellular energy supply in RASMCs supplemented with pyruvate and uridine.

![Fig. 1. Morphological and immunocytochemical analysis of rat airway smooth muscle cells (RASMCs). A: the typical “hills and valleys” morphological structures were noted in RASMCs in culture. B: a representative image showing smooth muscle-specific α-actin and nuclear staining. RASMCs were incubated with rat anti-α-actin antibody and Hoechst-33258. Green (α-actin) and blue fluorescence were observed in the cytoplasm and nucleus, respectively (×40). In α-actin immunostaining control experiments, only blue fluorescence was observed in nuclei (not shown).](http://ajplung.physiology.org/)
Effect of mitochondria depletion on agonist-induced \( \text{Ca}^{2+} \) signaling. In pilot experiments of this study, a series of concentrations of histamine from 0.1 to 10 \( \mu \text{M} \) were examined in fura 2-loaded RASMCs. Histamine (2 \( \mu \text{M} \)) induced biphasic \( \text{Ca}^{2+} \) elevations in most RASMCs examined. In 20 out of a total of 21 intact RASMCs examined, 2 \( \mu \text{M} \) histamine triggered \( \text{Ca}^{2+} \) increase from basal level of 97.4 \pm 7.5 nM to 538.6 \pm 127 nM of peak over 46.1 \pm 12.8 s, then \[ \text{Ca}^{2+} \] decreased from the peak to 244.3 \pm 25.8 nM over 111.3 \pm 41.9 s and stayed at this plateau during our experimental period (\( n = 20 \) cells for each, as shown in Fig. 3A); in the remaining one RASMC, histamine failed to trigger any \[ \text{Ca}^{2+} \] response. These results are roughly consistent with previous studies on histamine-induced \[ \text{Ca}^{2+} \] signaling including RASMC. Histamine (2 \( \mu \text{M} \)) triggered \[ \text{Ca}^{2+} \] increase with similar kinetics in 17 out of a total of 22 RASMCs cultured with pyruvate and uridine (Fig. 3B); there was no \[ \text{Ca}^{2+} \] response in the remaining five RASMCs. By contrast, in 20 out of a total of 23 mtDNA-depleted RASMCs examined, \[ \text{Ca}^{2+} \] increased upon 2 \( \mu \text{M} \) histamine stimulation from basal level of 100.7 \pm 9.8 nM to a peak of 528.2 \pm 75.9 nM over 8.7 \pm 2.2 s, then decreased from the peak to 100.73 \pm 14.2 nM over 39.7 \pm 9.5 s and stayed at this basal level (Fig. 3C); in the other three mtDNA-depleted RASMC cells examined, no \[ \text{Ca}^{2+} \] signaling upon histamine stimulation was noted.

Effect of mitochondria depletion on agonist-stimulated intracellular ROS production. Agonists like histamine and thrombin induced cellular \( \text{H}_2\text{O}_2 \) generation from different sources (12, 17) including mitochondria (12). Thus, we examined the effect of 2 \( \mu \text{M} \) histamine on intracellular ROS generation in RASMCs using DCF as a fluorescent indicator. Histamine (2 \( \mu \text{M} \)) exposure increased DCF fluorescence in...
tensity in 20 out of a total of 24 intact RASMCs from a base level of 6,120 ± 753 to a peak of 11,268 ± 811 within 1 min, which was followed by a plateau over >10 min (Fig. 4A). In the other four cells examined, histamine did not induce any response in DCF fluorescence. We then examined if mitochondria are the potential resource of ROS generation in histamine-stimulated RAMSCs using mtDNA depletion as the experimental model. Our results showed that 2 μM histamine slightly increased DCF fluorescence intensity in 19 out of 23 mtDNA-

Fig. 3. mtDNA depletion abolishes histamine-induced sustained [Ca\textsuperscript{2+}] elevations. A: a representative tracing showed that in fura 2-loaded RASMCs (cultured without pyruvate and uridine) in HEPES-buffered saline (HBS) containing 1.5 mM Ca\textsuperscript{2+}, upon exposure of 2 μM histamine, [Ca\textsuperscript{2+}], rapidly increased from the basal to a peak, then decreased and remained at a plateau above baseline (n = 20). B: a representative tracing showed that in fura 2-loaded RASMCs (cultured with pyruvate and uridine) in HBS containing 1.5 mM Ca\textsuperscript{2+}, upon exposure of 2 μM histamine, [Ca\textsuperscript{2+}], rapidly increased from the basal to a peak, then decreased and remained at a plateau above baseline (n = 17). C: a representative tracing showed that in mtDNA-depleted RASMCs loaded with fura 2 in HBS containing 1.5 mM Ca\textsuperscript{2+}, 2 μM histamine stimulation increased [Ca\textsuperscript{2+}], rapidly to a peak followed by a quick return to the basal level (n = 20).

Fig. 4. Histamine-triggered intracellular ROS generation is largely attenuated in mitochondria-depleted RASMCs. A: DCF-loaded RASMCs were examined under a fluorescent microscope as described in MATERIALS AND METHODS. Histamine (2 μM) exposure evoked a quick and sustained increase in DCF fluorescent intensity in RASMCs (○, averaged from 20 RASMCs), a response that is largely inhibited in mtDNA-depleted RASMCs (●, averaged from 20 RASMCs). B: statistical analysis showed that EB treatment and PEG-catalase, not PEG-SOD pretreatment, significantly inhibited histamine-evoked sustained elevation in DCF fluorescent intensity in RASMCs (*P < 0.05, vs. control, n = 20 for each). Additionally, a series of concentrations of H\textsubscript{2}O\textsubscript{2} on DCF fluorescent intensity was also examined on RASMCs. It was found that 0.1 and 0.30 μM H\textsubscript{2}O\textsubscript{2} increased DCF fluorescent intensity, and the sustained elevations of DCF were close to the level induced by histamine stimulation, whereas 0.01 or 0.03 μM H\textsubscript{2}O\textsubscript{2} failed to increase DCF fluorescent intensity (n = 17–22 for each).
depleted RASMCs from a base level of 5,847 ± 349 to 6,512 ± 398 within 1 min, which then declined to 6,341 ± 659 within 10 min (Fig. 4A). Histamine did not increase DCF fluorescence in the other four cells examined. Thus, in RASMCs in the present study, we validate that histamine stimulation induced ROS generation from mitochondria as previously reported in other types of cells like endothelial cells (12).

Experiments were also performed to examine the effect of free radical decompositions or scavengers on histamine-stimulated increase in DCF fluorescence intensity. For these experiments, RASMCs were incubated for 24 h with either of polyethylene glycol (PEG)-catalase (5,000 U/ml) or PEG-superoxide dismutase (SOD; 1,000 U/ml) before exposure to histamine. The concentrations of PEG-SOD and PEG-catalase were chosen on the basis of previous experiments (8, 9, 18) including ours (18). As summarized in Fig. 4B, PEG-SOD did not affect histamine-stimulated increase in DCF fluorescence intensity in RASMCs (DCF fluorescence plateau level increased to ~200% of baseline, n = 17, P = NS vs. control). In contrast, PEG-catalase pretreatment prevented the increase in DCF fluorescence intensity in RASMCs (DCF fluorescence plateau level remained at ~107% of baseline, n = 17, P < 0.05, vs. control). These results suggest the generation of H2O2 upon histamine stimulation in RASMCs. It is noted that the culture of RASMCs in the presence of pyruvate and uridine does not affect histamine-stimulated increase in DCF fluorescence intensity (DCF fluorescence plateau = 188.30 ± 11.27% of baseline, n = 20, P = NS vs. RASMCs cultured in the absence of pyruvate and uridine, Fig. 4B) or the employment of PEG-SOD and PEG-catalase (data not shown).

We then exposed DCF-loaded RASMCs to each of a series of concentrations of H2O2 including 0.01, 0.03, 0.1, and 0.3 μM. It was found that the DCF elevation induced by 0.1 and 0.3 μM H2O2 is close to the level induced by histamine stimulation (Fig. 4B, n = 17–22).

To investigate the potential contribution of other ROS-generating enzyme systems in histamine-stimulated H2O2 generation in this type of cell, RASMCs were pretreated with one of the following enzyme inhibitors for 30 min, and the inhibitors were present during histamine stimulation. They are: 1) the flavoprotein inhibitor diphosphopyridine nucleotide (DPN) (10 μM), which is a known inhibitor of the NADPH oxidase; 2) the xanthine oxidase inhibitor oxypurinol (100 μM); 3) the NO synthase inhibitor L-nitro-L-arginine methyl ester (G-nitro-L-arginine methyl ester (G-NAME), 1 mM); and 4) the cyclooxygenase inhibitor indomethacin (10 μM). In each case, the concentrations of the inhibitors used were previously shown to effectively block the enzyme activity in several types of cells (9, 14, 18) including SMCs (9, 14). None of the above inhibitors was found to prevent the increased level in DCF fluorescence intensity upon histamine stimulation in RASMCs (n = 18 for each, not shown).

Role of H2O2 in histamine-induced biphasic [Ca2+]i signaling. The agonist-stimulated ROS generation has been reported to be involved in some of the [Ca2+]i signaling processes (9, 12, 17). We explored to see if mitochondria-derived H2O2 is a potential mechanism underlying mitochondria depletion-altered biphasic [Ca2+]i signaling in RASMCs. Since mitochondria depletion did not abolish histamine-stimulated peak response in [Ca2+]i, mitochondria-derived ROS may therefore not trigger peak response directly. Our DCF fluorescence measurements show that 0.1 and 0.3 μM H2O2 are close to the level induced by histamine stimulation (Fig. 4B). We performed the following experiments to find the appropriate concentration(s) of H2O2 that does not trigger [Ca2+]i response directly in RASMCs and is close to the H2O2 level during histamine stimulation in RASMCs. In fura 2-loaded RASMCs, our experiments showed that [Ca2+]i signaling, including peak response, was induced by the exposure of 0.3 μM (Fig. 5A) and higher dosages of H2O2 (up to 100 μM, not shown), not by 0.1 μM H2O2 (n = 20 for each, Fig. 5B) or other lower concentrations of H2O2 (including 0.01 and 0.03 μM, n = 20 for each, not shown). H2O2 (0.1 μM) was therefore employed in the subsequent experiments.

Effect of coexposure of H2O2 and histamine on [Ca2+]i signaling in mtDNA-depleted RASMCs. By coexposure to 2 μM histamine and 0.1 μM H2O2, in 20 of a total of 23 mtDNA-depleted cells, [Ca2+]i increased from a basal level of 100.9 ± 6.6 nM to a peak of 541.6 ± 115.8 nM over 9.6 ± 1.7 s, then decreased from the peak to 201.7 ± 15.3 nM over 127.1 ± 30.5 s and kept in the plateau phase during the experimental period for over 10 min (Fig. 6C). In the other
three cells examined, no \([\text{Ca}^{2+}]\) response was noted. Thus \(\text{H}_2\text{O}_2\) at an appropriate concentration of 0.1 \(\mu\text{M}\), which is equivalent to DCF fluorescence elevation stimulated by 2 \(\mu\text{M}\) histamine and does not trigger \([\text{Ca}^{2+}]\), spike by itself, reverses the mitochondria depletion-abolished \([\text{Ca}^{2+}]\), plateau upon histamine stimulation in EB-pretreated RASMCs (representative \([\text{Ca}^{2+}]\), tracings in Fig. 4 are included here as Fig. 6, A and B, for comparison).

Comparisons of agonist-stimulated \([\text{Ca}^{2+}]\), kinetics in control and mitochondria-depleted RASMCs. As shown in Fig. 7A, there was no difference in basal or peak \([\text{Ca}^{2+}]\), level between control and mtDNA-depleted RASMCs. The most apparent difference of histamine-induced \([\text{Ca}^{2+}]\), signaling in mtDNA-depleted RASMCs was the disappearance of the \([\text{Ca}^{2+}]\), plateau following peak \([\text{Ca}^{2+}]\), response as noted in control RASMCs. In addition, the ascending and descending speeds of the peak \([\text{Ca}^{2+}]\), response in mtDNA-depleted RASMCs were faster than control RASMCs. The corresponding ascending and descending time durations of the peak \([\text{Ca}^{2+}]\), response in mtDNA-depleted RASMCs were shorter than control RASMCs (Fig. 7, B and C). However, except for alterations in ascending phase of the peak \([\text{Ca}^{2+}]\), response, the plateau and the descending speed and time duration of the peak \([\text{Ca}^{2+}]\), response in mtDNA-depleted RASMCs was reversed by coexposure to 2 \(\mu\text{M}\) histamine and 0.1 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\). It is noted that coexposure of 0.1 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) does not affect the \([\text{Ca}^{2+}]\), signal kinetics triggered by histamine in control RASMCs (not shown).

Role of \(\text{H}_2\text{O}_2\) in histamine-induced biphasic \([\text{Ca}^{2+}]\), signaling in intact RASMCs. To further explore the potential role of endogenous \(\text{H}_2\text{O}_2\) in histamine-induced \([\text{Ca}^{2+}]\), signaling, intact RASMCs were pretreated overnight with 1,000 U/ml PEG-SOD or 5,000 U/ml PEG-catalase. It was found that 2 \(\mu\text{M}\) histamine increased \([\text{Ca}^{2+}]\), from basal level at 102.6 ± 7.6 nM to 523.5 ± 45.7 nM of peak over 47.8 ± 11.0 s, then decreased from the peak to 110.4 ± 12.1 nM over 40.8 ± 9.6 s in 18 out of a total of 22 intact RASMCs pretreated overnight with PEG-catalase (Fig. 8A); no \([\text{Ca}^{2+}]\), response was noted in the remaining four cells. Whereas in 19 of 22 intact RASMCs pretreated overnight with PEG-SOD, 2 \(\mu\text{M}\) histamine increased \([\text{Ca}^{2+}]\), from basal level of 98.6 ± 8.6 nM to 542.3 ± 55.1 nM of peak over 44.8 ± 12.1 s, then decreased from the peak level to 230.2 ± 30.2 nM over 115.3 ± 25.7 s and remained at this plateau level over 10 min (Fig. 8B); no \([\text{Ca}^{2+}]\), response was observed in the other three cells. Thus, pretreatment with PEG-catalase, not PEG-SOD, which abolished histamine-stimulated \(\text{H}_2\text{O}_2\) generation, abolishes histamine-induced \([\text{Ca}^{2+}]\), plateau in intact RASMCs. Similarly, PEG-catalase pretreatment abolished histamine-induced \([\text{Ca}^{2+}]\), plateau in RASMCs cultured in the presence of pyruvate and uridine, the control for mtDNA depletion RASMCs (data not shown).

The treatment with either 10 \(\mu\text{M}\) DPI, 100 \(\mu\text{M}\) oxypurinol, 1 mM \(\text{L-NAME}\), or 10 \(\mu\text{M}\) indomethacin does not abolish histamine-induced \([\text{Ca}^{2+}]\), plateau in intact RASMCs (not shown).

Dependence of histamine-induced \([\text{Ca}^{2+}]\), plateau on STIM1. To explore the potential role of STIM1 in histamine-induced \([\text{Ca}^{2+}]\), plateau, RASMCs were transfected with either nonspecific shRNAi/GFP or STIM1-specific shRNAi/GFP construct. After fura 2 loading, \([\text{Ca}^{2+}]\), was continuously

Fig. 6. Coexposure of \(\text{H}_2\text{O}_2\) and histamine on \([\text{Ca}^{2+}]\), signaling in mtDNA-depleted RASMCs. A: a representative tracing showed that in fura 2-loaded RASMCs in HBS containing 1.5 mM \(\text{Ca}^{2+}\), upon exposure of 2 \(\mu\text{M}\) histamine, \([\text{Ca}^{2+}]\), rapidly increased from the basal to a peak, then decreased and remained at a plateau above baseline (n = 20). B: a representative tracing showed that in mtDNA-depleted RASMCs loaded with fura 2 in HBS containing 1.5 mM \(\text{Ca}^{2+}\), 2 \(\mu\text{M}\) histamine stimulation increased \([\text{Ca}^{2+}]\), rapidly to a peak followed by the quick return to the basal level (n = 20). C: mtDNA-depleted RASMCs were loaded with fura 2 in HBS containing 1.5 mM \(\text{Ca}^{2+}\), and \([\text{Ca}^{2+}]\), signal was measured. A representative tracing showed that by coexposure to 2 \(\mu\text{M}\) histamine and 0.1 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\), \([\text{Ca}^{2+}]\), increased immediately to a peak, and then decreased rapidly and kept in a plateau above basal level (n = 20). It is noted that Fig. 3, A and C, are included here as Fig. 6, A and B, respectively, for comparison.
monitored in the transfected RASMC as identified by GFP expression. As shown in Fig. 9, STIM1 knockdown abolished histamine-induced [Ca²⁺]i plateau in control RASMCs cultured in the absence or presence of pyruvate and uridine vs. nonspecific shRNAi construct, respectively, whereas EB pretreatment abolished the [Ca²⁺]i plateau (n = 18–20 cells for each).

Reversible effects of H₂O₂ on mtDNA depletion-abolished [Ca²⁺]i plateau and its dependence on STIM1. To further explore the mechanism underlying the involvement of H₂O₂ in histamine-induced [Ca²⁺]i signaling, RASMCs were pretreated overnight with 1,000 U/ml PEG-SOD or 5,000 U/ml PEG-catalase. Then, [Ca²⁺]i signaling was examined upon 2 μM histamine stimulation. A: a representative tracing showed that 2 μM histamine increased [Ca²⁺]i to a peak, then decreased to baseline in intact RASMCs pretreated with PEG-catalase (n = 18). B: a representative tracing showed that 2 μM histamine increased [Ca²⁺]i, from basal to a peak, then decreased and maintained at a sustained level above baseline over 10 min in intact RASMCs pretreated with PEG-SOD (n = 19).

Fig. 8. PEG-catalase abolishes histamine-induced sustained [Ca²⁺]i elevations in intact RASMCs. To explore the potential role of endogenous H₂O₂ in histamine-induced [Ca²⁺]i signaling, RASMCs were pretreated overnight with 1,000 U/ml PEG-SOD or 5,000 U/ml PEG-catalase. Then, [Ca²⁺]i signaling was examined upon 2 μM histamine stimulation. A: a representative tracing showed that 2 μM histamine increased [Ca²⁺]i, to a peak, then decreased to baseline in intact RASMCs pretreated with PEG-catalase (n = 18). B: a representative tracing showed that 2 μM histamine increased [Ca²⁺]i, from basal to a peak, then decreased and maintained at a sustained level above baseline over 10 min in intact RASMCs pretreated with PEG-SOD (n = 19).

Fig. 7. Comparisons of [Ca²⁺]i signaling kinetics in control and mtDNA-depleted RASMCs. Fura 2-loaded control (black) and mtDNA-depleted RASMCs (gray) were stimulated by 2 μM histamine, and mtDNA-depleted RASMCs were costimulated by 2 μM histamine and 0.1 μM H₂O₂ (brown). [Ca²⁺]i signaling before and after stimulation was monitored on the fluorescent microscope as described in MATERIALS AND METHODS. A: mtDNA depletion abolished histamine-induced [Ca²⁺]i plateau, and this was reversed by coexposure of 2 μM histamine and 0.1 μM H₂O₂ (brown). B: mtDNA depletion accelerated [Ca²⁺]i ascending and descending speed in histamine-stimulated RASMCs. The alteration of [Ca²⁺]i ascending and descending speed, not the ascending speed, was reversed in mtDNA-depleted RASMCs by costimulation of 2 μM histamine and 0.1 μM H₂O₂ (brown). C: mtDNA depletion decreased [Ca²⁺]i ascending and descending time periods in histamine-stimulated RASMCs. The alteration of [Ca²⁺]i ascending and descending time periods, not the ascending time period, in mtDNA-depleted RASMCs was reversed by costimulation of 2 μM histamine with 0.1 μM H₂O₂ (n = 20 for each, *P < 0.05 vs. control).
DISCUSSION

In this study, we successfully isolated, subcultured, and identified RASMCs. After treatment with EB, RASMCs with depleted mtDNA were generated. The RASMCs lacking mtDNA were maintained in culture medium supplemented with pyruvate and uridine, by which cellular ATP can be replenished (21, 22). Thus, this excludes a possible explanation that any alterations in \([\text{Ca}^{2+}]_i\) signal kinetics in mitochondria-depleted RASMCs are due to insufficient basal level of energy supply, since \([\text{Ca}^{2+}]_i\) signaling is an active process that consumes cellular energy. Mitochondria depletion can occur in a variety of cells due to either genetic mutation/defect or pathological processes. The employment of mitochondria-depleted cells is potentially (patho)biologically relevant.

As a \([\text{Ca}^{2+}]_i\) buffer during \([\text{Ca}^{2+}]_i\) response, the net effect of mitochondria in the descending phase of \([\text{Ca}^{2+}]_i\) peak response is to slow down the \([\text{Ca}^{2+}]_i\) removal speed and

![Diagram](http://ajplung.physiology.org/) by 10.220.33.1 on June 15, 2017
extend its time duration. However, it is noted that the recovery of the descending speed of peak Ca\textsuperscript{2+} response by extracellular application of 0.1 μM H\textsubscript{2}O\textsubscript{2} found in the present study may be due to H\textsubscript{2}O\textsubscript{2}-decreased activity of other cytosolic Ca\textsuperscript{2+} removal pathways like Ca\textsuperscript{2+}-ATPase in endoplasmic/sarcoplasmic reticulum membrane and/or even plasma membrane (37).

Our experiments provide a novel evidence to support the long-held understanding that mitochondria can serve as a Ca\textsuperscript{2+} buffer during the peak [Ca\textsuperscript{2+}]\textsubscript{i} response to agonist stimulation.

In human teratocarcinoma NT2 cells, mtDNA depletion eliminated receptor-mediated [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (38). In pancreatic acinar cells, mitochondria-targeted antioxidants quickly inhibited cholecystokinin-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (4), suggesting a necessary role of mitochondrial-derived oxidants in physiological [Ca\textsuperscript{2+}]\textsubscript{i}. The biphasic [Ca\textsuperscript{2+}]\textsubscript{i} elevations represent another major dynamical pattern of agonist-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} elevations (26). Whether and how mitochondria play any role in this type of Ca\textsuperscript{2+} signaling has not been studied previously. The findings reported in this study intend to fill this gap.

Our results showing that histamine stimulated ROS generation from mitochondria in RASMCs are generally consistent with previous studies (4, 12), including a very recent report demonstrating that receptor-linked mitochondrial ROS is essential for endothelial/leukocyte adherence (12). A low level of intracellular ROS as a signaling molecule in receptor-mediated cellular signaling processes has been shown in many types of cells (4, 12, 17) including our previous study (17). Agonist stimulates intracellular ROS generation from different resources, such as mitochondria (4, 12, 29), NADPH oxidase (17), and xanthine oxidase (29), depending on cell type studied and agonist employed (4, 12, 29). In the present study in RASMCs, mitochondria appear to be the major resource to generate ROS upon histamine stimulation, since mitochondria depletion almost completely abolished histamine-stimulated ROS generation (Fig. 4). However, this study cannot exclude

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**Fig. 10.** H\textsubscript{2}O\textsubscript{2} reverses EB pretreatment-abolished [Ca\textsuperscript{2+}]\textsubscript{i} plateau in an STIM1-dependent way. Representative fura 2 fluorescence obtained from 2 μM histamine-stimulated RASMCs pretreated by EB to deplete mitochondria. EB-pretreated RASMCs were transfected with either nonspecific shRNAi/GFP construct or STIM1-specific shRNAi/GFP construct. After fura 2 loading, the transfected RASMC was first identified by GFP expression under a fluorescent microscope with wavelength of 485 nm for excitation and 520 nm for emission. Then, the cell was excited alternatively by 340/380 nm, and the emitted fluorescence of 510 nm was continuously monitored before and after the challenge of 2 μM histamine. EB pretreatment abolished histamine-induced [Ca\textsuperscript{2+}]\textsubscript{i} plateau; the subsequent co-exposure of 0.1 μM H\textsubscript{2}O\textsubscript{2} reversed EB pretreatment-abolished [Ca\textsuperscript{2+}]\textsubscript{i} plateau in control (A) and nonspecific shRNAi-transfected RASMCs (B), but not in STIM1-knockdown cells (C) (n = 18–20 cells for each).
the potential involvement of other enzymatic pathways in the above process, since xanthine oxidase, nitric oxide synthase, and phospholipase A2 were recently suggested to generate ROS via mitochondria (29).

The external application of a low level of H2O2 that does not affect [Ca\(^{2+}\)], by itself reversed almost all the alterations of [Ca\(^{2+}\)], kinetics in mitochondria-depleted RASMCs except for the ascending speed and time duration of the [Ca\(^{2+}\)], ascending response (Fig. 7). This may exclude the role of mitochondria-derived H2O2 on the [Ca\(^{2+}\)], ascending phase, and the possible explanations can be the time lag between H2O2 generation (~1 min to peak, Fig. 4) and the [Ca\(^{2+}\)], ascending phase (~10–40 s, Fig. 7) and that the ascending phase of the peak [Ca\(^{2+}\)], response can be primarily governed by Ca\(^{2+}\) release from SR/ER (32). The role of mitochondria in shaping the ascending phase of the [Ca\(^{2+}\)], peak response, shown in Fig. 8, may be mediated by the coupling of SR and mitochondrial Ca\(^{2+}\) channels or their close linkage with SR in the space and sensing rapid Ca\(^{2+}\) changes in microdomains (28, 33) and triggering their Ca\(^{2+}\) uptake mechanism to slow down the speed and time duration of [Ca\(^{2+}\)], reaching its peak.

Our results of mitochondria depletion abolished-[Ca\(^{2+}\)], plateau are consistent with previous reports using mitochondrial pharmacological inhibitors (32, 35). A supportive line of evidence is the finding in dorsal root ganglion neurons (DRGN) showing that in the presence of CCCP, the plateau phase of [Ca\(^{2+}\)], elevation disappeared (32). Also in DRGN, [Ca\(^{2+}\)], transients elicited by depolarization in the presence of CCCP or the electron transport inhibitor antimycin A1, lacked the plateau (35). Similarly, antimycin A1, either alone or in combination with oligomycin, attenuated [Ca\(^{2+}\)], plateau in T cells (16). Our results are also consistent with a previous study in mitochondrial DNA-depleted neuroblastoma Rho cells showing that the plateau phase in Ca\(^{2+}\) decay was dependent on the extracellular Ca\(^{2+}\) (31). The finding from the current study that mtDNA depletion-attenuated mitochondrial ROS generation plays a critical role in diminishing histamine-stimulated Ca\(^{2+}\) plateau further provides an additional and novel insight into the above process. This conclusion is also supported by the blockage effect of PEG-catalase on histamine-evoked [Ca\(^{2+}\)], plateau in RASMCs, elucidating the role of endogenous ROS (H2O2) (Fig. 8).

It has been well established that histamine stimulates the turnover of IP\(_3\) signaling cascades that initiate the depletion of endoplasmic reticulum Ca\(^{2+}\) store(s) and thereafter activates store-operated Ca\(^{2+}\) entry. In the current study, we show that histamine stimulates intracellular H2O2 generation at a level equivalent to 0.1 \(\mu\)M extracellular H2O2 application. The effect of H2O2 on IP\(_3\) signaling cascade is diverse in previous studies. To the best of our knowledge, the lowest concentration of H2O2 that effectively activates PLC on cell membrane and subsequently initiates IP\(_3\) cascade was reported to be 30 \(\mu\)M in rat cortical astrocytes (15). H2O2 as high as 400 \(\mu\)M in rat hepatocytes (30) or 100 \(\mu\)M in endothelial cells (34) was reported in failure to induce PLC activation or the subsequent IP\(_3\) turnover (17, 34, 39). It appears that IP\(_3\) cascade turnover is not the mechanism underlying the role of 0.1 \(\mu\)M H2O2 in Ca\(^{2+}\) influx in RASMCs in the present study. It may be interesting to determine whether a low level of H2O2-induced sensitization of IP\(_3\) receptor, as previously suggested in endothelial cells (17, 18) or even the direct activation of IP\(_3\) receptor by low level of H2O2 (39), helps maintain the depletion status of endoplasmic reticulum Ca\(^{2+}\) store(s) and consequently facilitates STIM1-mediated extracellular Ca\(^{2+}\) influx in RASMCs.

It is well documented, particularly in T cells, that mitochondria maintains extracellular Ca\(^{2+}\) influx through both the Ca\(^{2+}\) buffering-dependent ability (16, 26) and the Ca\(^{2+}\) buffering-independent way as recently reported (25). In the latter case, mitochondrial-derived metabolites are most recently suggested to play complicated roles in the above processes (2, 25). To reveal the mechanism by which H2O2 contributes to the sustained phase of the histamine response, we performed experiments to explore the role of STIM1 pathway in this process. As summarized in Fig. 9, either STIM1 knockdown or mitochondria depletion abolished histamine-stimulated sustained [Ca\(^{2+}\)], response. Furthermore, the mitochondria depletion-abolished sustained [Ca\(^{2+}\)], response was reversed by extracellular application of H2O2 in the presence of STIM1, not in the absence of STIM1, as shown in Fig. 10. These results indicate that H2O2 may be necessary but insufficient for a histamine-stimulated sustained [Ca\(^{2+}\)], response. In other words, mitochondria-derived H2O2 may work as a cofactor together with STIM1 to mediate histamine-stimulated Ca\(^{2+}\) influx in RASMCs. H2O2 may therefore represent a novel candidate underlying mitochondria-maintained extracellular Ca\(^{2+}\) influx.

Our present study suggests H2O2 as an additional candidate in Ca\(^{2+}\) buffering-independent maintenance of Ca\(^{2+}\) influx by mitochondria. Future investigation, especially combining electrophysiology technique, will be needed to eventually elucidate how mitochondria-derived H2O2 cooperates with STIM1 to maintain Ca\(^{2+}\) influx in RASMCs.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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