Critical Role of NADPH Oxidase-derived Reactive Oxygen Species in Generating Ca\textsuperscript{2+} Oscillations in Human Aortic Endothelial Cells Stimulated by Histamine*

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There is increasing evidence that intracellular reactive oxygen species (ROS) play a role in cell signaling and that the NADPH oxidase is a major source of ROS in endothelial cells. At low concentrations, agonist stimulation of membrane receptors generates intracellular ROS and repetitive oscillations of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in human endothelial cells. The present study was performed to examine whether ROS are important in the generation or maintenance of [Ca\textsuperscript{2+}], oscillations in human aortic endothelial cells (HAEC) stimulated by histamine. Histamine (1 µM) increased the fluorescence of 2',7'-dihydrodichlorofluorescin diacetate in HAEC, an indicator of ROS production. This was partially inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI, 10 µM), by the far- nesyltransferase inhibitor H-Ampamb-Phe-Met-OH (2 µM), and in HAEC transiently expressing Rac1N17, a dominant negative allele of the protein Rac1, which is essential for NADPH oxidase activity. In indo 1-loaded HAEC, 1 µM histamine triggered [Ca\textsuperscript{2+}], oscillations that were blocked by DPI or H-Ampamb-Phe-Met-OH. Histamine-stimulated [Ca\textsuperscript{2+}], oscillations were not observed in HAEC lacking functional Rac1 protein but were observed when transfected cells were simultaneously exposed to a low concentration of hydrogen peroxide (10 µM), which by itself did not alter either [Ca\textsuperscript{2+}], or levels of inositol 1,4,5-trisphosphate (Ins-1,4,5-P\textsubscript{3}). Thus, histamine generates ROS in HAEC at least partially via NADPH oxidase activation. NADPH oxidase-derived ROS are critical to the generation of [Ca\textsuperscript{2+}], oscillations in HAEC during histamine stimulation, perhaps by increasing the sensitivity of the endoplasmic reticulum to Ins-1,4,5-P\textsubscript{3}.

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§ The abbreviations used are: ROS, reactive oxygen species; Ins-1,4,5-P\textsubscript{3}, inositol 1,4,5-trisphosphate; HAEC, human aortic endothelial cells; [Ca\textsuperscript{2+}], intracellular free calcium; ER, endoplasmic reticulum; DPI, diphenyleneiodonium; DCF-DA, 2',7'-dihydrodichlorofluorescin diacetate; HBS, HEPES-buffered saline; PLD, phospholipase D; PLC, phospholipase C; RyR, ryanodine receptor.

P\textsubscript{3}, inositol 1,4,5-trisphosphate; HAEC, human aortic endothelial cells; [Ca\textsuperscript{2+}], intracellular free calcium; ER, endoplasmic reticulum; DPI, diphenyleneiodonium; DCF-DA, 2',7'-dihydrodichlorofluorescin diacetate; HBS, HEPES-buffered saline; PLD, phospholipase D; PLC, phospholipase C; RyR, ryanodine receptor.

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loration and Ca$^{2+}$ signaling. The following study was therefore performed to determine whether NADPH oxidase-generated ROS affect [Ca$^{2+}$], oscillations during histamine stimulation in human endothelial cells.

**MATERIALS AND METHODS**

**Endothelial Cell Culture—**Human aortic endothelial cells (HAEC) were obtained as proliferating quaternary cultures (Clonetics, San Diego, CA) and were grown to confluence to passages 5–9 in endothelial cell growth medium supplemented with 2% fetal bovine serum, 10 µg/liter human recombinant epidermal growth factor, 1 mg/liter hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B, 12 µg/ml bovine serum albumin (Clonetics) in 27% humidified atmosphere of 95% air-5% CO$_2$.

To the Ca$^{2+}$-free medium containing 105/ml on 25-mm-diameter circular glass coverslips (VWR Scientific, Media, PA) pre-coated with 2% gelatin solution (Sigma Chemical Co.) for at least 2 h at 37 °C. The glass coverslips were washed three times with phosphate-buffered saline (Quality Biological, Inc., Gaithersburg, MD) before cell seeding. After exposure to a solution of 0.025% trypsin and 0.01% EDTA (Sigma), HAECs were plated at an approximate concentration of 1 × 10$^5$/ml on the glass coverslips. Cells were used for experiments after reaching ~70% confluence after incubation for 1–2 days at 37 °C in a humidified atmosphere of 95% air-5% CO$_2$.

**Measurement of Intracellular ROS Generation in HAEC—**Detection of intracellular ROS was performed by a previously established method using the ROS-sensitive fluorescent probe 2′,7′-dihydrodichlorofluorescin diacetate (DCF-DA) and confocal microscopy (1, 36). For measurement of intracellular ROS, HAECs were plated in a chamber slide system (Fisher Sciences, Newark, DE) at a density of 1 × 10$^5$ cells/ml and then cultured for 3 days. The cells were washed with HEPES-buffered saline (HBS) and were loaded with 5 µg/ml of DCF-DA (Molecular Probes, Eugene, OR) for 5 min at 37 °C. The fluorescent dichlofluorescin was quantified by using a laser-scanning confocal microscope (TCS-4D, Heidelberg, Germany) with the excitation and emission wavelengths of 488 and 520 nm, respectively.

**Measurement of [Ca$^{2+}$]i—**HAEC (Ca$^{2+}$i) was measured as previously described (37) using the fluorescent Ca$^{2+}$ probe indo 1. Briefly, HAEC in tissue culture dishes were exposed to a solution of 0.025% trypsin and 0.01% EDTA (Sigma Chemical Co., St. Louis, MO) and were then plated at a concentration of ~1 × 10$^6$/ml on 25-mm-diameter circular glass coverslips (VWR Scientific, Media, PA) pre-coated with 2% gelatin solution (Sigma) for 2 h at 37 °C. Cells were used for experiments after reaching ~70% confluence after incubation for 1–2 days at 37 °C in a humidified atmosphere of 95% air-5% CO$_2$. To measure [Ca$^{2+}$]i, HAEC monolayers on glass coverslips were incubated with culture dishes were exposed to a solution of 0.025% trypsin and 0.01% EDTA (Sigma Chemical Co., St. Louis, MO) and were then plated at a concentration of ~1 × 10$^6$/ml on 25-mm-diameter circular glass coverslips (VWR Scientific, Media, PA) pre-coated with 2% gelatin solution (Sigma) for 2 h at 37 °C. Cells were used for experiments after reaching ~70% confluence after incubation for 1–2 days at 37 °C in a humidified atmosphere of 95% air-5% CO$_2$. To measure [Ca$^{2+}$]i, HAEC monolayers on glass coverslips were incubated with culture medium containing 10 µM of the ester derivative (acetoxymethyl ester form) of indo 1 (Molecular Probes, Eugene, OR) in a room temperature 95% air-5% CO$_2$ for 30 min. The coverslips were washed three times with indicator-free HBS of the following composition (in millimolar): NaCl 137, KCl 4.9, CaCl$_2$ 1.5, MgSO$_4$ 1.2, NaH$_2$PO$_4$ 1.2, glucose 15, HEPES 20 (pH adjusted to 7.40 at room temperature with NaOH). The cells were maintained in HBS for at least 30 min before the beginning of the experiment to allow for de-esterification of the indicator. The fluorescence of indo 1 was recorded from single HAEC on coverslips in a perfusion chamber mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope. The fluorescence of indo 1 was excited at 350 ± 50 nm using a xenon short arc lamp (UXL-75 XE, Ushio Inc., Japan), and bandpass interference filters (Omega Optical, Brattleboro, VT) with selected wavelength bands of the excitation of indo 1 was excited at 350 ± 50 nm using a xenon short arc lamp (UXL-75 XE, Ushio Inc., Japan), and bandpass interference filters (Omega Optical, Brattleboro, VT) with selected wavelength bands of the excitation and emission wavelengths of 488 and 520 nm, respectively.

**Measurement of Intracellular Ins-1,4,5-P3 Levels—**To measure Ins-1,4,5-P3 levels, HAECs were incubated with culture medium containing 10 µM of the ester derivative (acetoxymethyl ester form) of indo 1 (Molecular Probes, Eugene, OR) in a room temperature 95% air-5% CO$_2$ for 30 min. The coverslips were washed three times with indicator-free HBS of the following composition (in millimolar): NaCl 137, KCl 4.9, CaCl$_2$ 1.5, MgSO$_4$ 1.2, NaH$_2$PO$_4$ 1.2, glucose 15, HEPES 20 (pH adjusted to 7.40 at room temperature with NaOH). The cells were maintained in HBS for at least 30 min before the beginning of the experiment to allow for de-esterification of the indicator. The fluorescence of indo 1 was recorded from single HAEC on coverslips in a perfusion chamber mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope. The fluorescence of indo 1 was excited at 350 ± 50 nm using a xenon short arc lamp (UXL-75 XE, Ushio Inc., Japan), and bandpass interference filters (Omega Optical, Brattleboro, VT) with selected wavelength bands of the excitation and emission wavelengths of 488 and 520 nm, respectively.

**Regulation of Histamine-stimulated [Ca$^{2+}$i] Oscillations by Intracellular ROS—**As previously demonstrated by our laboratory (39) and others (30), 1 µM histamine triggered [Ca$^{2+}$i] oscillations in all control HAEC studied (Fig. 2A). By contrast, [Ca$^{2+}$i] oscillations were not observed in any HAEC expressing the dominant negative form of Rac1 (n = 16). In 14 of 16 cells,
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no change in [Ca\(^{2+}\)], was noted, and, in the other 2 HAEC expressing the dominant negative form of Rac1, only a single [Ca\(^{2+}\)] spike was observed. Expression of the Rac1\^N17 mutant did not appear to have a more general effect on Ca\(^{2+}\) signaling, because even in those cells lacking any [Ca\(^{2+}\)], response to 1 \(\mu\)M histamine, the response to 100 \(\mu\)M histamine or to 1 \(\mu\)M ionomycin (Fig. 2B) was preserved. To determine whether expression of the dominant negative isoform of Rac1 inhibited [Ca\(^{2+}\)] oscillations by blocking ROS generation, experiments were performed to determine whether [Ca\(^{2+}\)] oscillations would be triggered by histamine in cells lacking functional Rac1 protein in the presence of exogenous hydrogen peroxide (H\(_2\)O\(_2\)). H\(_2\)O\(_2\) was employed for these studies rather than a superoxide-generating system, because our previous work showed that the increased sensitivity of intracellular Ca\(^{2+}\) stores to Ins-1,4,5-P\(_3\) stimulated by NADPH oxidase activity was also blocked by catalase but was unaffected by superoxide dismutase (29). As shown in Fig. 2C (top), 10 \(\mu\)M H\(_2\)O\(_2\) alone did not affect [Ca\(^{2+}\)] in HAEC, as previously shown in our laboratory (43). Whereas 1 \(\mu\)M histamine did not stimulate Ca\(^{2+}\) signaling in HAECs expressing the dominant negative form of Rac1, [Ca\(^{2+}\)] oscillations were observed after the simultaneous addition of 10 \(\mu\)M H\(_2\)O\(_2\) in 5 of 7 HAECs examined (Fig. 2C, bottom), and in another a single [Ca\(^{2+}\)] spike was observed. To determine whether the effect of H\(_2\)O\(_2\) was related to an increase in the sensitivity of intracellular Ca\(^{2+}\) stores to Ins-1,4,5-P\(_3\) (29) or to an effect on Ins-1,4,5-P\(_3\) levels, Ins-1,4,5-P\(_3\) levels were measured in HAEC treated with 1 \(\mu\)M histamine or 10 \(\mu\)M H\(_2\)O\(_2\) alone or with the two together. As shown in Fig. 2D, 1 \(\mu\)M histamine alone produced a rapid increase in Ins-1,4,5-P\(_3\) levels, with a 1.56 \(\pm\) 0.25-fold increase evident 1 min after stimulation. H\(_2\)O\(_2\) did not affect Ins-1,4,5-P\(_3\) levels by itself and did not alter the effect of histamine on Ins-1,4,5-P\(_3\) levels (1.63 \(\pm\) 0.16-fold increase at 1 min, \(p = NS\) compared with histamine alone, \(n = 3\) for each).

Additional experiments were performed to assess whether blocking the generation of ROS by the NADPH oxidase affects histamine-stimulated [Ca\(^{2+}\)] oscillations in HAEC. In these experiments (Fig. 3), [Ca\(^{2+}\)] oscillations were generated and then cells were exposed to histamine-free buffer either alone (Fig. 3A), with DPI (Fig. 3B), or with the FTase inhibitor (Fig. 3C) before a second exposure to histamine. After [Ca\(^{2+}\)] oscillations were generated in the presence of histamine, the washout of histamine resulted in the cessation of oscillations. When the cell was stimulated again with histamine after approximately a 10-min washout period, [Ca\(^{2+}\)] oscillations recurred without any significant difference in oscillation amplitude (indo 1 ratio = 1.20 \(\pm\) 0.21 versus 1.14 \(\pm\) 0.24, \(p = NS\)) or frequency (0.28 \(\pm\) 0.02 versus 0.23 \(\pm\) 0.04 min\(^{-1}\), \(p = NS\)) when compared with that observed before the 10-min washout (Fig. 3A). By contrast, repetitive [Ca\(^{2+}\)] oscillations were not observed when either 10 \(\mu\)M DPI (Fig. 3B) or 2 \(\mu\)M FTase inhibitor (Fig. 3C) was present during and after histamine washout. Of

![Fig. 1. Effect of histamine on ROS generation in HAEC. A, representative changes in the fluorescence of 2',7'-dihydrodichlorofluorescin diacetate (DCF-DA) in HAEC treated with 100 \(\mu\)M H\(_2\)O\(_2\) (top), with 1 \(\mu\)M histamine (middle) or unstimulated (bottom) examined by confocal microscopy (\(\times100\)) and photographed at 0, 5, and 10 min. B, averaged data showing that the increase in DCF fluorescence stimulated by histamine (solid circles) is inhibited by diphenyleneiodonium (DPI, 10 \(\mu\)M, open circles) or a farnesyltransferase (FTase) inhibitor.

(H-Ampamb-Phe-Met-OH, 2 \(\mu\)M, solid triangles). The change in DCF fluorescence is expressed as a percentage increase above baseline fluorescence after subtraction of the small increase in DCF fluorescence observed in control cells over time (data represent mean \(\pm S.E.\) of four experiments in each group; *, \(p < 0.05\) versus control). C, averaged data showing that the increase in DCF fluorescence stimulated by histamine (solid bars) is less in dominant negative Rac transfected cells which lack functional Rac1 protein (open bars). The change in DCF fluorescence is expressed as a percentage increase above baseline fluorescence after subtraction of the small increase in DCF fluorescence observed in control cells over time (data represent mean \(\pm S.E.\) of four experiments in each group; *, \(p < 0.05\) versus control).
**DISCUSSION**

This study shows that histamine, like many other agonists (15–28), stimulates the production of ROS in endothelial cells.
Histamine-stimulated ROS production results, at least in part, from activation of the NADPH oxidase and Rac1 signaling, because it is inhibited by the NADPH oxidase inhibitor DPI, by an FTase inhibitor, and in cells lacking functional Rac1 protein.

The generation of ROS by the NADPH oxidase was previously reported when endothelial cells were stimulated by thrombin (28), vascular endothelial growth factor (21), or tumor necrosis factor-α (7, 22–25). In the case of tumor necrosis factor, NADPH oxidase-derived ROS were found to be important in activating nuclear factor-kB (NF-kB) in endothelial cells (24). NF-kB activation was previously shown to be redox-sensitive in HeLa cells (36). In HeLa cells, transient expression of a constitutively active Rac1 mutant increased NF-kB transcrip-tional activity, whereas basal and cytokine-stimulated NF-kB activity was inhibited in dominant negative Rac1 mutants. This is interesting, because we previously showed that [Ca²⁺], oscillations regulate NF-kB activity during histamine stimulation in HAEC (39), and now show that NADPH oxidase-derived ROS are critical to the generation of [Ca²⁺], oscillations during histamine stimulation.

The effect of NADPH oxidase activation on [Ca²⁺], oscillations may derive from the sensitization of the ER Ins-1,4,5-P₃ receptor by oxidase-derived ROS. Ins-1,4,5-P₃ is critical to the generation of [Ca²⁺], oscillations in non-excitable cells (33). Like many other agonists, histamine binds to membrane receptors and activates phospholipase C to hydrolyze phosphatidyl-inositol 4,5-bisphosphate and to generate diacylglycerol and Ins-1,4,5-P₃ (44). The generation of Ins-1,4,5-P₃ after agonist stimulation leads to the release of ER Ca²⁺, [Ca²⁺], oscillations are believed to depend on Ins-1,4,5-P₃ receptors releasing Ca²⁺ in “hotspots” in the ER (45) and the subsequent diffusion of this Ca²⁺ to adjacent sites in the ER, increasing the local sensitivity of the Ins-1,4,5-P₃ receptor and inducing further Ca²⁺ release. Changes in the sensitivity of the ER to Ins-1,4,5-P₃ are likely to be important in the generation of repetitive [Ca²⁺] spikes. Redox sensitivity of the Ins-1,4,5-P₃ receptor has previously been reported in hepatocytes (46–48), and we previously showed that NADPH oxidase activation increases the sensitivity of intracellular Ca²⁺ stores to Ins-1,4,5-P₃ in HAEC. The finding, that 10 μM H₂O₂ “restores” [Ca²⁺], oscillations in histamine-stimulated HAEC expressing the dominant negative form of Rac1 but does not affect Ins-1,4,5-P₃ levels in histamine-stimulated HAEC, is consistent with the notion that histamine-stimulated ROS increase Ins-1,4,5-P₃ receptor sensitivity and thereby affect the generation of [Ca²⁺], oscillations.

Histamine-stimulated ROS may also affect upstream signaling pathways in HAEC. For example, we recently showed that activation of phospholipase D (PLD), which exhibits redox sensitivity in endothelial cells (49), regulates [Ca²⁺], oscillation frequency in HAEC during histamine stimulation (50). It is not likely that the effect of histamine-stimulated ROS on the generation of [Ca²⁺], oscillations is related to PLD signaling, however, because time-dependent activation of PLD by histamine in HAEC is not rapid enough to affect generation of oscillations. Stimulation of HAEC by histamine activates PLD by 5 min, but no significant effect is observed 1 min after stimulation. Moreover, inhibition of PLD decreases oscillation frequency, but does not inhibit the generation of [Ca²⁺], oscillations during histamine stimulation (50). Alternatively, histamine-stimulated ROS may modulate [Ca²⁺], oscillations by an effect on PLC-γ, because generation of Ins-1,4,5-P₃ by agonists like bradykinin appears to be secondary to tyrosine phosphorylation of PLC-γ1 (51) and H₂O₂ is known to activate PLC-γ (52). We do not believe this mechanism is likely to play a role during histamine stimulation, because 1 μM histamine stimulated only

**Fig. 3.** Histamine-stimulated [Ca²⁺], oscillations are blocked by NADPH oxidase inhibition. A, representative tracing of five similar experiments from indo 1-loaded HAEC stimulated sequentially by 1 μM histamine with a 10-min washout period between exposures. Repetitive [Ca²⁺], oscillations were observed during histamine stimulation but not during washout. B, representative tracing of four similar experiments from indo 1-loaded HAEC stimulated by 1 μM histamine first in control buffer and then a second time in the presence of 10 μM DPI. Only a single [Ca²⁺], spike and no oscillations were observed in the presence of DPI. C, representative tracing of three similar experiments from indo 1-loaded HAEC stimulated by 1 μM histamine first in control buffer and then a second time in the presence of 2 μM FTase inhibitor. Only a single [Ca²⁺], spike was observed in two of three HAEC; in a third, several irregularly occurring [Ca²⁺], spikes with decreasing amplitude were observed over ~15 min, and then no further increases in [Ca²⁺], occurred during the observation period.
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weak tyrosine phosphorylation of PLC-γ1 and histamine-stimulated tyrosine phosphorylation of PLC-γ2 was not inhibited by DPI, by an FTase inhibitor, or in cells lacking functional Rac1 protein (data not shown).

ROS may also be important in the generation of [Ca\textsuperscript{2+}i] oscillations, because of an effect on other redox-sensitive Ca\textsuperscript{2+} release mechanisms that are activated by histamine. For example, the ryanodine receptor (RyR) may be important in histamine-stimulated Ca\textsuperscript{2+} signaling. It was previously shown that blocking ryanodine-sensitive Ca\textsuperscript{2+} release inhibits [Ca\textsuperscript{2+}i] oscillations in endothelial cells stimulated by histamine (53). Redox regulation of the RyR is well-established in cardiac and skeletal muscle, with sulfhydryl oxidation, S-nitrosylation, or modification of sulfhydryl groups of the RyR by other oxidants increasing Ca\textsuperscript{2+} release channel activity (54). Hyper-reactive cysteine moieties may represent biochemical components of a transmembrane redox sensor within the RyR channel complex that conveys information about localized changes in redox potential induced by different stimuli (55, 56).

The finding that ROS play a role in agonist-stimulated Ca\textsuperscript{2+} signaling is novel and important in cell biology, reinforcing the potential produced by different stimuli (55, 56).

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