NADPH Oxidase Activation Increases the Sensitivity of Intracellular Ca\(^{2+}\) Stores to Inositol 1,4,5-Trisphosphate in Human Endothelial Cells*

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Many stimuli that activate the vascular NADPH oxidase generate reactive oxygen species and increase intracellular Ca\(^{2+}\), but whether NADPH oxidase activation directly affects Ca\(^{2+}\) signaling is unknown. NADPH stimulated the production of superoxide anion and H\(_2\)O\(_2\) in human aortic endothelial cells that was inhibited by the NADPH oxidase inhibitor diphenyleneiodonium and was significantly attenuated in cells transiently expressing dominant negative alleles of the small GTP-binding protein Rac1 which is required for oxidase activity. In permeabilized Mag-indo 1-loaded cells, NADPH and H\(_2\)O\(_2\) each decreased the threshold concentration of inositol 1,4,5-trisphosphate (InsP\(_3\)) required to release intracellularly stored Ca\(^{2+}\) and shifted the InsP\(_3\)-Ca\(^{2+}\) release dose-response curve to the left. Concentrations of H\(_2\)O\(_2\) as low as 3 \(\mu\)M increased the sensitivity of intracellular Ca\(^{2+}\) stores to InsP\(_3\) and decreased the InsP\(_3\) EC\(_{50}\) from 423.2 \(\pm\) 54.9 to 276.9 \(\pm\) 14.4 nM. The effect of NADPH on InsP\(_3\)-stimulated Ca\(^{2+}\) release was blocked by catalase and by diphenyleneiodonium and was not observed in cells lacking functional Rac1 protein. Thus, NADPH oxidase-derived H\(_2\)O\(_2\) increases the sensitivity of intracellular Ca\(^{2+}\) stores to InsP\(_3\) in human endothelial cells. Since Ca\(^{2+}\)-dependent signaling pathways are critical to normal endothelial function, this effect may be of great importance in endothelial signal transduction.

The endothelial cell membrane contains an NADPH oxidase-like H\(_2\)O\(_2\)-generating enzyme (1, 2) that is stimulated by posthypoxic reoxygenation (3), cyclic stretch (4, 5), and low density lipoprotein (6). The signal transduction pathways stimulated following activation of the NADPH oxidase in the vascular endothelium have not been completely characterized. Many stimuli that activate the oxidase also increase endothelial cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) (7–10), but whether activation of the NADPH oxidase affects endothelial Ca\(^{2+}\) signaling is unknown.

We recently showed that H\(_2\)O\(_2\) stimulates [Ca\(^{2+}\)]\(_i\) oscillations in human aortic endothelial cells (HAEC)\(^1\) (11). In contrast to other agonists that stimulate [Ca\(^{2+}\)]\(_i\) oscillations in endothelial cells like bradykinin (12), histamine (13), and adenosine triphosphate (12), H\(_2\)O\(_2\) does not increase levels of inositol 1,4,5-trisphosphate (InsP\(_3\)) at the concentrations that produce [Ca\(^{2+}\)]\(_i\) oscillations (14). Since the H\(_2\)O\(_2\)-generating enzyme xanthine oxidase has been shown to decrease luminal Ca\(^{2+}\) content in vascular endothelial cells (15) and since redox sensitivity of the InsP\(_3\) receptor has been demonstrated in other cell types (16, 17), we hypothesized that activation of the NADPH oxidase affects endothelial Ca\(^{2+}\) signaling by increasing the sensitivity of intracellular Ca\(^{2+}\) stores to InsP\(_3\)-stimulated Ca\(^{2+}\) release. To test this hypothesis, the effect of NADPH oxidase stimulation on intracellular Ca\(^{2+}\) stores was examined in permeabilized HAEC using the low affinity (micromolar range) Ca\(^{2+}\)-sensitive fluorescent indicator Mag-indo 1. To characterize the specific role of NADPH oxidase stimulation, diphenyleneiodonium was used to pharmacologically inhibit the oxidase, and studies were performed using HAEC transiently expressing Rac1\(^1\)\(^17\) (18), a dominant negative allele of the small GTP-binding protein Rac1 that is required for oxidase activity.

**MATERIALS AND METHODS**

**Culture of HAEC—**HAEC were obtained as proliferating quaternary cultures (Clonetics, San Diego, CA) and were grown to confluence to passaged 5–9 in endothelial cell growth medium supplemented with 2% fetal bovine serum, 10 \(\mu\)g/liter human-recombinant epidermal growth factor, 1 ng/liter hydrocortisone, 50 \(\mu\)g/ml gentamicin, 50 ng/ml amphotericin-B, and 12 \(\mu\)g/ml bovine brain extract (Clonetics) in a 37 °C humidified atmosphere of 95% air, 5% CO\(_2\). For Ca\(^{2+}\) measurements, HAEC were plated at an approximate concentration of 1 \(\times\) 10\(^5\)/ml on 25-mm diameter circular glass coverslips (WVR Scientific, Media, PA), which were precoated with 2% gelatin solution (Sigma) and washed three times with phosphate-buffered saline (Quality Biological, Inc., Gaithersburg, MD) before cell seeding. 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\(_2\).

**Determination of Superoxide Generation by HAEC—**Superoxide (O\(_2^{-}\)) production by suspensions of permeabilized HAEC (~8 \(\times\) 10\(^5\) cells/ml) was detected by lucigenin luminescence using a Berthold Multi-Biolumat LB-9505C luminometer (Nashua, NH). Lucigenin luminescence was recorded at room temperature continuously over 30–45 min from test tubes of HAEC with 25 \(\mu\)M lucigenin in intracellular-like medium (ICM) containing 125 mM KCl, 19 mM NaCl, 10 mM HEPES, 1 mM EGTA (Sigma), and 0.33 mM CaCl\(_2\) (free Ca\(^{2+}\) concentration was 50 nM, pH adjusted to 7.20 at room temperature with KOH). Chemiluminescence counts were integrated over 3–4 min from the time of the addition of 100 \(\mu\)M NADPH to plateau. In some experiments, HAEC were preincubated for 15 min with either superoxide dismutase (SOD; 200 units/ml) or with the NADPH oxidase inhibitor diphenyleneiodonium (DPI; Color cell’s); InsP\(_3\), inositol 1,4,5-trisphosphate; DPI, diphenyleneiodonium; NS, not significant; SOD, superoxide dismutase.

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\(^{1}\) The abbreviations used are: HAEC, human aortic endothelial...
Your Enzyme, Ontario Canada; 10 μM), which were each also present during the chemiluminescence recording.

Measurement of H₂O₂ Production by HAEC—H₂O₂ production by HAEC was measured fluorometrically using the Amplex™ Red Hydrogen Peroxide Assay Kit (Molecular Probes, Inc., Eugene, OR). Amplex™ Red is a fluorogenic substrate with very low background fluorescence, which reacts with H₂O₂ with a 1:1 stoichiometry to produce highly fluorescent resorufin (19). Measurements of H₂O₂ production after the addition of NADPH were performed using suspensions (3.1 μM) of permeabilized HAEC. Fluorescence intensity was measured in a Cytofluor 2300 System (Millipore Corp., Bedford, MA) at an excitation wavelength of 530 ± 25 nm and an emission wavelength of 590 ± 35 nm at room temperature. After subtracting background fluorescence, cumulative H₂O₂ concentrations (μM/10⁶ cells) were calculated using a resorufin-H₂O₂ standard calibration curve generated from cell-free experiments using H₂O₂ and Amplex™ Red. In some experiments, HAEC were preincubated for 30 min prior to NADPH addition with either DPI (10 μM), catalase (1000 units/ml), or SOD (200 units/ml). These inhibitors were also present during fluorescence measurements.

Measurement of Ca²⁺ Release from Intracellular Ca²⁺ Stores—HAEC monolayers on glass coverslips were incubated in a HEPES (Sigma)-buffered saline containing 10 μM of the ester derivative (acetoxyethyl ester form) of Mag-indo 1 (Molecular Probes) at room temperature for 45 min. The HEPES-buffered saline contained 137 mM NaCl, 4.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 15 mM d-glucose, 20 mM HEPES (pH adjusted to 7.40 at room temperature with NaOH). All salt solutions were made with pure water (free Ca²⁺). The pH of the Ca²⁺-free acid). The pH of the Ca²⁺-free medium was 7.20 (data not shown). These findings are consistent with the known characteristics of Mag-indo 1, whose sensitivity to Mg²⁺ is 100-fold less than to Ca²⁺ (Kd = 31 μM for Mg²⁺ and 35 μM for Ca²⁺, both at 22°C, pH 7.40) (22). The
Mag-indo 1 ratio was sensitive to application of InsP₃, and the rate of the increase in the Mag-indo 1 ratio during filling of intracellular Ca²⁺ stores was inhibited by the endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin (data not shown). HAECTransiently Expressing the Dominant Negative Allele of Rac1—An adenosin receptor encoding the Myc epitope-tagged-dominant negative Rac1 cDNA containing a substitution at position 17 (R17N) was used as described previously (18). Expression of the R17N mutant was confirmed by protein immunoblotting with an antibody to the Myc epitope (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistical Analysis—Data are reported as mean ± S.E. Statistical comparisons were made using Student’s t test for the paired and the unpaired groups. An analysis of variance was used when multiple comparisons were performed. A difference was considered significant at p < 0.05.

RESULTS

NADPH-stimulated O₂⁻ Generation in HAEC—Neither HAEC suspensions alone nor NADPH alone generated detectable chemiluminescence in the presence of 25 μM lucigenin. Following the addition of 100 μM NADPH to HAEC suspensions (Fig. 1), a rapid increase in O₂⁻ production occurred (1.92 ± 0.11 cm⁻¹ pm⁻¹ 10⁶ cells, n = 11), which peaked at approximately 15 min. Significant inhibition of NADPH-stimulated O₂⁻ generation was observed in the presence of SOD (0.72 ± 0.03 cm⁻¹ pm⁻¹ 10⁶ cells, p < 0.05, n = 4) or DPI (0.02 ± 0.01 cm⁻¹ pm⁻¹ 10⁶ cells, p < 0.05, n = 5).

Effect of NADPH on H₂O₂ Production by HAEC—The effect of NADPH on H₂O₂ production by HAEC was examined by measuring resorufin fluorescence intensity from HAEC suspensions after NADPH addition (1–300 μM). NADPH stimulated production of H₂O₂ in a time- and concentration-dependent manner (Fig. 2A), with a threshold of ~30 μM. H₂O₂ production reached a plateau after ~12.5 min. The apparent maximum rate of H₂O₂ production was 0.46, 0.86, and 1.11 μM/min/10⁶ cells after the addition of 30, 100, and 300 μM, respectively. No measurable spontaneous H₂O₂ production was observed in the absence of NADPH. H₂O₂ production was significantly inhibited by pretreatment with either catalase or with the NADPH oxidase inhibitor DPI at each concentration of NADPH examined (Fig. 2B). In contrast, SOD did not significantly affect H₂O₂ production at any NADPH concentration (p = NS, n = 3).

Effect of H₂O₂ on InsP₃-sensitive Intracellular Ca²⁺ Stores—In permeabilized HAEC in which Ca²⁺ stores are filled by perfusion with Ca²⁺-, Mg²⁺-, and ATP-containing ICM, 100 μM H₂O₂ did not affect intracellular Ca²⁺ stores during a 15-min exposure (Δ ratio = 0.01 ± 0.00, n = 3, p = NS versus 0.01 ± 0.01, n = 8 for time control). In the same HAEC, however (Fig. 3A), the addition of InsP₃ stimulated a rapid decrease in the content of intracellular Ca²⁺ stores. A higher concentration of H₂O₂ (1 μM for 10 min) in the absence of InsP₃ also did not affect intracellular Ca²⁺ stores in Ca²⁺-replete, permeabilized HAEC (Δ ratio = 0.01 ± 0.01, n = 3, p = NS versus time control).

As shown in Fig. 3B, when HAEC were exposed to a submaximal concentration of InsP₃ (300 nM), the Mag-indo 1 ratio decreased from 1.06 ± 0.05 to 0.92 ± 0.05 (n = 4, p < 0.001) and was then maintained at this level for more than 10 min of observation. The decrease in the content of intracellular Ca²⁺ stores stimulated by InsP₃ was reversibly inhibited by heparin (Δ ratio = 0.02 ± 0.01 with heparin versus 0.01 ± 0.01 for time control, p = NS, n = 8 for each). While 100 μM H₂O₂ did not affect intracellular Ca²⁺ stores in permeabilized HAEC in the absence of InsP₃, during an established response to a submaximal InsP₃ concentration of 300 nM (Fig. 3C), 100 μM H₂O₂ further decreased the content of intracellular Ca²⁺ stores (Δ ratio = 0.31 ± 0.05, n = 10, in the presence of H₂O₂ plus InsP₃ versus 0.16 ± 0.03, n = 10, in the presence of InsP₃ alone, p < 0.001). This effect was also abolished by pretreatment with heparin (Δ ratio = 0.02 ± 0.01, n = 4, in the presence of H₂O₂, InsP₃, and heparin, p = NS versus time control, Fig. 3D).

Effect of H₂O₂ on the InsP₃ Dose-Response Relationship—After filling of Ca²⁺ stores in Mag-indo 1-loaded, permeabilized HAEC, InsP₃ stimulated a dose-dependent decrease in the content of intracellular Ca²⁺ stores with a threshold concentration of >10 nM and a maximal concentration of approximately 1 μM (Fig. 4A). At a concentration of 10 nM InsP₃, the change in the Mag-indo 1 ratio was not significant (0.01 ± 0.00, p = NS, n = 5). Higher concentrations of InsP₃ reduced the content of intracellular Ca²⁺ stores (Δ ratio = 0.09 ± 0.01 for 100 nM InsP₃, 0.17 ± 0.01 for 300 nM, and 0.50 ± 0.05 for 1 μM, n = 5 for each). A concentration of 3 μM InsP₃ did not produce an additional decrease in the content of intracellular Ca²⁺ stores (Δ ratio = 0.54 ± 0.06, p = NS versus 1 μM InsP₃, n = 5). The addition of ionomycin stimulated an additional decrease in the content of intracellular Ca²⁺ stores following a maximal concentration of InsP₃, demonstrating the presence of addi-
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A

B

C

D
tional InsP₃-insensitive intracellular Ca²⁺ stores in HAEC (Fig. 4A).

H₂O₂ (3–300 μM) shifted the InsP₃-stimulated intracellular Ca²⁺ release dose-response curve to the left, decreased the threshold concentration to 10 nMInsP₃, and decreased the EC₅₀ in response to InsP₃ (Fig. 4, B and C). In the presence of 100 μM H₂O₂, a significant decrease in the Mag-indo 1 ratio was observed during exposure to 10 nMInsP₃ (Δ ratio = 0.10 ± 0.01, n = 4, p < 0.001 versus 10 nMInsP₃ alone). When permeabilized HAEC were exposed to 100 and 300 nMInsP₃ in the presence of 100 μM H₂O₂, the decrease in the content of intracellular Ca²⁺ stores was greater than that observed in the absence of H₂O₂ (Δ ratio = 0.24 ± 0.04 versus 0.09 ± 0.01 for 100 nMInsP₃, 0.43 ± 0.05 versus 0.17 ± 0.01 for 300 nMInsP₃, p < 0.05 at each concentration, n = 4). In the presence of 100 μM H₂O₂, the decrease in the Mag-indo 1 ratio stimulated by 1 μMInsP₃ (Δ ratio = 0.64 ± 0.03) and by 3 μMInsP₃ (Δ ratio = 0.65 ± 0.03) was not significantly different from that stimulated by these concentrations of InsP₃ in the absence of H₂O₂ (p = NS, n = 4 for each concentration). Similarly, the change in the Mag-indo 1 ratio upon stimulation by ionomycin was not different in the presence (0.75 ± 0.03) versus the absence of H₂O₂ (0.67 ± 0.06, p = NS, n = 4 for each). As shown in Fig. 4C, H₂O₂ produced a dose-dependent decrease in the EC₅₀ of InsP₃. At a concentration of 100 μM H₂O₂, the EC₅₀ of InsP₃-stimulated Ca²⁺ release was decreased from 423.2 ± 54.9 nM to 145.2 ± 23.3 nM (p < 0.05, n = 6). A significant effect of H₂O₂ on InsP₃-stimulated Ca²⁺ release was observed at a concentration of H₂O₂ as low as 3 μM. At this concentration, H₂O₂ decreased the EC₅₀ of InsP₃-stimulated Ca²⁺ release by more than 30% to 276.9 ± 14.4 nM (p < 0.05, n = 6). No additional effect on the EC₅₀ of InsP₃ was observed at H₂O₂ concentrations above 100 μM.

Effect of NADPH on InsP₃-sensitive Intracellular Ca²⁺ Stores—To determine the effect of NADPH oxidase stimulation on InsP₃-sensitive intracellular Ca²⁺ stores, Mag-indo 1-loaded HAEC were stimulated by the addition of 100 μM NADPH. This concentration of NADPH has previously been used to stimulate the oxidation in endothelial cells (2, 3, 23). The addition of 100 μM NADPH to permeabilized HAEC rapidly and irreversibly decreased the Mag-indo 1 ratio by 0.08 ± 0.01 (n = 4) over 10 min, due primarily to an increase in the fluorescence intensity of Mag-indo 1 at the 485-nm wavelength. This appeared to be an effect on the indicator itself, rather than an effect on intracellular Ca²⁺ stores, since in a cell-free system, the ratio of Mag-indo 1 free acid (10 μM in 100 μM free Ca²⁺) was similarly decreased (0.08 ± 0.02, n = 2). NADPH shifted the InsP₃ dose-response relationship to the left, reduced the threshold concentration of InsP₃, and decreased the EC₅₀ in response to InsP₃. At a concentration of 10 nM InsP₃, which does not decrease the content of intracellular Ca²⁺ stores in HAEC in the absence of NADPH, the Mag-indo 1 ratio decreased by 0.10 ± 0.01 in the presence of 100 μM NADPH, n = 4, p < 0.001 versus 10 nMInsP₃ alone, Fig. 5A). The effects of 100 and 300 nMInsP₃

Fig. 3. Effect of H₂O₂ on intracellular store [Ca²⁺]. A, H₂O₂ (100 μM) did not affect intracellular store [Ca²⁺] when added to permeabilized, Ca²⁺-filled HAEC. After wash-out of H₂O₂, the subsequent addition of 3 μMInsP₃ and 1 μMionomycin decreased intracellular store [Ca²⁺] (n = 4). B, addition of 300 nMInsP₃ decreased intracellular store [Ca²⁺]. C, following the decrease in intracellular store [Ca²⁺] by a submaximal addition of InsP₃ (300 nM), 100 μM H₂O₂ decreased intracellular store [Ca²⁺] in the continued presence of InsP₃. D, the effects of both submaximal (300 nM) InsP₃ and H₂O₂ (100 μM) were blocked by pretreatment with 0.5 mg/ml heparin. In all tracings, both the Mag-indo 1 fluorescence ratio (left) and the approximate intracellular store [Ca²⁺] (right) are shown.
on intracellular Ca\textsuperscript{2+} release were similarly enhanced by 100 μM NADPH (Δ ratio = 0.22 ± 0.04 for 100 nM InsP\textsubscript{3}, p < 0.01 versus 100 nM InsP\textsubscript{3} alone and Δ ratio = 0.31 ± 0.05 for 300 nM InsP\textsubscript{3}, p < 0.05 versus 300 nM InsP\textsubscript{3} alone, n = 4 for each concentration). The decrease in the content of intracellular Ca\textsuperscript{2+} stores stimulated by 1 and 3 μM InsP\textsubscript{3} was not affected by the presence of NADPH.

As shown in Fig. 6, NADPH produced a dose-dependent decrease in the EC\textsubscript{50} of InsP\textsubscript{3}, which became significant at a concentration of 100 μM. At this concentration, NADPH decreased the EC\textsubscript{50} in response to InsP\textsubscript{3} by more than 50% from 423.2 ± 54.9 to 170.6 ± 23.2 nM. No additional effect on the EC\textsubscript{50} of InsP\textsubscript{3} was observed at NADPH concentrations above 100 μM. The effect of 100 μM NADPH on InsP\textsubscript{3}-stimulated intracellular Ca\textsuperscript{2+} release was blocked by pretreatment with either 1000 units/ml catalase (EC\textsubscript{50} = 372.9 ± 28.7 nM, n = 4, p = NS versus control, Fig. 5B) or by 10 μM DPI (EC\textsubscript{50} = 330.5 ± 41.7 nM, n = 4, p = NS versus control, Fig. 5C) but was unaffected by 200 units/ml SOD (EC\textsubscript{50} = 198.7 ± 37.3 nM, p < 0.05 versus control, p = NS versus 100 μM NADPH alone). Neither catalase, DPI, nor SOD themselves affected the sensitivity to InsP\textsubscript{3}-stimulated intracellular Ca\textsuperscript{2+} release (InsP\textsubscript{3} EC\textsubscript{50} = 383.7 ± 31.8 for catalase, 418.2 ± 32.8 nM for DPI, 389.8 ± 42.4 nM for SOD, n = 3, p = NS versus control for each).

Importance of Rac1 in NADPH-induced H\textsubscript{2}O\textsubscript{2} Production and Increased Sensitivity to InsP\textsubscript{3}—To further characterize the role of NADPH oxidase activation in stimulating the production of H\textsubscript{2}O\textsubscript{2} and increasing the sensitivity to InsP\textsubscript{3}-stimulated intracellular Ca\textsuperscript{2+} release, studies were performed in HAEC transiently expressing Rac1 N17, a dominant negative allele of Rac1 (18). When transfected cells were stimulated with NADPH, H\textsubscript{2}O\textsubscript{2} production was significantly reduced in comparison with vector controls (Fig. 7A) at all three NADPH concentrations examined. At 100 μM NADPH, H\textsubscript{2}O\textsubscript{2} production in
vector controls (4.8 ± 0.5 μM/10^5 cells) was similar to that observed in nontransfected cells (5.3 ± 0.4 μM/10^5 cells, n = 4, p = NS; see Fig. 2). In contrast, H_2O_2 production in Rac^−/− HAEC after exposure to 100 μM NADPH was significantly decreased (1.7 ± 0.2 μM/10^5 cells, p < 0.01 versus vector control).

To determine the importance of functional Rac1 protein in the increased sensitivity of intracellular Ca^{2+} stores to InsP_3 stimulated by NADPH, both Rac^−/− HAEC and vector control cells were permeabilized and pretreated with 100 μM NADPH for 30 min before being stimulated by InsP_3. As shown in Fig. 7B, the decrease in the EC_{50} to InsP_3 produced by 100 μM NADPH in vector control cells (394.8 ± 46.5 versus 175.0 ± 30.5 nM, p < 0.05) was not observed in Rac^−/− HAEC (412.4 ± 51.2 versus 386.8 ± 58.1 nM, p = NS). In contrast, the effect of 100 μM H_2O_2 on InsP_3-sensitive intracellular Ca^{2+} release was not different in Rac^−/− and vector controls.

**DISCUSSION**

This study shows that NADPH oxidase activation stimulates the production of H_2O_2 and increases the sensitivity of intracellular Ca^{2+} stores to InsP_3 in human endothelial cells. A vascular NADH/NADPH oxidase is a major source of reactive oxygen species in the vasculature (24), and previous studies have demonstrated the expression of one or more components of the NADPH oxidase in endothelial cells (1, 2). Although it has been suggested that reactive oxygen species produced upon activation of the vascular oxidase may play a role in endothelial dysfunction (25–27), the signal transduction pathways stimulated following activation of the endothelial NADPH oxidase are largely unknown. Many stimuli that activate the oxidase increase endothelial [Ca^{2+}], but this is the first study to show a direct effect of NADPH oxidase activation on endothelial Ca^{2+} signaling.

In the present study, stimulation of the endothelial NADPH oxidase by NADPH (2, 3, 23) produced H_2O_2 in a time- and concentration-dependent manner. That NADPH stimulated H_2O_2 production via NADPH oxidase activation is supported by the finding that H_2O_2 production was inhibited by DPI and was significantly reduced in cells lacking a functional GTP-binding protein Rac1, which is required for oxidase activity. NADPH oxidase stimulation generated O_2^{•−} in the present study, and this is most likely the source of oxidase-derived H_2O_2. Although neither NADPH nor H_2O_2 alone affected intracellular Ca^{2+} stores, each stimulated additional release of intracellular Ca^{2+} stores during an established response to a submaximal concentration of InsP_3. In the presence of either H_2O_2 or NADPH, the threshold concentration of InsP_3-stimulated intracellular Ca^{2+} release was decreased to <10 nM, the InsP_3 dose-response curve was shifted to the left, and the EC_{50} to InsP_3 was significantly decreased. Heparin, a competitive inhibitor of InsP_3-induced Ca^{2+} release (28), blocked the effect of H_2O_2 in the presence of a submaximal concentration of InsP_3. This suggests that the addition of H_2O_2 or the generation of H_2O_2 by NADPH affects intracellular Ca^{2+} stores by increasing the sensitivity of the InsP_3 receptor in HAEC. The finding that the effect of NADPH on the sensitivity of intracellular Ca^{2+} stores to InsP_3 was blocked by catalase but was not affected by SOD suggests a specific effect of H_2O_2 on the InsP_3 receptor. Redox sensitivity of the InsP_3 receptor has been reported previously in other cell types (16, 17).

The present study suggests that small changes in the intracellular concentration of H_2O_2 are likely to have significant effects on InsP_3-sensitive intracellular Ca^{2+} stores, given the effects on the EC_{50} of InsP_3 of relatively small changes in the concentration of either H_2O_2 or NADPH. Such a narrow range between subthreshold and maximal responses suggests that the intracellular concentration of H_2O_2 is tightly regulated in vivo. Since the concentrations of H_2O_2 that are generated by vascular cells as a result of NADPH oxidase activation are not accurately known, it is difficult to determine how the concentrations of H_2O_2 observed following NADPH addition to permeabilized cell suspensions in the present study compare with that which occurs in vivo. Importantly, however, even concentrations of H_2O_2 in the low micromolar range increased the sensitivity of intracellular Ca^{2+} stores to InsP_3 in HAEC.

Our data indicate that NADPH oxidase-derived H_2O_2 decreases the threshold concentration for intracellular Ca^{2+} release in endothelial cells to <10 nM InsP_3. Since basal intracellular levels of InsP_3 are in the 50 nM range (29, 30), stimuli that activate the oxidase may affect endothelial Ca^{2+} signaling,
even without altering levels of InsP$_3$. It may be of interest in this regard that we recently showed that H$_2$O$_2$, at concentrations that do not increase levels of InsP$_3$ (14), stimulate Ca$^{2+}$ oscillations in human endothelial cells (11). Since intracellular Ca$^{2+}$ oscillations can occur even at constant levels of InsP$_3$ (31), the increased sensitivity of intracellular Ca$^{2+}$ stores to InsP$_3$ resulting from NADPH oxidase activation may facilitate the generation of intracellular Ca$^{2+}$ oscillations or the regulation of their frequency. Such an effect may underlie recent observations in platelet-derived growth factor-stimulated vascular smooth muscle cells. In this cell type, platelet-derived growth factor induces intracellular Ca$^{2+}$ oscillations (32), generates O$_2$. and stimulates the activity of the proinflammatory transcription factor NF-$\kappa$B (33). Activation of NF-$\kappa$B by platelet-derived growth factor is inhibited by both SOD and DPI, suggesting a link between NADPH oxidase activation and proinflammatory signaling pathways in the vasculature. Since the frequency of intracellular Ca$^{2+}$ oscillations regulates the activity of proinflammatory transcription factors like NF-$\kappa$B (34, 35), the effect of NADPH oxidase activation on InsP$_3$-mediated Ca$^{2+}$ release reported in this study may be of great importance in vascular signal transduction.

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