Hydrogen Peroxide, Potassium Currents, and Membrane Potential in Human Endothelial Cells

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Background—Hydrogen peroxide (H₂O₂) and reactive oxygen species are implicated in inflammation, ischemia-reperfusion injury, and atherosclerosis. The role of ion channels has not been previously explored.

Methods and Results—K⁺ currents and membrane potential were recorded in endothelial cells by voltage- and current-clamp techniques. H₂O₂ elicited both hyperpolarization and depolarization of the membrane potential in a concentration-dependent manner. Low H₂O₂ concentrations (0.01 to 0.25 μmol/L) inhibited the inward-rectifying K⁺ current (K_ir). Whole-cell K⁺ current analysis revealed that H₂O₂ (1 mmol/L) applied to the bath solution increased the Ca²⁺-dependent K⁺ current (KCa) amplitude. H₂O₂ increased KCa current in outside-out patches in a Ca²⁺-free solution. When catalase (5000 μ/mL) was added to the bath solution, the outward-rectifying K⁺ current amplitude was restored. In contrast, superoxide dismutase (1000 u/mL) had only a small effect on the H₂O₂-induced K⁺ current changes. Next, we measured whole-cell K⁺ currents and redox potentials simultaneously with a novel redox potential-sensitive electrode. The H₂O₂-mediated KCa current increase was accompanied by a whole-cell redox potential decrease.

Conclusions—H₂O₂ elicited both hyperpolarization and depolarization of the membrane potential through 2 different mechanisms. Low H₂O₂ concentrations inhibited inward-rectifying K⁺ currents, whereas higher H₂O₂ concentrations increased the amplitude of the outward K⁺ current. We suggest that reactive oxygen species generated locally increases the KCa current amplitude, whereas low H₂O₂ concentrations inhibit K_ir via intracellular messengers. (Circulation. 1999;13:1719-1725.)

Key Words: nitric oxide ▪ potassium ▪ free radicals ▪ endothelium

Hydrogen peroxide (H₂O₂) is important in inflammation, ischemia-reperfusion injury, and atherosclerosis. The endothelium is exposed to local reactive oxygen species generated by neutrophils and monocytes. In vitro, oxidative stress can be produced either by direct application of H₂O₂ or by continuous H₂O₂ generation in enzymatic systems. An interaction between H₂O₂ and the endothelium may modulate the cellular redox balance. H₂O₂ may also trigger multiple functions, such as the release of lactate dehydrogenase and tissue factor. Exposure of endothelial cells to H₂O₂ decreases cellular ATP and inhibits protein synthesis. H₂O₂ stimulates adherence of neutrophils to isolated canine carotid arteries, veins, and cultured endothelial cells. Reactive oxygen species (H₂O₂, O₂⁻, and OH⁻) disturb Ca²⁺ homeostasis in renal tubular epithelial cells and mammalian ovarian cells. In addition, H₂O₂ was recently found to regulate intracellular Ca²⁺ signaling by stimulating Ca²⁺ release from the inositol triphosphate–sensitive stores in venous endothelial cells, bovine pulmonary endothelial cells, and human endothelial cells. Although H₂O₂ has been shown to regulate intracellular and intercellular signal transduction, few data are available on the regulation of endothelial cell membrane potential by H₂O₂. Membrane potential contributes to the regulation of the release of vasoactive compounds and influences the contractile state of underlying vascular smooth muscle. Furthermore, control of the membrane potential is an essential requirement in cell growth. We investigated the effects of H₂O₂ on endothelial cell membrane potential, ion currents, and membrane polarization. Because H₂O₂ can oxidize and reduce the cellular membrane components, we also examined links between ionic currents and the redox potential of whole endothelial cells.

Materials

Iberotoxin was obtained from RBI (Natick). DIDS (4,4'-diisothiocyanato-2,2'-disulfonyl acid stilbene), niflumic acid, EGTA, 4-aminopyridine, HEPES, and A23187 were purchased from Sigma-Aldrich. All salts and H₂O₂ were obtained from Merck. Human umbilical vein endothelial cells were isolated from umbilical cords by chymotrypsin treatment. Cells were subcultured in EGM (Clonetics) with 2% FCS and were used between passages 1 and 2.

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**K⁺ CurrentRecordings**

Whole-cell K⁺ currents were measured by conventional patch clamp3 or by perforated patch with nystatin.24 The external solution E1 contained (in mmol/L) 140 NaCl, 1.8 CaCl₂, 1 MgCl₂, 5.4 KCl, and 10 Na-HEPES, pH 7.4. The E2 solution contained (in mmol/L) 90.4 NaCl, 1.8 CaCl₂, 1 MgCl₂, 50 KCl, and 10 Na-HEPES, pH 7.4. The patch pipette, filled with a solution containing (in mmol/L) 80 K-aspartate, 30 KCl, 20 NaCl, 1 MgCl₂, 3 Mg-ATP, 10 EGTA, and 5 K-HEPES, pH 7.4. The Cs⁺-dialyzing pipette solution contained (in mmol/L) 80 Cs-aspartate, 40 CsCl, 10 tetraethylammonium chloride, 1 MgCl₂, 3 Mg-ATP, 10 EGTA, and 5 Cs-HEPES, pH 7.4. In the experiments recording single channel activity in the outside-out configuration, 1.5 mmol/L EGTA was used in the intracellular solution. Ca²⁺ was added to the solution to obtain 3 μmol/L free Ca²⁺ concentration. Na⁺ ions replaced Ca²⁺ ions when Ca²⁺-free solution was used. Solutions were perfused through the chamber by gravity at a rate of 1 mL per 10 seconds. Experiments were done at room temperature. Nystatin (Sigma) was dissolved in dimethyl sulfoxide and diluted in the pipette solution to give a final concentration ranging from 50 to 100 μg/mL. Whole-cell access was achieved by nystatin within 10 to 20 minutes of seal formation. Whole-cell K⁺ currents were recorded at 5 to 10 kHz with an Axopatch 200A or a List EPC-7 amplifier, filtered at 1 kHz with an 8-pole low-pass Bessel filter instrument (Frequency Devices), digitized with a CED1401 interface (Cambridge Electronic Design Ltd), and analyzed with CED Patch and Voltage Clamp Software version 6.08. We performed 2 electrode recordings from 1 endothelial cell with 2 amplifiers. One was used in the voltage-clamp mode to record K⁺ currents. The second was connected to the redox sensor in the current-clamp mode. Series resistance and total cell capacitance were calculated from uncompensated capacitive transients, from 10-ms hyperpolarizing linear ramp pulses (10 mV), or by adjustment of the series resistance and whole-cell capacitance controls of the Axopatch 200A amplifier to eliminate the resulting current transitions. The membrane input resistance of the cells was measured with small hyperpolarizing voltage pulses (10 mV for 10 ms) from a holding potential of –40 mV. A UV lamp (Braun HUV1) was used to expose endothelial cells treated with H₂O₂ to UV flashes for 2 to 6 seconds. Voltage ramps were used routinely to measure instantaneous current-voltage relations (IV curves). Ramps from –150 to 150 mV for 500 ms were applied from a holding potential of 0 mV.

**Redox SensorPreparation**

Redox-sensitive glass electrodes were synthesized by the melting of appropriate quantities of transition metal and glass-forming oxides in a furnace at 800°C to 900°C for 12 to 30 hours with a subsequent quenching of the melt.25-26 The synthesized glasses were annealed for 24 hours at 20°C to 30°C below the glass transition temperature to remove the internal stress of vitreous membranes. Bulk sensor membranes were cut from the melt and thoroughly polished with an alumina powder and then a diamond-polishing paste. Platinum or gold was sputtered on the back side of the membrane, and a metallic wire was attached to it by a silver microadhesive. The membranes were then sealed into PVC tubes. Redox microsensors for single cell measurements were prepared by use of a metallic wire coated with a redox-sensitive thin-layer glass electrode. Two techniques were used for coating: sputtering of the bulk glass and dipping of a metallic wire into the glass-forming melt with subsequent quenching and annealing. Redox calibration measurements of the prepared bulk glass sensors and redox microsensors were performed with potassium hexacyanoferrate (II)/(III) solutions K₃[Fe(CN)₆] with a ratio of oxidized to reduced species of 0.01, 0.1, 1, 10, and 100. The total redox concentration varied between 0.05 and 5×10⁻⁹ mol/L.

**Data Analysis**

All values are given as mean±SEM. Wilcoxon rank sum or Mann-Whitney-Wilcoxon tests were used to determine significant differences. A value of p<0.05 was considered statistically significant. The Nernst equation, \( E = \frac{RT}{F} \log [A]/[A]_0 \), with \( A \) and \( A_0 \) denoting the extracellular and intracellular concentrations of an ion, respectively, was used to calculate the equilibrium potentials.

**Results**

**K⁺ Currents in Cultured Endothelial Cells**

The current- voltage relationship for the unstimulated endothelial cells was characterized by a pronounced inward rectification at potentials negative to the K⁺ reversal potential (\( E_{rev} \)) and relatively small outward currents at potentials positive to the \( E_{rev} \) (Figure 1A). The inward K⁺ current (\( I_{K} \)) showed strong rectification at potentials negative to –80 mV with a voltage dependency reported elsewhere.20,24,27 The K⁺ current was recorded in 64 of the 75 cells examined. Second K⁺ current amplitude increased at voltages positive to 27±12 mV under physiological K⁺ gradient and had strong outward rectification. Reversal potential of both the \( I_{K} \) and outward K⁺ current was dependent on the extracellular K⁺ concentration. Varying the [K⁺]o from 5.4 to 50 mmol/L shifted the K⁺ current reversal potential from –75±8 to 28±5 and 0±0.2 mV, respectively (n=20). The whole-cell inward rectifier K⁺ current was reversibly inhibited by Ba²⁺ (100 μmol/L), a typical blocker of the \( I_{K} \) current, to 8±6% at –120 mV. Tetramethylammonium (TEA) was added to the bath solution after the inward rectifier
inhibition by 100 μmol/L Ba²⁺. TEA (1 mmol/L) blocked the outward K⁺ current up to 17±6% (n=6) in the resting endothelial cells. Ca²⁺ ionophore A23187 (1 μmol/L) added to the bath solution ([Ca²⁺]₀=1.8 mmol/L) increased the K⁺ current amplitude to 187±10% at 100 mV (n=10) (Figure 1B). Increased intracellular Ca²⁺ concentration elicited an increase in K⁺ current amplitude and shifted the apparent threshold of the current-voltage relation from 27±4 to −14±8 mV. Measurements were performed in perforated patch configuration. Iberiotoxin (100 μmol/L) inhibited the Kᵣ current stimulated by Ca²⁺ influx (n=8) to 15±3% of the control values.

**H₂O₂-Induced Inhibition of Kᵣ**

Cells were pretreated with 100 μmol/L iberiotoxin to prevent activation of the large-conductance BKCa channels. Experiments were performed in 140 mmol/L of K⁺-containing and Ca²⁺-free solution. Cells were dialyzed with 10 mmol/L EGTA to prevent the interfering influence of the intracellular Ca²⁺ transients to K⁺ currents. Kᵣ was obtained by 10-mV voltage steps over the range of 0 to −150 mV at a holding potential of 0 mV or by ramp changing from −150 to 150 mV. First, Kᵣ was recorded under physiological K⁺ gradients (5.4 mmol/L₀ · 140 mmol/Lᵣ⁻). Then, the extracellular solution was changed to symmetrical K⁺ solution (140 mmol/L₀ · 140 mmol/Lᵣ⁻). H₂O₂ applied to the bath solution decreased Kᵣ in a dose-dependent manner (Figure 2B). H₂O₂ (250 μmol/L) decreased the Kᵣ peak and steady-state current (Figure 2C). The amplitude of the current was measured at −150 mV and plotted against the corresponding H₂O₂ concentration (Figure 2A). IC₅₀ and curve slope were estimated from the best fit of the logistic function and were 110±3 μmol/L and 0.83±0.07, respectively. Interestingly, low H₂O₂ concentrations (0.25 mmol/L) decreased the slope of the I/V curve. In contrast, the high H₂O₂ concentrations (up to 0.5 mmol/L) elicited, in addition to the decreasing slope, a parallel shift of the current-voltage relation toward negative potentials. However, this shift of about 13±4 mV was independent of the extracellular ionic composition. The shift was observed when the bath solution contained 5.4 mmol/L K⁺. When cells were pretreated with iberiotoxin, H₂O₂ elicited an increase in the K⁺ current amplitude from 52±4 to 81±7 pA at 150 mV. Stimulation of the iberiotoxin-insensitive K⁺ current was observed in 4 of the 19 cells examined.

**H₂O₂- and Ca²⁺-Dependent K⁺ Channels**

Next, we examined the effect of H₂O₂ on iberiotoxin-sensitive K⁺ current. The amplitude of the whole-cell K⁺ current induced by H₂O₂ was concentration dependent. The dose-response relationship to the amplitude of the Kᵣ current was examined in the Ca²⁺-free solution containing 140 mmol/L K⁺. Cells were dialyzed with 10 mmol/L EGTA. Kᵣ current was blocked by 100 μmol/L Ba²⁺. Niflumic acid (100 μmol/L) and DIDS were used to block Cl⁻ currents that were described previously in human endothelial cells.²⁰ DIDS (100 μmol/L) elicited an inhibition in the same range as niflumic acid. Thus, we used niflumic acid in subsequent experiments to prevent possible activation of the Cl⁻ current. Niflumic acid (100 μmol/L) in the bath solution inhibited the outward current to 41±5 from 58±4 pA (Figure 3A). These subsequent experiments were performed in the presence of 100 μmol/L Ba²⁺ and 100 μmol/L niflumic acid in the bath solution. H₂O₂ activated the outward-rectifying K⁺ current under these conditions in a dose-dependent manner. The current amplitude was measured at 150 mV and plotted against the corresponding H₂O₂ concentration with a semilogarithmic scale (Figure 3C). The slope and half-maximal activation, calculated from the best fit, were 1.3±0.01 and 0.71±0.05 mmol/L, respectively. Three repetitive H₂O₂ applications in the Ca²⁺-free bath solution elicited repetitive increases in the outward K⁺ current amplitude with subsequent washout.

**Effects of Superoxide Dismutase and Catalase**

We then investigated the effects of superoxide dismutase (SOD) and catalase on these 2 different K⁺ currents using a current-voltage relationship elicited by ramp from −150 mV to 150 mV applied from 0-mV holding potential. We used 1 mmol/L H₂O₂ because at that concentration we
observed all effects described previously. SOD (1000 μmol/L) did not prevent inhibition of the K\textsubscript{IR} current elicited by 1 mmol/L H\textsubscript{2}O\textsubscript{2} (Figure 4A). The amplitude of the outward component of the K\textsubscript{1} current decreased slightly to 88±6% of the maximal activation level. In contrast to SOD, catalase (5000 u/mL) decreased the amplitude of the outward-rectifying K\textsubscript{1} current to control values (Figure 4B). Catalase also inhibited the parallel shift of the K\textsubscript{IR} current altered by H\textsubscript{2}O\textsubscript{2}.

In the next series of experiments, we examined the stimulatory effect of H\textsubscript{2}O\textsubscript{2} on BK\textsubscript{Ca} channels in the membrane patches using the outside-out configuration. The BK\textsubscript{Ca} currents were recorded under a physiological K\textsubscript{1} gradient in Ca\textsuperscript{2+}-free solution. A single channel activity was recorded in 6 patches (Figure 5A). Amplitudes of the single K\textsubscript{1} channels were plotted against the membrane potential. The data were fitted with linear regression analysis (Figure 5B). The slope (181±4 pS) indicated that we recorded large-conductance Ca\textsuperscript{2+}-dependent K\textsubscript{1} channels. The single channel activity was recorded continuously at 0-mV holding potential. H\textsubscript{2}O\textsubscript{2} (0.5 mmol/L) elicited a sustained increase in BK\textsubscript{Ca} channel activity (Figure 5C) without changing the amplitude (Figure 5D). Catalase (5000 u/mL) decreased sustained H\textsubscript{2}O\textsubscript{2}-mediated activation of the BK channels.

**Depolarization and Hyperpolarization of Whole Endothelial Cells**

The membrane potential was recorded in the current-clamp mode. The values at rest ranged from -70 to -20 mV. The
The average membrane potential was \(-52 \pm 17\) mV (n=60). H\(_2\)O\(_2\) (0.5 to 2 mmol/L) added to the bath solution elicited fully reversible hyperpolarization (Figure 6A). The rate of hyperpolarization depended on the H\(_2\)O\(_2\) concentration. We estimated the H\(_2\)O\(_2\) effect on the membrane potential by plotting the difference between membrane potential under control conditions and after H\(_2\)O\(_2\) application to the bath solution. The semilogarithmic plot (Figure 6B) showed a near-Nernstian response of the membrane potential hyperpolarization elicited by H\(_2\)O\(_2\), with a slope of \(64 \pm 6.6\) mV per decade. The repetitive H\(_2\)O\(_2\) application to the bath solution elicited repetitive hyperpolarizations of the membrane potential. H\(_2\)O\(_2\) applied to the bath solution at concentrations <0.25 mmol/L elicited depolarization of the membrane potential from \(-59 \pm 11\) to \(-32 \pm 7\) mV (n=11) (Figure 6C). Barium (100 \(\mu\)mol/L) elicited depolarization from \(-57 \pm 14\) to \(-28 \pm 9\) mV (n=6).

Whole-Cell Redox Potential and Steady-State K\(^+\) Currents With H\(_2\)O\(_2\)

We hypothesized that H\(_2\)O\(_2\) might act through the generation of free radicals, in part by producing the superoxide ion. H\(_2\)O\(_2\) or products of its degradation could oxidize some components of the endothelial cell plasma membrane and thereby modulate the K\(^+\) currents. To examine this possibility, we used UV flash. UV light triggered H\(_2\)O\(_2\) destruction and thereby increased the amount of superoxide ion in the bath solution.28 Outside-out configuration was used to perform the following experiment. H\(_2\)O\(_2\) (0.5 mmol/L), at a concentration lower than the previously estimated IC\(_{50}\), was added first to the bath solution. H\(_2\)O\(_2\) elicited an increase in the KCa current. Next, we exposed the experimental chamber to a UV flash for 2 to 6 seconds, when the KCa current activation reached a
steady-state level (Figure 7A). We observed an increase in the K\text{\textsubscript{Ca}} current amplitude of 291\(\pm\)42% at 150 mV (n=6). UV light alone, without H\textsubscript{2}O\textsubscript{2}, did not augment the K\textsuperscript{+} current amplitude.

We also recorded the K\textsuperscript{+} current and redox potential of whole cells simultaneously with a novel electrode, selectively sensitive to the redox pairs ratio, in aqueous solutions. The redox glass electrode was calibrated in standard solutions containing different ratios of oxidizing to reducing species. The redox potential formed by the Fe\textsubscript{II}/Fe\textsubscript{III} redox pair was measured with the measurement mode of the zero current to record redox potential from endothelial cells. The steady-state K\textsuperscript{+} current was recorded at 0-mV holding potential in the physiological K\textsuperscript{+} gradient. Application of H\textsubscript{2}O\textsubscript{2} (1 mmol/L) to the bath solution elicited an increase in K\textsuperscript{+} current and simultaneously decreased the redox potential (Figure 6C) (n=5). However, the kinetics of the responses were different. The K\textsuperscript{+} current increased exponentially, whereas the redox potential decreased in a stepwise fashion. The redox potential decreased by about \(-28\pm5\) mV (n=5). The K\textsuperscript{+} current increase and the decrease in redox potential elicited by H\textsubscript{2}O\textsubscript{2} were fully reversible.

**Discussion**

H\textsubscript{2}O\textsubscript{2} secretion by activated neutrophils suggests that H\textsubscript{2}O\textsubscript{2} serves as an intercellular messenger. How much H\textsubscript{2}O\textsubscript{2} is produced by neutrophils in vivo is unknown; however, within the microenvironment, the concentration might be quite high and could reach 1 to 2 mmol/L if such an area represents a limited diffusion space. The H\textsubscript{2}O\textsubscript{2} concentrations used in our study are similar to those used in previous studies on cell function. We focused only on very early events in response to H\textsubscript{2}O\textsubscript{2}, which occurred within the first 5 minutes after application of H\textsubscript{2}O\textsubscript{2} to the bath solution. Two main K\textsuperscript{+} currents were present in our cells: the inward-rectifying K\textsubscript{IR} and Ca\textsuperscript{2+}-dependent K\textsuperscript{+} currents. Both currents have been characterized in endothelial cells previously. We found that H\textsubscript{2}O\textsubscript{2} inhibited K\textsubscript{IR} and elicited an increase in the K\textsubscript{Ca} current. Thus, H\textsubscript{2}O\textsubscript{2} could regulate the membrane potential by 2 different mechanisms. Low H\textsubscript{2}O\textsubscript{2} concentrations inhibited K\textsubscript{IR} and depolarized the membrane. Inward-rectifying K\textsuperscript{+} currents were previously reported to regulate the resting membrane potential of endothelial cells. H\textsubscript{2}O\textsubscript{2} at concentrations \(>0.5\) mmol/L increased the amplitude of the K\textsubscript{Ca} current and thus elicited membrane potential hyperpolarization.

H\textsubscript{2}O\textsubscript{2} induced membrane depolarization in cells that had hyperpolarized membrane potentials from \(-55\) to \(-75\) mV. The hyperpolarization elicited by high H\textsubscript{2}O\textsubscript{2} concentrations was resolute in cells with intermediately polarized membrane potentials from \(-25\) to \(-50\) mV. An H\textsubscript{2}O\textsubscript{2}-elicited increase in intracellular Ca\textsuperscript{2+} could lead to K\textsubscript{Ca} current activation. However, we also observed K\textsubscript{Ca} current stimulation by H\textsubscript{2}O\textsubscript{2} in cells dialyzed with 10 mmol/L EGTA in a Ca\textsuperscript{2+}-free solution. High intracellular EGTA concentration buffered all Ca\textsuperscript{2+} release from the intracellular stores triggered by \(50\) \(\mu\)mol/L histamine. We examined 10 cells, and histamine elicited no changes in K\textsubscript{Ca} current amplitude when cells were dialyzed with 10 mmol/L EGTA. We also observed stimulation of BK\textsubscript{Ca} channels by H\textsubscript{2}O\textsubscript{2} in the outside-out membrane patches in a Ca\textsuperscript{2+}-free solution. BK\textsubscript{Ca} channels with similar...
conductances have been found in other endothelial cell preparations.\textsuperscript{29} However, whether BK\textsubscript{Ca} is indeed the unique Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel stimulated by H\textsubscript{2}O\textsubscript{2} is still unknown. Three different types of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels (large, intermediate, and small conductance) were found previously in different preparations of endothelial cells.\textsuperscript{2,8,29,30} 

K\textsubscript{IR} was inhibited by H\textsubscript{2}O\textsubscript{2}, in contrast to the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} current. However, the IC\textsubscript{50} concentration for the inward rectifier was 10 times smaller than for the K\textsubscript{Ca} current. We hypothesized that superoxide ion, the product of H\textsubscript{2}O\textsubscript{2} degradation in aqueous solutions, might increase the K\textsubscript{Ca} current amplitude. Superoxide ion may oxidize some plasma membrane components and thereby could modulate membrane permeability. Recently, oxidation of voltage-gated K\textsuperscript{+} channels was demonstrated.\textsuperscript{31} Concentration of the superoxide ion in bath solution is significantly lower than the H\textsubscript{2}O\textsubscript{2} concentration, which might explain why high H\textsubscript{2}O\textsubscript{2} concentrations stimulated K\textsubscript{Ca} current, compared with inward-rectifying K\textsuperscript{+} current. The experiments performed with UV light support this hypothesis. UV flash dramatically increased the amplitude of the K\textsubscript{Ca} current, whereas H\textsubscript{2}O\textsubscript{2} applied to the bath solution at low concentrations did not increase it. The next line of evidence for a chemical redox reaction came from the simultaneous measurement of the whole-cell K\textsubscript{Ca} current and redox potential. Simultaneous measurements of the K\textsuperscript{+} current and redox potential revealed a strong correlation between K\textsuperscript{+} current increase and redox response. The kinetics of the K\textsuperscript{+} current activation and the increase in redox potential were different. The current increased in an exponential fashion, whereas the redox potential changed in a stepwise manner. The effect was fully reversible and could be repeated. Future studies of endothelial cell responses to different reactive oxygen species, including H\textsubscript{2}O\textsubscript{2}, will clarify under which conditions H\textsubscript{2}O\textsubscript{2}-mediated effects are physiological or injurious to endothelial cells.

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