Contrasting Effects of Hypochlorous Acid and Hydrogen Peroxide on Endothelial Permeability
Prevention with cAMP Drugs

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Activated polymorphonuclear leukocytes generate a cascade of reduced oxygen metabolites. In addition to their antimicrobial role, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) function as inflammatory mediators and increase the protein permeability of the vascular endothelium. The objectives of the present study were to compare the effects of H_2O_2 and HOCl with respect to relative potencies and the time course and magnitude of changes in cell shape and permeability of endothelial cell monolayers derived from bovine pulmonary artery, to determine if HOCl produced by conversion of H_2O_2 with myeloperoxidase and Cl^- produces comparable results as the direct administration of HOCl, and to show that adenosine 3′,5′-cyclic monophosphate (cAMP)–enhancing agents can prevent the increased endothelial permeability induced by HOCl and H_2O_2. HOCl given directly or produced by myeloperoxidase, H_2O_2, and Cl^- caused faster and greater changes in cell shape (cell retraction), electrical resistance, and protein permeability (^{125}I-labeled albumin clearance) of endothelial cell monolayers than induced by H_2O_2. HOCl (10 to 100 μM) induced these changes within 1 to 3 min, whereas H_2O_2 (50 to 400 μM) required $\sim$ 30 min. 8-Bromo-cAMP prevented the increased endothelial protein permeability induced by HOCl or H_2O_2, but isoproterenol only prevented the H_2O_2 response. Thus, HOCl at a much lower concentration caused a faster and greater increase in endothelial permeability *in vitro* than H_2O_2, and an increased intracellular level of cAMP prevented the increased permeability induced by either oxidant. Ochoa L, Waypa G, Mahoney JR, Jr., Rodriguez L, Minnear FL. Contrasting effects of hypochlorous acid and hydrogen peroxide on endothelial permeability: prevention with cAMP drugs. AM J RESPIR CRIT CARE MED 1997;156:1247–1255.

An increase in vascular endothelial permeability to protein and the development of pulmonary edema are characteristic features of the lung pathophysiology associated with inflammation, embolism, sepsis, and trauma. Polymorphonuclear leukocytes have been implicated in the pathogenesis as these cells when activated produce toxic, reactive oxygen metabolites and proteases that can increase the pulmonary vascular permeability to the movement of water and protein leading to pulmonary edema and the onset of acute respiratory distress syndrome. Upon activation, these cells assemble a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the cell surface and generate a cascade of reactive oxygen metabolites that include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($^{\cdot}OH$), and peroxynitrite radical ($ONOO^-$). The first four of these oxidants have been reported to increase endothelial permeability (1–5).

H_2O_2, produced by the spontaneous or enzymatic dismutation of O_2^-, is lipid soluble and readily diffuses into endothelial cells, where it can react with ferrous iron to produce the extremely toxic $^{\cdot}OH$. Approximately 40% of the H_2O_2 formed by activated polymorphonuclear leukocytes can be oxidized by myeloperoxidase (MPO) in the presence of Cl^-, the most abundant halide in plasma, to HOCl (6), which is highly reactive and does not accumulate in biologic systems. HOCl is a powerful oxidant that is $\sim$ 100-fold more potent as an antimicrobial compound than H_2O_2 (7) and that can degrade structural proteins and inactivate enzymes (8). Although the effects of H_2O_2 on vascular endothelial permeability have been studied extensively, the effects of HOCl are represented by only one study (5). The purpose of the present study was to compare and contrast the effects of H_2O_2 and HOCl on the permeability of the vascular endothelium.

The first objective was to compare the time course and the degree of alterations in endothelial cell monolayers of protein permeability, electrical resistance, and cell shape induced directly by H_2O_2 or HOCl. The second objective was to determine if the generation of HOCl by H_2O_2 and MPO in the presence of Cl^- would yield similar results as the direct administration

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of HOCl. This protocol has been used successfully to induce glomerular injury (9) and simulates the *in vivo* condition where the release of H_2O_2 and MPO from polymorphonuclear leukocytes would react in the presence of Cl^- to form HOCl at localized sites on the endothelial cell surface. A third objective was to determine if the adenosine 3′,5′-cyclic monophosphate (cAMP)–enhancing agents, isoproterenol and 8-bromo-cAMP, could prevent the alterations in endothelial protein permeability induced by HOCl given directly or generated by H_2O_2 and MPO as well as by H_2O_2 alone (4, 10).

Diffusive permeability was assessed by the clearance of ^{125}I-labeled albumin across cell monolayers of bovine pulmonary artery endothelial cells. Electrical resistance of these cell monolayers was monitored on-line using a novel methodology known as ECIS (for electric cell-substrate impedance sensor) (11, 12). Alterations in cell shape were viewed live with differential interference-contrast (DIC) microscopy.

METHODS

Endothelial Cell Preparation

Description of the endothelial cells has been outlined in detail elsewhere (13, 14). Briefly, bovine pulmonary artery endothelial cells (CCL-209; American Type Culture Collection, Rockville, MD) were obtained at passage 16; cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), 20% donor calf serum (GIBCO), gentamycin (50 mg/ml; M.A. Bioproducts, Walkerville, MD), and modified Eagle's nonessential amino acids (10 mM; GIBCO); and used between passages 19 and 24.

Albumin Clearance Measurement

Endothelial cells (75,000) were grown to confluence (3 to 4 d) on sterilized, Transwell filters (0.33 cm^2, 0.4-μm pore size; Costar, Cambridge, MA). Twenty-four hours before the experiment, DMEM was removed by aspiration and replaced with DMEM without phenol red. The well or luminal compartment containing cells and filter was floated in a stirred 25-ml abluminal compartment, with the compartments separated by the polycarbonate filter lined with the confluent, endothelial cell monolayer. Both compartments contained DMEM without phenol red, 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 20 mM HEPES and were heated to 37° C. This experimental apparatus was designed to study transendothelial transport in the absence of hydrostatic and oncotic pressure gradients, as described previously (13–15). Measurement of the clearance (microliters per minute) of ^{125}I-labeled albumin was used as an assessment of changes in the diffusive permeability of albumin across the endothelial cell monolayers.

Clearance of ^{125}I-labeled albumin was determined after treatment of the endothelial cells with Hanks' balanced salt solution (HBSS, medium control; GIBCO), H_2O_2 (10 to 200 μM; Sigma), HOCl (1 to 100 μM; Sigma), MPO (0.75 U/ml; 1 U is the amount of enzymatic activity that causes a change of 1 absorbance unit of the substrate, guaiacol, per minute) then 10 to 100 μM H_2O_2, isoproterenol hydrochloride (10 μM; Sigma), 8-bromo-cAMP (1 mM; Sigma), and MPO (a gift from Dr. Beulah Gray, University of Minnesota, Minneapolis, MN). The concentration of H_2O_2 was determined by spectrophotometric analysis at 240 nm with an extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ (16). H_2O_2 was measured in HBSS (1.26 mM $CaCl_2$, 5.37 mM KCl, 0.441 mM KH_2PO_4, 0.811 mM $MgSO_4$, 137 mM NaCl, 0.342 mM Na_2HPO_4, 4.17 mM $NaHCO_3$; pH 7.4) using the dye xylenol orange (16). The concentration of HOCl was determined using the spectrophotometric assay for chloride-containing compounds, as described by Chesney and colleagues (17). HOCl was incubated directly with the cells for 10 min after the wells containing the cell monolayers were immersed three times in HBSS to remove serum proteins in the medium. The cell monolayers were immersed a fourth time to remove any residual HOCl and then ^{125}I-labeled albumin clearance was measured for 30 min. This same experimental procedure was used when HOCl was produced by the reduction of H_2O_2 by MPO.

Analysis of the transvascular clearance of ^{125}I-labeled albumin has been described in detail elsewhere (13). Briefly, the total activity of the abluminal chamber was expressed as a volume of the luminal chamber that was cleared of the albumin tracer into the lower chamber. The clearance volume of albumin over time, equal to the clearance in microliters per minute, was determined by weighted least-square non-linear regression (BMDP Statistical Software, Berkeley, CA). The radioactivity of all samples was measured with an LKB/Wallac 1272 Clinigamma Counter (Gaithersburg, MD).

^{125}I-labeled albumin was prepared with Na-^{125}I and bovine serum albumin by the chloramine-T procedure, as described previously (18). Bovine serum albumin was passed over a Blue Sepharose affinity column to remove immunoglobulin contamination. To ensure that monomeric albumin was used in these experiments, albumin was passed over a Sephacryl 200 column and the purity checked using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After iodination, the ^{125}I-labeled albumin was separated from free ^{125}I by dialysis (25,000 Kd cutoff; Spectrapore, Spectrum, Houston, TX) against normal saline (0.9%) and was maintained under dialysis until the day of the experiment. The ^{125}I-labeled albumin was used only if the percent free was $<$ 0.1%, as determined by comparing isotope stock with filtrate from ultrafiltration cones (CF30; 30,000 Kd cutoff; Amicon, Beverly, MA).

Measurement of the Resistive Portion of Electrical Impedance

Measurement of electrical impedance of endothelial cells grown as a monolayer is based on a technique developed to study dynamic behavior of cells in culture. This novel methodology uses an electric cell-substrate impedance sensor (ECIS) (Applied Biophysics, Troy, NY) (11, 12). Endothelial cells are cultured on small gold electrodes, and culture medium is used as the electrolyte. The small gold electrode, covered by confluent endothelial cells, and a larger gold counter electrode are connected to a phase-sensitive, lock-in amplifier. A 1-V, 4,000-Hz ac signal is supplied through a 1-MΩ resistor to approximate a constant current source of 1 μA. Treating the cell-electrode system as a simple series RC circuit, the measured changes in electrical impedance can be partitioned into a measured in-phase voltage proportional to the resistance and an out-of-phase voltage proportional to capacitive reactance. The resistive portion of impedance is related to the resistance between adjacent cells (or paracellular resistance). Voltage and phase data are stored and processed with a personal computer that controls the output of the amplifier and switches the measurement to different electrodes in the five different wells during the course of an experiment.

Endothelial cells (80,000) were grown to confluence (3 to 4 d) on 1% gelatin–coated wells (8 mm in diameter), with each of the five wells containing a small gold electrode (10^{-3} cm^2). Twenty-four hours before the experiment, DMEM was removed by aspiration and replaced with DMEM without phenol red. On the day of the experiment, DMEM without phenol red was removed from each well by aspiration and replaced with 200 μl of HBSS. ECIS wells were placed in the ECIS incubator for 10 min to equilibrate to 37° C and 5% CO_2. Cells were preincubated for 10 min with either MPO or HBSS, then electrical impedance measurements, reported in the present study as the resistive portion of impedance, were acquired for 5 min before the addition of HBSS or H_2O_2. Electrical resistances were then obtained every minute for 1 h after treatment of the endothelial cells with HBSS, 100 μM H_2O_2, 10-min pretreatment with 0.75 U/ml of MPO followed by 100 μM H_2O_2 to produce HOCl, and 0.75 U/ml of MPO alone.

Differential Interference-Contrast (DIC) Microscopy

Cell shape was viewed over a 30-min period after incubation with 10 μM HOCl administered directly or by conversion of 400 μM H_2O_2 with 0.75 U/ml of MPO or with 400 μM H_2O_2 alone. Cultured endothelial cells seeded on glass coverslips were used for live observations upon reaching confluence (3 to 4 d), as judged by a cuboidal appearance and fluorescent staining with Calcein AM. The cell-seeded coverslips were inverted on glass slides, supported on and elevated from the slides on two sides of the coverslips by tape, and viewed with a Nikon Microphot SA microscope (Nikon Inc., Melville, NY) using 20, 40, 60, and 100× objectives equipped with DIC optics. Incubation of

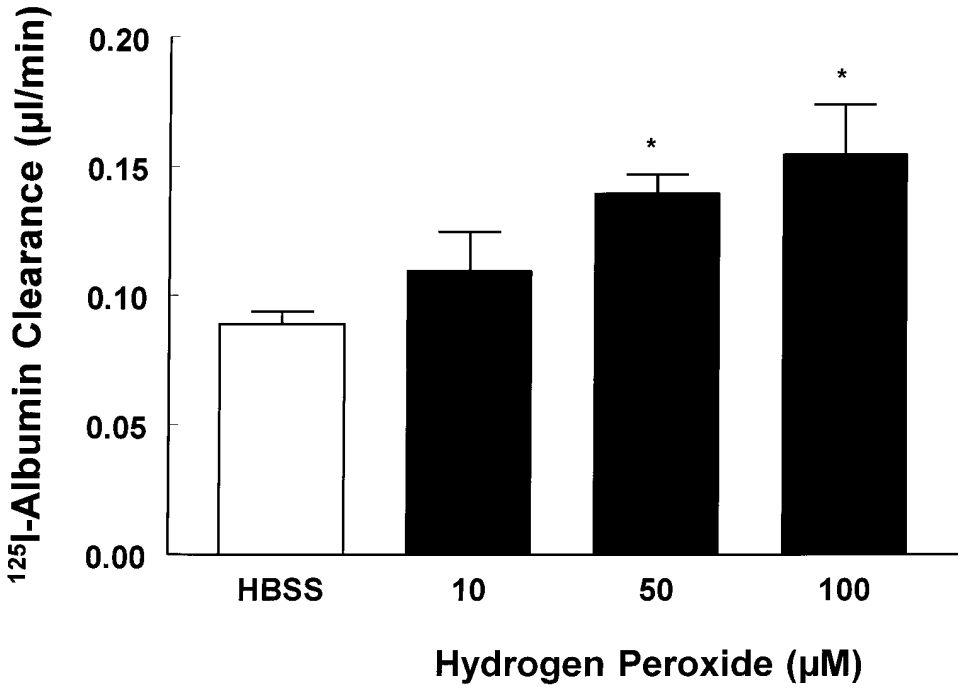

Figure 1. H_2O_2 induced a dose-response increase in ^{125}I-labeled albumin clearance upon incubation with pulmonary artery endothelial cells for 30 min. Measurement of albumin clearance coincided with incubation of H_2O_2. Bars are means ± SE with at least nine cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from HBSS.

the cell monolayers at 37° C with the test oxidants was performed on the microscope stage by applying 40 µl of the solution on one side of the inverted coverslip while gently withdrawing it by means of filter paper on the opposite side.

To observe reproducible changes in cell shape with H_2O_2, cell-seeded coverslips were incubated directly in the incubator while lying in a 35-mm petri dish. Different coverslips were then viewed every 5 min for 30 min to record changes in cell shape.

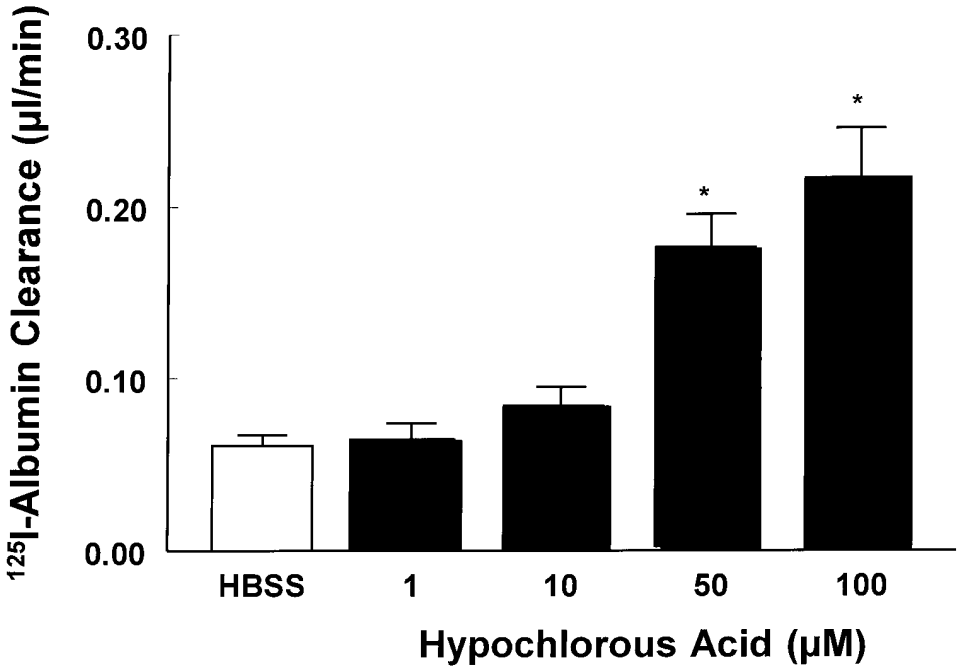

Figure 2. HOCl induced a dose-response increase in ^{125}I-labeled albumin clearance upon incubation with endothelial cells for 10 min. Albumin clearance was measured for 30 min immediately after incubation of endothelial cells with HOCl for 10 min in serum-free medium. Bars are means ± SE with 24 cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from HBSS.

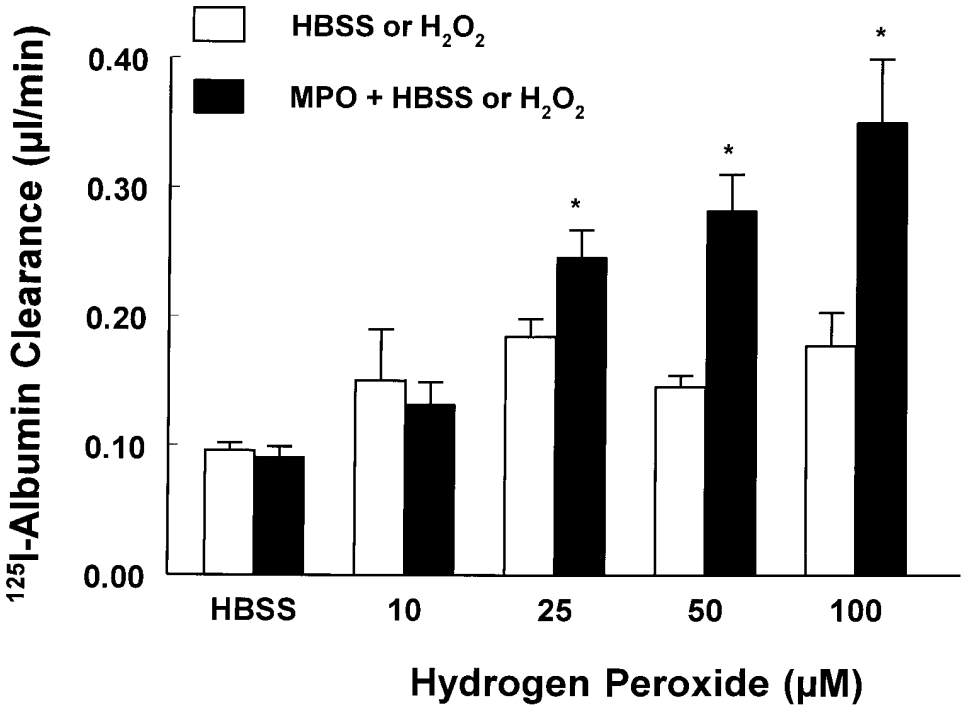

Figure 3. HOCl produced from H_2O_2 by pretreatment with 0.75 U/ml of MPO and Cl^- induced a dose-response increase in ^{125}I-labeled albumin clearance upon incubation with endothelial cells for 10 min. Measurement of albumin clearance for H_2O_2 coincided with incubation of H_2O_2 and for HOCl occurred immediately after 10-min production of HOCl by H_2O_2 and MPO. Bars are means ± SE with 10 cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from corresponding dose of H_2O_2.

Statistics

Each albumin clearance study consisted of at least three experiments done on different days with four to six cell monolayers per group. The electrical resistance study consisted of three experiments done on different days with one to two cell monolayers per group. Data were analyzed in two ways, as raw data by two-way analysis of variance and as normalized data after log transformation by one-way analysis of variance (19). Differences among treatments or groups within treatments were analyzed further by the Newman-Keuls multiple range test, and differences among treatments or groups from the DMEM-control group were analyzed by the Dunnett's multiple range test. Statistical significance was set at $p < 0.05$.

RESULTS

^{125}I-labeled Albumin Clearance Measurements

Incubation of endothelial cell monolayers for 60 min with 10, 50, and 100 µM H_2O_2 produced a dose-related increase in the transendothelial clearance of ^{125}I-labeled albumin. Doses of H_2O_2 of 50 and 100 µM consistently increased ($p < 0.05$) albumin clearance when compared with the HBSS control value (Figure 1). This increase in the diffusive permeability of the endothelium to albumin occurred within the first 30 min of the experiment and persisted for an additional 30 min, as determined from analysis of the data from 10 to 30 min and from 30 to 60 min, respectively (data not shown).

The effect of HOCl on albumin clearance was assessed using two different protocols, both of which yielded similar results. Firstly, HOCl at doses of 1, 10, 50, and 100 µM was incubated directly with endothelial cell monolayers for 10 min and albumin clearance assessed for 30 min. HOCl consistently increased ($p < 0.05$) albumin clearance at doses of 50 and 100 µM, whereas 10 µM produced a variable response (Figure 2). Secondly, HOCl was produced by the reduction of H_2O_2 at 10, 25, 50, and 100 µM by 0.75 U/ml of MPO in the presence of Cl^- in HBSS. After the 10-min incubation of MPO with the four doses of H_2O_2, no detectable H_2O_2 remained, suggesting complete conversion of H_2O_2 to HOCl. The direct incubation of H_2O_2 at these four doses caused only slight increases in albumin clearance from the HBSS control value (Figure 3). The conversion to 25, 50, and 100 µM HOCl by MPO and H_2O_2 increased ($p < 0.05$) albumin clearance by as much as twofold greater than the H_2O_2 control groups (Figure 3).

Electrical Resistance Measurements

Real-time measurements of electrical resistance of endothelial monolayers incubated with 100 µM H_2O_2 or 100 µM HOCl (produced by MPO, H_2O_2, and Cl^-) revealed that H_2O_2 caused a slower and smaller decrease in resistance in comparison with HOCl (Figure 4). H_2O_2 decreased ($p < 0.05$) resistance by 30% within 31 min, whereas HOCl decreased ($p < 0.05$) resistance by 50% within 7 min. Electrical resistances were significantly different between the two groups at all time points.

Cell Morphology

Changes in cell shape were visualized live with DIC microscopy. The time course and degree of change in cell shape induced by the two oxidants correlated well with the ECIS measurements. H_2O_2 (400 µM) caused a relatively slow change (~ 25 min) in cell shape (Figure 5). In contrast, cells retracted from one another within 3 min when HOCl was administered directly (10 µM HOCl) or produced indirectly (~ 400 µM HOCl) via conversion of 400 µM H_2O_2 by MPO (Figure 5). HOCl caused more cells as viewed in an objective field to change shape, greater retraction of cells, and the formation of more intercellular strands.

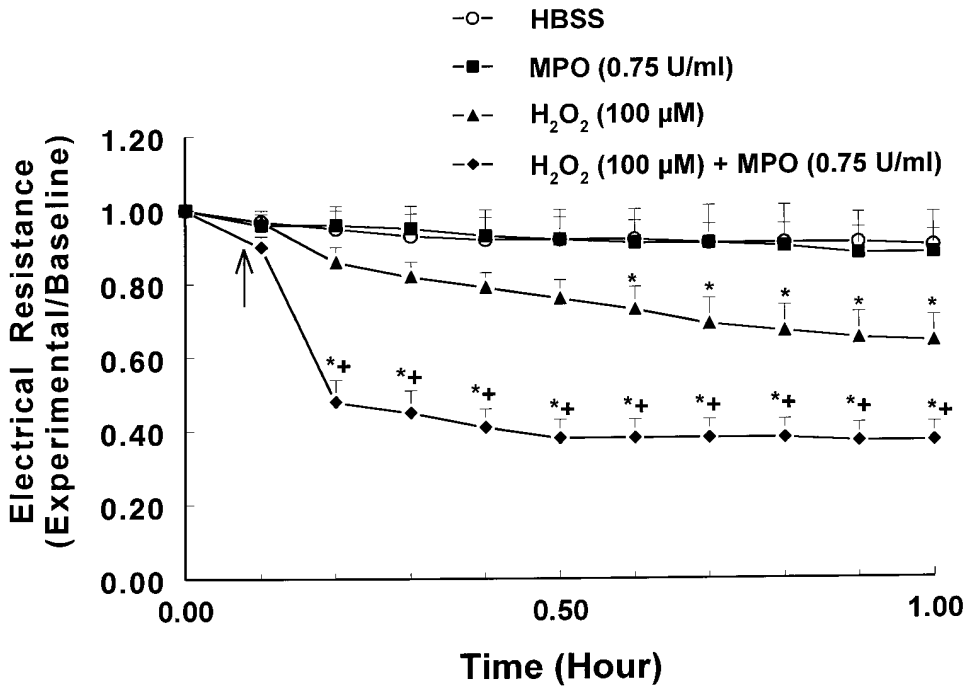

Figure 4. HOCl, produced from H_2O_2 and MPO, rapidly and H_2O_2 slowly decreased electrical resistance of endothelial cell monolayers. Values are means ± SE of temporal changes in resistance for each of four groups with three cell monolayers per group. *Arrow* depicts time (5 min) of addition of H_2O_2 or production of HOCl. HBSS: vehicle control. *$p < 0.05$ from 0 min. $^{+}p < 0.05$ from H_2O_2 group (*triangles*).

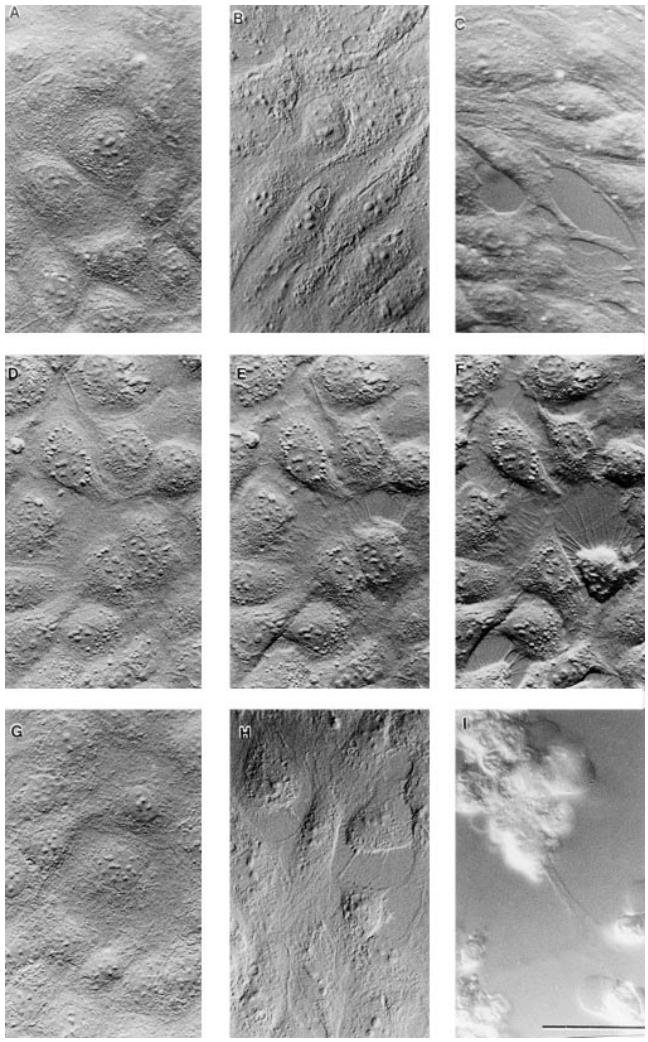

Figure 5. DIC micrographs show that 400 μM H_2O_2 (*B*: 20 min; *C*: 25 min) induced a relatively slow change in cell shape within 25 min (*C*) with fewer intercellular strands compared with 10 μM HOCl given directly (*E*: 2 min; *F*: 7 min) or ~ 400 μM HOCl produced by conversion of 400 μM H_2O_2 with 0.75 U/ml of MPO (*H*: 1 min; *I*: 5 min). *A*: HBSS control; *D*: HOCl control; *G*: MPO control. Original magnification: ×500. Bar = 50 μM.

Attenuation of the Oxidant Response with Isoproterenol and 8-Bromo-cAMP

Co-incubation of 10 μM isoproterenol or 1 mM 8-bromo-cAMP prevented the increase in albumin clearance induced by 50 and 200 μM H_2O_2 (Figure 6). The permeability-decreasing effect of isoproterenol and 8-bromo-cAMP was not as dramatic when HOCl was incubated directly with the endothelial cell monolayers. 8-Bromo-cAMP prevented the increase in albumin clearance induced by 25 μM HOCl and attenuated ($p < 0.05$) the increase induced by 50 μM HOCl (Figure 7). Isoproterenol slightly attenuated the response at both doses, but the differences were not significant (data not shown). However, 8-bromo-cAMP almost completely prevented the increase in albumin clearance when HOCl was generated by conversion of 50 μM H_2O_2 by MPO (Figure 8).

DISCUSSION

The objectives of the present study were: (*1*) to compare the effects of H_2O_2 and HOCl with respect to relative potencies and the time course and magnitude of changes in protein permeability, electrical resistance, and shape change; (*2*) to determine if the production of HOCl by the conversion of H_2O_2 by MPO in the presence of Cl^- would yield similar results as the

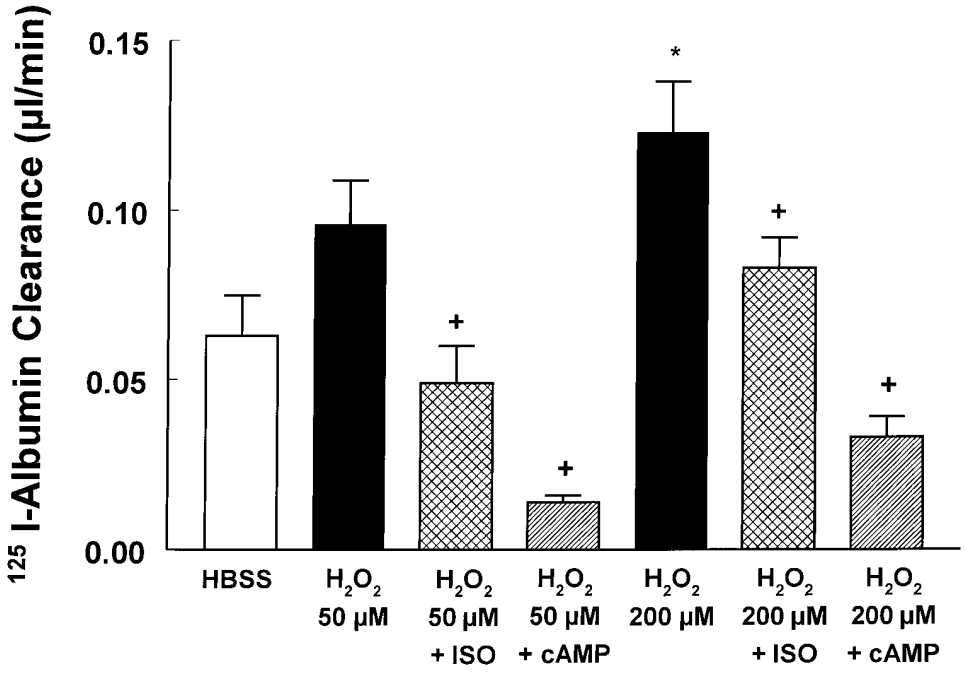

Figure 6. Co-treatment of 10 µM isoproterenol (ISO) or 1 mM 8-bromo-cAMP (cAMP) prevented the increase in ^{125}I-labeled albumin clearance induced by H_2O_2. Bars are means ± SE with at least 15 cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from HBSS. $^{+}p < 0.05$ from corresponding dose of H_2O_2.

direct administration of HOCl; and (*3*) to determine if cAMP-enhancing agents could prevent the increase in endothelial protein permeability induced by HOCl given directly or generated by H_2O_2 and MPO as well as by H_2O_2 alone. HOCl caused rapid, within 1 to 3 min, changes in cell shape and electrical resistance, whereas H_2O_2 required ~ 30 min to induce these changes. HOCl produced greater increases in endothelial protein permeability and decreases in electrical resistance

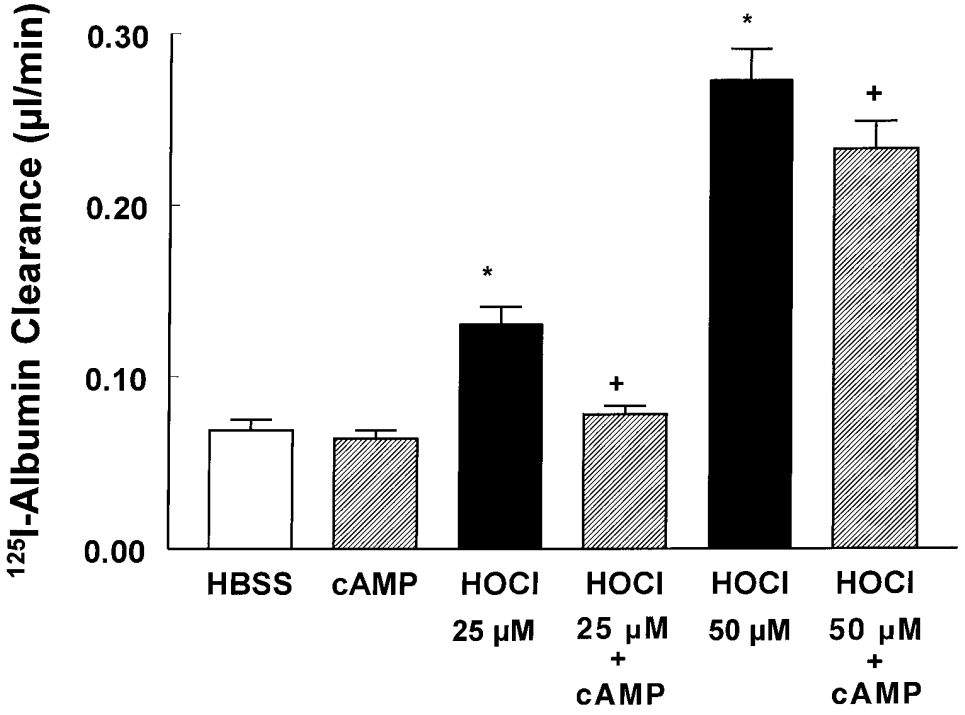

Figure 7. Co-treatment of 1 mM 8-bromo-cAMP (cAMP) prevented and attenuated, respectively, the increase in ^{125}I-labeled albumin clearance induced by 25 and 50 µM HOCl. Albumin clearance was measured for 30 min immediately after co-incubation of HOCl and 8-bromo-cAMP with endothelial cells for 10 min in serum-free medium. Bars are means ± SE with 15 cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from HBSS. $^{+}p < 0.05$ from corresponding dose of HOCl.

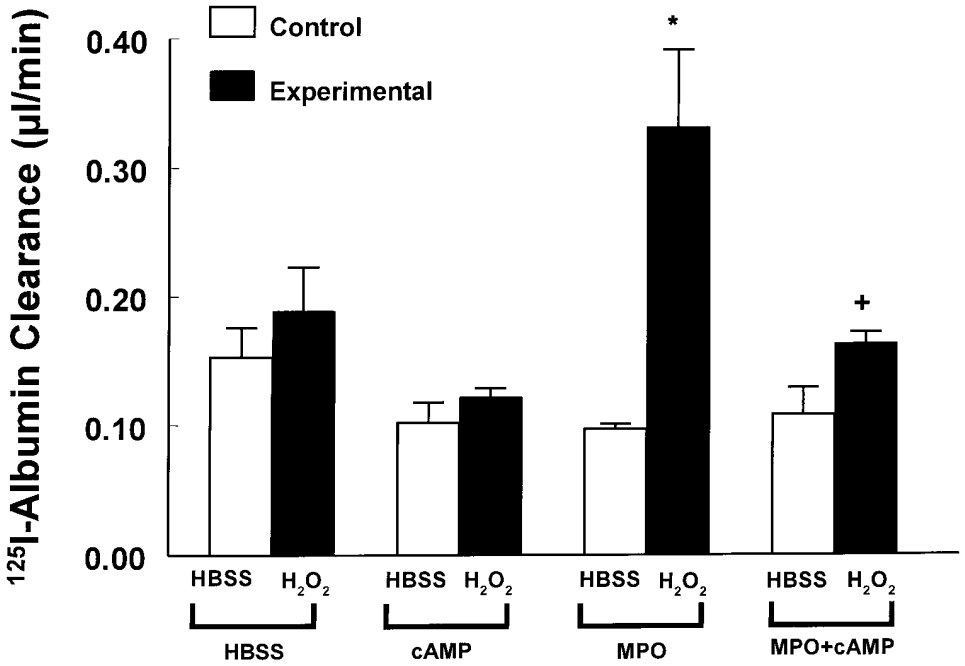

Figure 8. Pretreatment with 1 mM 8-bromo-cAMP (cAMP) prevented the increase in ^{125}I-labeled albumin clearance induced by HOCl produced by conversion of 25 µM H_2O_2 by MPO. *Brackets* differentiate pretreated agents (*below*) and post-treated agents (*above*) for each group of cell monolayers. Bars are means ± SE with 10 cell monolayers per group. HBSS: vehicle control. *$p < 0.05$ from corresponding MPO/HBSS control. $^{+}p < 0.05$ from corresponding MPO/H_2O_2 experimental group.

and more extensive cell retraction at much lower concentrations than H_2O_2. Generation of HOCl by the conversion of H_2O_2 with MPO produced results similar to the direct administration of HOCl. The increase in endothelial protein permeability induced by H_2O_2 was prevented by isoproterenol and 8-bromo-cAMP, but the increased permeability induced by HOCl was prevented only by 8-bromo-cAMP. These findings demonstrate that HOCl is a more potent oxidant than H_2O_2 in altering endothelial cell shape and permeability and that an elevation in the intracellular level of cAMP can prevent the increase in endothelial protein permeability induced by either oxidant.

Previous studies have shown that both H_2O_2 and HOCl increase endothelial protein permeability *in vitro* (1, 5) and that cAMP-enhancing agents can prevent the increased permeability induced by H_2O_2 (4, 10). H_2O_2 has been reported to increase permeability in a dose range from 50 to 100 µM, with doses $>$ 100 µM causing cellular damage as assessed by release of lactate dehydrogenase or ^{51}Cr (1, 20, 21). HOCl was found to increase permeability at doses equal to and greater than 25 µM (5). The present study confirms these findings and, in addition, directly compares the potency and temporal response of these two oxidants using four different methodologies. First, the potency of these two oxidants was compared by the measurement of endothelial protein permeability. The direct administration of HOCl produced a variable increase in permeability at 10 µM and consistent increases at 25, 50, and 100 µM, whereas H_2O_2 produced consistent increases at 50 and 100 µM. Secondly, HOCl produced by conversion of H_2O_2 with MPO significantly increased endothelial protein permeability at doses of 25, 50, and 100 µM compared directly to the same doses of H_2O_2 administered alone as the control groups. At 50 and 100 µM, HOCl doubled the protein permeability of the H_2O_2 control groups. Thirdly, the potency and time course of changes in endothelial electrical resistance were compared directly using the ECIS apparatus. HOCl caused almost an immediate decrease in electrical resistance, whereas H_2O_2 required $\sim$ 30 min to induce a significant change in resistance. Furthermore, HOCl decreased endothelial electrical resistance to a twofold greater extent than H_2O_2. Fourthly, HOCl caused a more rapid (within 3 min) and extensive alteration in cell shape than H_2O_2 ($\sim$ 30 min), as depicted by a greater number of and wider intercellular gaps and more extensive formation of intercellular strands. The dose of HOCl (10 µM) that readily produced cell retraction was much lower than that required for H_2O_2 (400 µM). These four comparisons demonstrate that HOCl induces a more rapid and potent change in electrical resistance, protein permeability, and shape change of endothelial cell monolayers than H_2O_2.

Similar findings with regard to the time course leading to cellular damage and potency of these two oxidants have been reported for murine macrophage-like tumor cells (P388D1) exposed to HOCl and H_2O_2 (8). Low concentrations (10 to 20 µM) of HOCl caused oxidation of plasma membrane sulfhydryls and disturbances of plasma membrane functions such as inactivation of glucose uptake, loss of cellular K^+, and an increase in cell volume. Higher concentrations led to generalized oxidation of sulfhydryl, methionine, and tryptophan residues, ATP depletion, and cell lysis. H_2O_2 on the other hand, induced sulfhydryl oxidation of glyceraldehyde-3-phosphate dehydrogenase, depletion of ATP, degradation of NAD, and strand breaks in DNA. Compared with H_2O_2, which caused cell death after several hours, HOCl induced cell lysis within an hour at one-tenth of the molar concentration (8).

HOCl increased endothelial protein permeability when administered either directly or by conversion of H_2O_2 with

MPO. Enzymatic production of HOCl was used to mimic the *in vivo* production of HOCl by polymorphonuclear leukocytes. A similar protocol has been used to induce glomerular injury in rats (9). MPO was shown by electron microscopy to localize, presumably by electrostatic interactions, to the glomerular capillary wall. Subsequent administration of H_2O_2 produced a greater than threefold increase in urine protein excretion from the MPO and H_2O_2 control groups. These investigators concluded that HOCl produced by the conversion of H_2O_2 by MPO and Cl^- may be important in polymorphonuclear leukocyte–mediated glomerulonephritis.

Under ideal conditions, the conversion of H_2O_2 to HOCl catalyzed by MPO in the presence of Cl^- should be one-to-one. Under physiologic conditions and in the present study, probably not all of the H_2O_2 was converted to HOCl. Some of the H_2O_2 was inaccessible to MPO because it had already diffused into the cell. Furthermore, HOCl interferes with its own production by inhibiting the reduction of H_2O_2 by MPO. Although the added H_2O_2 was not detectable, using the xylenol orange assay, after conversion by MPO to HOCl, most likely all of the H_2O_2 was not converted to HOCl. Thus, the comparisons of potency and temporal changes between H_2O_2 and HOCl produced indirectly are probably limited by the fact that not all of the H_2O_2 was converted by MPO to HOCl. However, this indirect method of production of HOCl is probably required for studies involving intact animals or isolated lungs perfused with protein, since proteins scavenge HOCl. Furthermore, this indirect production of HOCl simulates the *in vivo* condition whereby polymorphonuclear leukocytes produce HOCl via the release of MPO, which binds electrostatically to endothelial cells, and H_2O_2.

We noted that the doses of H_2O_2 from 10 to 200 μM did not always produce consistent increases in protein permeability. This measurement is determined by linear regression of radioactive counts taken by sampling the abluminal chamber every 5 min for 60 min. Linear regression analysis from the first and second 30-min periods of the 1-h experiments resulted in similar increases in endothelial protein permeability induced by H_2O_2. With the introduction of the ECIS apparatus in the laboratory, it became apparent that the slower onset and reversible nature of the H_2O_2 response could influence our measurement of protein permeability. Both H_2O_2 and HOCl have been shown by other investigators to produce reversible changes in endothelial cell shape. For H_2O_2, changes in cell shape were maximal within 4 to 6 h and reversible by 24 h (22). HOCl caused changes in cell shape that were noticeable within 2 to 6 min and reversible by 4 h (5). The reversible response of HOCl was observed with noncytotoxic concentrations of 10 and 25 μM and when the HOCl was incubated with the cells for 2 min but not when incubated for 6 min. We have also observed using the ECIS apparatus that the H_2O_2-induced decrease in electrical resistance is reversible within 1 to 2 h (unpublished observations). Thus, a slow and reversible change in endothelial cell shape could readily explain the variable nature of the H_2O_2 response as assessed by the measurement of endothelial protein permeability.

With both oxidants, the changes in endothelial cell shape have been reported to include cell retraction, the formation of thin intercellular strands connecting the separated cells early in the retraction process, and the reorganization of actin microfilaments (1, 5, 22). We also observed cell retraction and the formation of intercellular strands, but more so with HOCl than H_2O_2. Even more intercellular strands are produced upon incubation of endothelial cells with α-thrombin, which induces cell contraction within a minute (unpublished observations). Fluorescence microscopy indicates that these intercellular strands contain cytoplasm, as they take up the vital cytoplasmic dye, Calcein AM, and contain the adherens junctional proteins, actin, vinculin, and plakoglobin (unpublished observations). Although both oxidants caused cell retraction, the differences in time of onset and the extent of intercellular strand formation would suggest that these two oxidants function by different mechanisms. Schraufstatter and associates (8) have demonstrated that HOCl and H_2O_2 interact with similar and different cellular targets. For example, HOCl reacts with the closest targets, often components of the plasma membrane, whereas H_2O_2 diffuses freely into cells and causes site-directed damage presumably due to the formation of metal-dependent $^{\cdot}OH$.

A variety of cAMP-enhancing agents have been used to attenuate the H_2O_2-induced increase in endothelial permeability *in vitro* (4, 10). Pretreatment with prostaglandin E_1, forskolin, type III and IV phosphodiesterase inhibitors, and β_2-adrenergic agonists have been effective in the prevention of the H_2O_2-induced increase in endothelial permeability. There are a number of mechanisms whereby an increase in the intracellular level of cAMP might protect against the H_2O_2 response. H_2O_2 has been shown to reduce intracellular levels of ATP (21, 23, 24), NAD (24), and glutathione (20) and activate the inositol phosphate pathway which increases intracellular Ca^{2+} (25, 26) and activates protein kinase C (1). In an 18-h model of asbestos-induced increase in endothelial permeability to protein, the attenuation of this response by cAMP was associated with a decrease in the level of ATP depletion and the proliferation of endothelial cells but not by any effects on glutathione depletion or production of $^{\cdot}OH$ (27). Inhibition of poly(ADP-ribose) polymerase by 3-aminobenzamide, nicotinamide, or theophylline prevented cytotoxicity of leukocyte cell lines exposed to 1 mM H_2O_2 (24). The mechanism of action of these inhibitors is not clear but may ultimately lead to an elevation in intracellular cAMP by inhibition of cAMP-dependent phosphodiesterases or by activation of a cAMP-dependent protein kinase (24). cAMP-enhancing agents also reduce the activity of myosin light chain kinase and it is this step in the cell signaling pathway that cAMP probably antagonizes when cAMP functions to block the permeability response induced by thrombin and histamine as well as other agents that work via cell contraction (28, 29).

8-Bromo-cAMP prevented the increased protein permeability induced by either oxidant, whereas isoproterenol failed to modify the HOCl response. Administered as a co-treatment, HOCl as well as H_2O_2 could directly alter the structure of isoproterenol and/or oxidize the sulfhydryl groups in the β-adrenergic receptor and subsequently depress the function of the receptor (30). That HOCl may react directly with isoproterenol and is a more potent oxidant than H_2O_2 might explain why isoproterenol was protective against H_2O_2 but not HOCl.

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