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Original Contribution

VASCULAR ENDOTHELIAL CELL KILLING BY COMBINATIONS OF MEMBRANE-ACTIVE AGENTS AND HYDROGEN PEROXIDE

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Abstract—Previous studies have demonstrated that a number of membrane-active agents are capable of binding to the surface of polymorphonuclear leukocytes (PMN) resulting in an augmentation of superoxide anion and hydrogen peroxide (H_2O_2) production in response to soluble stimuli. It is now demonstrated that these same membrane-active agents can bind to the surface of endothelial cells and enhance their susceptibility to killing by H_2O_2. Membrane-active agents which are capable of synergizing with H_2O_2 include cationic proteins, cationic poly-amino acids, lysophosphatides and enzymes which are capable of degrading membrane phospholipids (e.g., phospholipase C, phospholipase A_2 and streptolysin S). In each case, treatment of the target cells with the membrane-active agent and H_2O_2 produces greater damage than the sum of the damage produced by either agent separately. Since inflammatory lesions, particularly sites of bacterial infection, may contain a rich mixture of cationic substances, phospholipases and phospholipid breakdown products, these substances may contribute to the tissue damage observed at sites of inflammation by enhancing endothelial cell sensitivity to PMN-generated H_2O_2 as well as by augmenting the generation of H_2O_2 by PMNs.

Keywords—Free radicals, Hydrogen peroxide, Cationic proteins, Lysophosphatides, Phospholipases, Streptolysin S, Membrane-active agents, Endothelial cell killing

INTRODUCTION

Stimulation of polymorphonuclear leukocytes (PMNs) with a variety of agonists leads to activation of the membrane NADPH oxidase and the generation of superoxide anion (O_2^-). The generated O_2^- is converted to hydrogen peroxide (H_2O_2) either spontaneously or following interaction with superoxide dismutase. H_2O_2 can then be converted to the highly toxic hydroxyl radical (HO·) in the presence of ferrous iron or can be converted through interaction with myeloperoxidase to hypo-halous (e.g., hypochlorous, hypobromous) acids. The generation of these metabolites and their roles in tissue damage is well established.[1–5]

Recent studies from our laboratories have shown that cationic poly-alpha amino acids[6–9] and lysophosphatides[10] bind to the surface of PMNs to greatly increase the amount of O_2^- generated following stimulation with soluble agonists. Since inflammatory sites, particularly sites of bacterial infection, are rich in cationic polypeptides (e.g., of bacterial and host origins)[9,11,12] and microbial phospholipases and hemolysins,[13,14] we have speculated that the presence of these agents could contribute to the inflammatory process by their stimulatory effect on the PMN respiratory burst.[9,10] In the present report we describe a second mechanism whereby these same agents may enhance tissue injury. We show that these agents bind to target (endothelial) cells and enhance their susceptibility to the cytotoxic effects of PMN-derived oxygen radicals.

MATERIALS AND METHODS

Endothelial cells

Rat pulmonary artery endothelial cells were isolated from the pulmonary vasculature by perfusion of microcarrier beads into the vessels and subsequent retrieval of the beads with endothelial cells attached by retrograde perfusion.[15,16] Upon isolation, the cells ex-

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hibited the typical cobblestone morphology of endothelial cells. They were positive for factor VIII by immunofluorescence, bound acetylated low-density lipoprotein and had high levels of angiotensin-converting enzyme (ACE) ($3.2 \times 10^4 - 1.9 \times 10^5$ molecules/cell) as measured with the synthetic substrate, ^{3}H-Benzoyl-phe-ala-pro.[17–19] The cells were maintained in monolayer culture using minimal essential medium of Eagle with Earle's salts (MEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin as culture medium. They were passaged by scraping with a rubber policeman without exposure to proteases. Growth was at 37°C and 5% CO_2. Stocks were kept frozen in liquid N_2. All experiments were conducted on cells passaged less than 34 times since isolation. Throughout the course of the study, the endothelial cells maintained their cobblestone morphology and levels of ACE activity.

H_2O_2

H_2O_2 was obtained as a 30% solution or generated from a mixture of glucose + glucose oxidase. Both the reagent H_2O_2 and the glucose oxidase were obtained from Sigma Chemical Company (St. Louis, MO). To generate H_2O_2 using glucose oxidase, 0.128-0.512 units/ml of glucose oxidase were used. In the presence of excessive glucose, this amount of glucose oxidase was able to generate 500–1000 nmols of H_2O_2 in 15 min in Hanks' balanced salt solution (HBSS) (37°C, pH 7.3). The method of Thurman et al.[20] employing ferrous ammonium sulfate and sodium thiocyanate was employed to quantitate H_2O_2 production.

Agents employed

The following compounds were examined for ability to synergize with H_2O_2 in the killing of rat pulmonary artery endothelial cells: lysophosphatidylcholine (1–5 μg/ml), streptolysin S (50–500 hemolytic units/ml) and streptolysin 0 (100–500 hemolytic units/ml), both from group A streptococci, phospholipase C from *Clostridium welchii* (0.01–0.15 units/ml), phospholipase A_2 from bee venom (25 units/ml), nuclear histone (Type IIA) rich in both lysine and arginine, histone Type III, rich in lysine, histone Type VIII rich in arginine, (all at 10–100 μg/ml), poly-L-arginine (MW = 40,000), poly-L-lysine (MW = 26,000) (1–10 μg/ml), protamine chloride (50–100 μg/ml) and polymyxin B (100–500 μg/ml). These reagents were obtained from Sigma.

Prior to use with endothelial cells, activity of the two phospholipases and bacterial hemolysins was assessed using a hemolytic assay employing human red blood cells.[13] Briefly, 1 ml aliquots of a 1% red blood cell suspension in HBSS were mixed with various amounts of the phospholipase or streptolysin preparation. In the case of phosphalipase A_2, 500 μg/ml of fatty acid-free albumin was added 10 min after the phospholipase. The hemolysis titer was determined after incubation for 30 min. One hemolytic unit was defined as the smallest amount which caused a 50% hemolysis of the red blood cell suspension, based on the spectrophotometric analysis (540 nm) of hemoglobin in solution. The hemolytic agents were employed in amounts that were by themselves sublytic to the endothelial cells.

Cytotoxicity assay

Cytotoxicity was assessed using a ^{51}Cr-release assay as described previously.[5] Endothelial cells were harvested by trypsinization, resuspended at 1.6×10^5 cells/ml in MEM supplemented with 10% fetal bovine serum and 10 μCi/ml of $Na^{51}CrO_4$ and seeded into the wells of a 24 well dish (1 ml/well). The cells were incubated at 37°C and 5% CO_2 until the cells formed a confluent monolayer (usually one day). When the cells reached confluence, the wells were washed five times to remove excess radioactivity. One ml of serum-free MEM was then added per well and the cells were treated with H_2O_2 (e.g., either reagent H_2O_2 or glucose + glucose oxidase) alone or in combination with the various agonists described above. The cultures were further incubated for 2 h (unless otherwise stated) at 37°C and 5% CO_2. At the end of the incubation period, the cells were observed under phase-contrast microscopy for morphological changes characteristic of injury. The amount of radioactivity released into the culture fluid was simultaneously determined. For this, 0.8 ml of culture medium was removed from the wells and centrifuged at 2000 × G for 20 min. A 0.4 ml sample was then obtained from the supernatant and counted in a gamma counter. In all experiments, the amount of radioactivity released in response to treatment was compared to the amount released in cultures incubated with buffer alone (spontaneous release) and the amount released following treatment with 1% triton X-100 (total release).

RESULTS

Interaction of polycations and H_2O_2 in endothelial cell killing

In the first series of experiments rat pulmonary artery endothelial cells were exposed to sublytic concentrations of glucose oxidase (0.256 units/ml) alone or in combination with increasing amounts of histone.

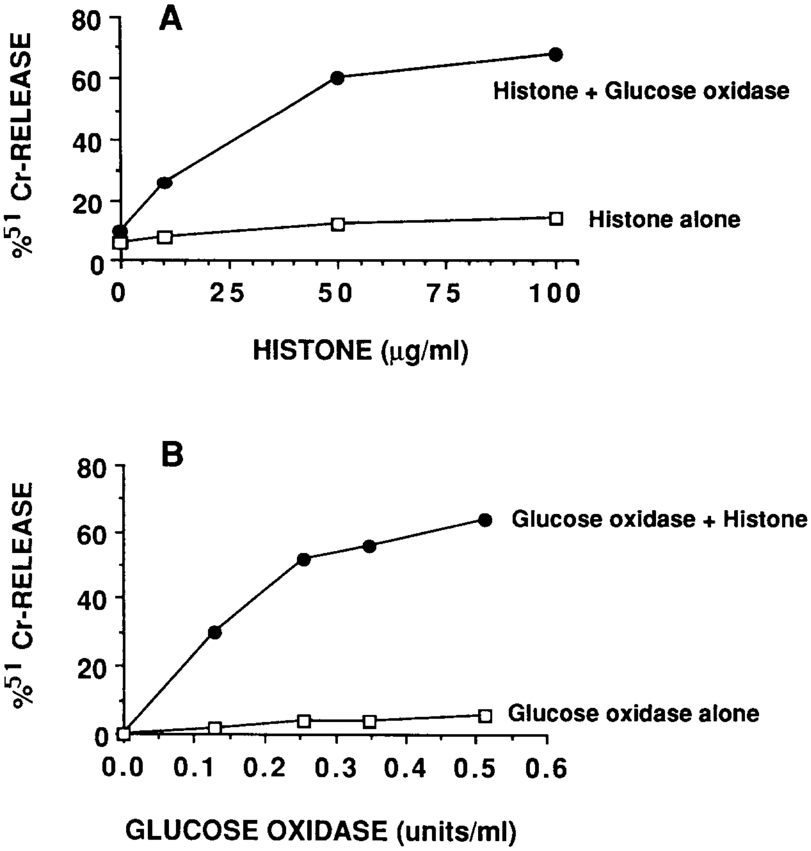

Fig. 1. Sensitivity of rat pulmonary artery endothelial cells to killing by combinations of H_2O_2 and histone. (A) Cells were incubated for 2 h in the presence of various concentrations of histone either alone or in combination with 0.256 units/ml of glucose oxidase. (B) Cells were incubated for 2 h in the presence of various concentrations of glucose oxidase either alone or in combination with 50 μg/ml of histone. At the end of the incubation period, the cytotoxicity was determined using ^{51}Cr-release as indicator. The values shown are averages based on duplicate samples in a single experiment where the differences between individual values and averages were less than 10%. The experiment was conducted five times with similar results.

^{51}Cr released into the supernatant fluid 2 hours later demonstrated that the two agents were synergistic in their effect on the target cells. While neither agent alone resulted in a significant amount of cell killing, the two agents together were highly cytotoxic (Fig. 1A). The data in Figure 1A were obtained by mixing a constant amount of glucose oxidase with increasing amounts of histone. Figure 1B demonstrates a similar synergy when the endothelial cells were treated with a constant amount of histone and increasing amounts of glucose oxidase.

In addition to using ^{51}Cr-release as an indicator of injury, endothelial cells treated with histone and glucose oxidase alone and in combination were also examined under phase-contrast microscopy for evidence of injury. Cells treated with concentrations of histone (30 μg/ml) and glucose oxidase (0.256 units/ml) that by themselves were sublytic based on ^{51}Cr-release did not show evidence of lethal injury (Fig. 2b and 2c). In combination, however, the two agents produced morphological changes in the endothelial cells that are characteristic of lethal injury (Fig. 2d). As an additional criterion of injury, endothelial cells in monolayer were stained with trypan blue after exposure to histone and/or glucose oxidase. When untreated monolayers of endothelial cells or monolayers of cells treated with histone or glucose oxidase alone were stained, only an occasional cell was positive by 2 hours. In contrast, the great majority of cells treated with histone and glucose oxidase together were positive (>75%) (not shown).

To determine whether the simultaneous presence of H_2O_2 and histone were required for synergistic killing, endothelial cells were treated with two reagents sequentially. In one experiment the cells were first treated with varying concentrations of glucose oxidase (0.128–0.512 units/ml). After incubation for 40 min, the glucose oxidase-generated H_2O_2 was inactivated with catalase (1800 units/ml) followed by the addition of 75 μg/ml of histone and incubation for an additional two hours. In a second experiment, the cells were treated for 40 minutes with varying amounts of histone. This

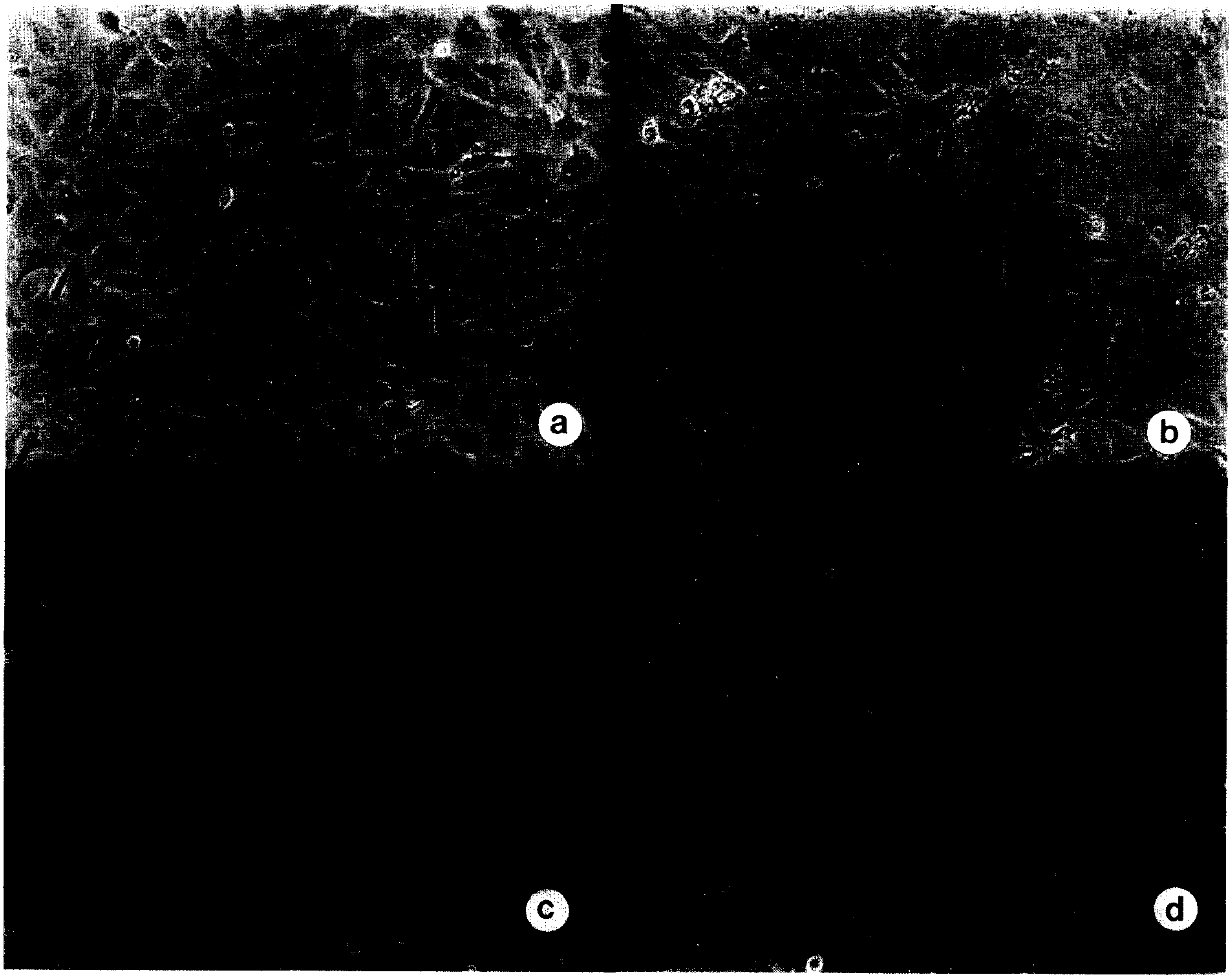

Fig. 2. Phase-contrast photomicrographs of rat pulmonary artery endothelial cells under control conditions (a) and after 2-h exposure to glucose oxidase (0.256 units/ml) (b), histone (30 μg/ml) (c), and combinations of glucose oxidase and histone (0.256 units and 30 μg/ml, respectively) (d). (X185).

was followed by the addition of polyanethole sulfonate (500 μg/ml) to neutralize the cationic charge on the histone and incubation with glucose oxidase (0.128 units/ml) for 2 h. The results of these experiments (Fig. 3 A and B) clearly indicate that the polycationic substance and the H_2O_2 must be present simultaneously in a functionally active form to produce synergistic killing of endothelial cells.

Another set of experiments was conducted to determine how long H_2O_2 and histone had to be simultaneously present to induce synergistic killing. Endothelial cells were treated with 75 μg/ml of histone and 0.128 units/ml of glucose oxidase. At 10 min intervals thereafter, catalase (1800 units/ml) or polyanethole sulfonate (500 μg/ml) was added. Addition of either inhibitor to the reaction mixture up to 40 minutes after the start of the incubation provided substantial inhibition of the synergistic killing (Fig. 4). Addition of the inhibitors at later time points were much less effective.

In other experiments (not shown) we found that both lysine-rich and arginine-rich histones had similar effects. Other polycations, for example, poly-arginine (5–10 μm/ml), poly-lysine (5–10 μg/ml), protamine chloride (50–100 μg/ml) and polymyxin B (100–500 μg/ml) all interacted with H_2O_2 to induce endothelial cell killing under conditions in which they were nonlytic by themselves.

Interaction of lysophosphatidylcholine or phospholipases/hemolysins with H_2O_2 in endothelial cell killing

In our recent study it was found that lysophosphatidylcholine (with $\geq$ 10 carbon fatty acid chains) as well as several other lysophosphatides markedly en-

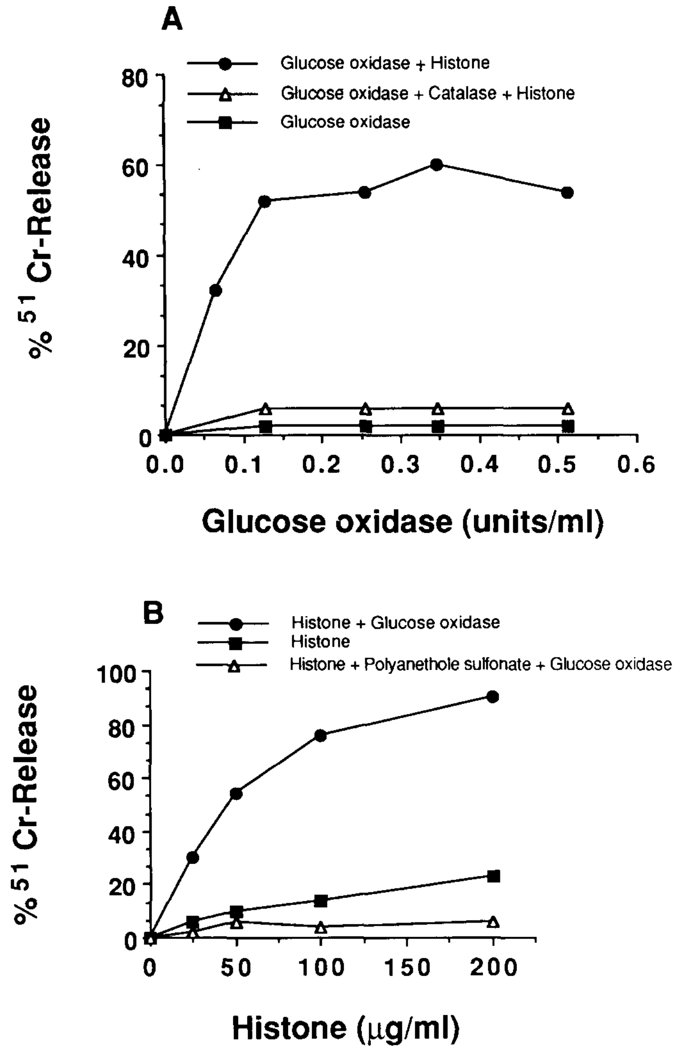

Fig. 3. Sensitivity of rat pulmonary artery endothelial cells to sequential treatment with H_2O_2 and histone. (A) Cells were treated for 40 minutes with various concentrations of glucose oxidase. Excess catalase (1800 units/ml) was then added to half of the cultures. This was followed by addition of 75 μg/ml of histone to all the cultures. Cytotoxicity was assessed after an additional 2-h incubation. (B) Cells were treated for 40 min with various concentrations of histone. Polyanethole sulfonate (500 μg/ml) was then added to half of the cultures. This was followed by addition of 0.128 units/ml of glucose oxidase. Cytotoxicity was assessed after an additional 2-h incubation. Values are averages based on duplicate samples in a single experiment in which the differences between the individual values and averages were less than 10%. The experiment was repeated three times with similar results.

hanced the generation of O_2^- by PMNs in response to a number of agonists.[10] It was of interest, therefore, to determine if the same compounds could also interact with H_2O_2 to enhance the killing of endothelial cells. Figure 5 shows that treatment of rat pulmonary artery endothelial cells with 4 μg/ml of lysophosphatidylcholine (which was only slightly cytotoxic by itself) greatly enhanced killing by H_2O_2. The capacity of lisophosphatidylcholine to synergize with H_2O_2 in producing endothelial cell cytotoxicity was inhibited in the presence of phosphatidylcholine. When 50 μg of phosphatidylcholine was added to the endothelial cells along with the lysophosphatidylcholine and H_2O_2, cytotoxicity was reduced from 56 to 23% (Table 1). Phosphatidylcholine had no effect on the synergy between histone and H_2O_2 (Table 1).

Four different enyzmes which affect membrane lipid composition were also examined for ability to interact with H_2O_2 in the killing of endothelial cells. These were phospholipase C, phospholipase A_2, streptolysin S and streptolysin O. Phospholipid moieties are the primary targets of the first three of these enzymes; cholesterol moieties are the primary targets of streptolysin O.[14] Table 2 shows that phospholipase C, phospholipase A_2 and streptolysin S were all capable of interacting with H_2O_2 to kill endothelial cells. In contrast, no synergy was observed between streptolysin O and H_2O_2. Although only a single concentration of

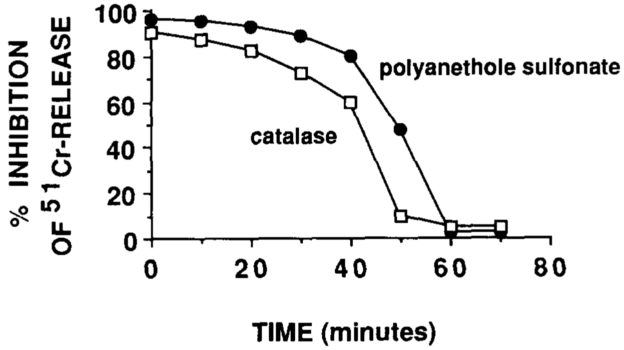

Fig. 4. Time-dependence of catalase-mediated and polyanethole sulfonate-mediated inhibition of endothelial cell killing. Endothelial cells were treated with 0.128 units/ml of glucose oxidase and 75 μg of histone. At 10-min intervals thereafter, 1800 units/ml of catalase or 500 μg/ml of polyanethole sulfonate was added to duplicate cultures. Cytotoxicity was assessed after 2 h of incubation and the percentage of inhibition determined. Values are averages based on duplicate samples in a single experiment in which differences between individual values and averages were less than 10%. The experiment was repeated three times with similar results.

Table 1. Phosphatidycholine Inhibits Syngergistic Killing of Endothelial Cells by H_2O_2 and Lysophosphatidylcholine

Treatment[a]	Percent ^{51}Cr-release[b]
H_2O_2 (1000 nmols)	1 ± 1
lysophosphatidylcholine (1 μg)	8 ± 6
H_2O_2 + lysophosphatidylcholine	56 ± 11
H_2O_2 + lysophosphatidylcholine + 50 μg phosphatidylcholine	23 ± 10
histone (75 μg)	12 ± 1
H_2O_2 + histone	66 ± 5
H_2O_2 + histone + 50 μg phosphatidylcholine	66 ± 6

[a]Cells were treated with various combinations of reagents and incubated at 37°C. Cytotoxicity was assessed after 2 h.

[b]Values are averages ± standard deviations based on 4 samples in 2 independent experiments.

streptolysin O is shown in Table 2, a wide range of concentrations was tested with similar results. Even at concentrations of streptolysin O that were cytotoxic by themselves, we saw no synergy with H_2O_2. The simultaneous addition of high concentrations of phosphatidylcholine (100 μg/ml) to the reaction mixture completely inhibited synergistic killing by phospholipase C, phospholipase A_2, streptolysin S and H_2O_2 (not shown).

DISCUSSION

Inflammatory lesions, particularly those involving bacterial infection, contain a mixture of bioactive substances. Bacterial products may include a number of distinct proteases, phospholipases and hemolysins as well as polycationic substances such as polyamines. Components of injured resident cells include cationic proteins such as nuclear histones and various hydrolytic enzymes. Host inflammatory cells contribute their own hydrolytic enzymes, oxygen radicals and cytokines. Each of these substances may independently contribute to the tissue destruction that often characterizes an inflammatory lesion. In addition to their independent action, however, these various agents may interact with one another to magnify damage. Previous studies by others[21–23] have shown that oxygen metabolites can interact synergistically with leukocyte cationic proteins to effect cytolysis of neoplastic cells and *Schistosoma mansoni*. It has also been shown that cytokines such as tumor necrosis factor-α and interleukin-1 may produce damage to cells by synergizing with other products of inflammation.[24–26] In recent studies from our laboratory, we demonstrated that a number of different membrane-active agents including cationic

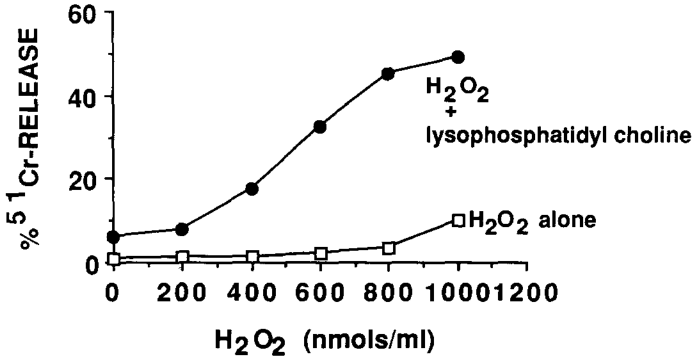

Fig. 5. Sensitivity of rat pulmonary artery endothelial cells to killing by combinations of lysophosphatidylcholine and H_2O_2. Cells were treated with various concentrations of H_2O_2 in the presence and absence of 4 μg/ml of lysophosphatidylcholine. Cytotoxicity was assessed after 2 h. The values shown are averages based on duplicate samples in a single experiment in which differences between individual values and averages were less than 10%. The experiment was repeated five times with similar results.

Table 2. Interaction of Enzymes Which Affect Membrane Lipid Composition and Glucose Oxidase in Endothelial Cell Killing

Treatment[a]	Percent ^{51}Cr-release[b]
glucose oxidase (0.348 units/ml)	8 ± 1
phospholipase C (0.06 units/ml)	6 ± 1
phospholipase + glucose oxidase	54 ± 5
phospholipase A_2 (25 units/ml)	9 ± 2
phospholipase + glucose oxidase	68 ± 7
streptolysin S (250 hemolytic units/ml)	6 ± 1
streptolysin + glucose oxidase	56 ± 4
streptolysin O (250 hemolytic units/ml)	12 ± 4
streptolysin + glucose oxidase	10 ± 5

[a]Enzyme activity of each prepartion was verified using red blood cell hemolysis as indicator. Enzyme concentrations used were chosen on the basis of preliminary studies which showed these concentrations to produce minimal toxicity when used alone. Endothelial cells were treated with the various enzymes for 10 min at 37°C. In the case of phospholipase A_2, 0.5 mg/ml of fatty acid-free bovine serum albumin was added 10 min later. Ten minutes after addition of the enzymes, glucose oxidase was added to the reaction mixture. Release of ^{51}Cr into the culture medium was measured after an additional 2-h incubation.

[b]Values are means ± differences between individual values and means based on duplicate samples in a single experiment. Each enzyme was examined in three or more separate experiments which similar results.

proteins[6–9] and lysophosphatides[10] could bind to the surface of neutrophils and greatly enhance the respiratory burst initiated by soluble stimuli. Thus, one of the possible ways in which these products of inflammation could contribute to the developing lesion is by enhancing the activity of host effector cells.

The present study suggests a second mechanism. The same agents which enhance H_2O_2 production by PMNs can also interact with endothelial cells and increase the sensitivity of these cells to H_2O_2-mediated injury. How these interactions occur at the molecular level is not known at present. In all cases we presume an important role for H_2O_2 since endothelial cell injury mediated by PMNs as well as by reagent H_2O_2 can be almost completely blocked with catalase.[5,27–29] Possibly, agents such as cationic proteins and lysophosphatides alter the integrity of the plasma membrane in such a manner that H_2O_2-sensitive structures become more available. Alternatively, some of these same agents can be directly cytotoxic if cells are exposed to sufficiently high concentrations for extended periods of time. Cells normally repair sub-lytic damage to their membrane caused by these agents. Perhaps the simultaneous exposure to H_2O_2 along with these membrane-damaging agents produces lethal injury by preventing the cells from repairing a normally sub-lethal injury. The ability of H_2O_2 to reduce cellular ATP levels[30,31] is consistent with this idea. Whatever this mechanism, the ability of H_2O_2 to synergize with a number of membrane-active agents to produce lethal injury to intact endothelial cells allows us to hypothesize that the tissue injury seen at sites of inflammation results from the combined effects of different inflammatory mediators rather than from the independent activities of each.

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