Hydrogen Peroxide Stimulates the Synthesis of Platelet-activating Factor by Endothelium and Induces Endothelial Cell-dependent Neutrophil Adhesion

Michael S. Lewis, Ralph E. Whatley, Patricia Cain, Thomas M. McIntyre, Stephen M. Prescott, and Guy A. Zimmerman
Nora Eccles Harrison Cardiovascular Research and Training Institute, and Departments of Internal Medicine and Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84112

Abstract

Oxidant-induced damage to the intima of pulmonary and systemic vessels is thought to be an important mechanism of injury in a variety of syndromes of vascular damage. Hydrogen peroxide (H_2O_2) is an active oxygen metabolite that may induce intimal injury by cytolytic attack or by inducing biochemical and functional alterations in the endothelial cells (EC); however, mechanisms involved in noncytolytic perturbation of EC are largely unknown. We found that H_2O_2 stimulated the synthesis of platelet-activating factor (PAF) by primary cultures of bovine pulmonary artery endothelium (BPAEC) and by human umbilical vein endothelium (HUVEC). In each cell type the incorporation of [^{3}H]acetate into [^{3}H-*acetyl*]PAF was concentration- and time-dependent and was temporally dissociated from severe plasma membrane disruption and cytolytic cell injury; the newly synthesized PAF remained associated with the EC. H_2O_2 caused permeabilization of EC to $^{45}Ca^{2+}$ and an increase in intracellular Ca^{2+}, suggesting that a transmembrane Ca^{2+} flux is the signal that initiates PAF synthesis. H_2O_2 also induced the endothelial cell-dependent adhesion of neutrophils to HUVEC monolayers. This response was rapid, with an onset within minutes and a subsequent time course that paralleled the time course of PAF accumulation, and was dependent on extracellular Ca^{2+} but not on *de novo* protein synthesis. These studies demonstrate that H_2O_2 can induce two rapid activation responses of endothelium, PAF synthesis and EC-dependent neutrophil adhesion, events that may be important in physiologic and pathologic inflammation.

Introduction

It is currently thought that oxidant-induced injury to endothelium is an important mechanism of vascular damage (1–3). Injury to the vascular intima caused by oxidants can result from endogenously produced active O_2 metabolites that are generated in response to high partial pressures of O_2, ionizing radiation, or drugs, or by oxidant species released by other cells, such as leukocytes (1–12). Hydrogen peroxide (H_2O_2), in particular, may be a key molecular species in such injury: it has been implicated in in vitro endothelial cell (EC)[1] injury (4–13), increased vascular permeability in ex vivo and whole-animal lung models (14–17), and in postischemic tissue reperfusion injury (18). One mechanism of this injury is lysis of EC (4–11). However, it is also possible that H_2O_2 causes changes in the endothelium that initiate or amplify vascular responses in the absence of, or before, cytolytic injury, such as the synthesis of biologically active molecules.

EC synthesize a number of substances that may be involved in local mechanisms of coagulation and inflammation (19). One of these is platelet-activating factor (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) (20, 21), which is produced when EC are stimulated by agonists that interact with specific plasma membrane receptors (22, 23). PAF is a phospholipid autacoid with known vasoactive and proinflammatory effects (24–26). Although it may be important in local vascular homeostasis (20, 26), it has also been implicated as a mediator of vascular injury (20, 27–32). For example, it causes pulmonary hypertension and lung edema under some conditions (29–32). Since H_2O_2 also causes these vascular responses (14–17), we examined the possibility that PAF may be produced by endothelium that has been perturbed by H_2O_2. Our findings demonstrate that H_2O_2 induces the synthesis and accumulation of PAF by bovine pulmonary artery endothelial cells (BPAEC) and human umbilical vein endothelial cells (HUVEC) in a concentration-, time-, and calcium-dependent manner that is temporally dissociated from lytic cell injury. In addition, H_2O_2 stimulates endothelial cell-dependent neutrophil adherence that is tightly coupled with PAF synthesis, suggesting that PAF may mediate the cell–cell interaction.

Methods

Materials

PAF (> 99% pure) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Calcium ionophore A23187 (IoA), cycloheximide, actinomycin D, bradykinin, catalase, and superoxide dismutase (SOD) were from Sigma Chemical Co. (St. Louis, MO), [^{3}H]acetate (3.4 Ci/mmol) and [$^{45}Ca^{2+}$]chloride from New England Nuclear (Boston, MA), medium 199 and antibiotic solutions from M. A. Bioproducts (Walkersville, MD) or KC Biologicals (Lenexa, KS), hydrogen peroxide (30% solution) from Mallinckrodt, Inc. (Paris, KY), Hanks' balanced salt solution (HBSS) from M. A. Bioproducts or Life Technologies (Grand Island, NY), Hepes from Behring Diagnostics (La Jolla, CA), fetal bovine serum from Hyclone Laboratories (Logan, UT),

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Address reprint requests to Dr. Zimmerman.

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1. *Abbreviations used in this paper:* BPAEC, bovine pulmonary artery endothelial cell(s); EC, endothelial cell(s); Fn, fibronectin; HUVEC, human umbilical vein endothelial cell(s); IoA, ionophore A23187; PAF, platelet-activating factor; pBPB, para-bromophenacyl bromide; SOD, superoxide dismutase; vWF, von Willebrand factor.

collagenase from Cooper Biomedical, Inc. (Malvern, PA), precoated plates of silica gel 60 from Merck (Darmstadt, Federal Republic of Germany), and EDTA from Fisher Scientific Co. (Fairlawn, NJ). Purified human thrombin was a gift from Dr. John Fenton (Albany, NY). Polyclonal rabbit antisera to human von Willebrand factor (vWF) and human fibronectin (Fn) were from Calbiochem-Behring Corp. (San Diego, CA) and Cappell (Cooper Biomedical, Malvern, PA), respectively, and were shown to interact with vWF and Fn associated with EC by indirect immunofluorescent staining.

EC culture

Pulmonary artery. Cultures of tightly confluent bovine pulmonary artery EC were prepared utilizing methods described in detail elsewhere (33).

Human umbilical vein. Primary cultures of tightly confluent HUVEC were prepared as previously described (20, 22).

EC cultured by these methods were characterized using morphologic (phase-contrast microscopy), immunologic (staining for vWF), and functional (uptake of acetylated LDL, angiotensin-converting enzyme activity, PGI_2 synthesis) criteria as previously described (20, 22, 33). Only primary cultures were used for these experiments.

Assay of PAF production

Production of PAF in BPAEC was measured by incorporation of [^{3}H]acetate into PAF by a modification of the method of Mueller et al. (34) as described in detail elsewhere (22, 33). This has been shown to be an accurate method of measuring PAF accumulation in a variety of cell types (20, 22, 33–36) and to correlate closely with quantitation by gas chromatography-mass spectroscopy (35) or by bioassay (20, 22, 36). Briefly, the medium was removed from confluent EC monolayers and replaced with 1 ml of HBSS/10 mM Hepes (pH 7.4), containing 25 μCi of carrier-free [^{3}H]acetate, and the appropriate concentration of H_2O_2 or control buffer. In some incubations catalase or SOD was included in the incubation mixture. The incubations were performed at 25°C and were stopped at the indicated times by the addition of 0.5 ml of 50 mM acetic acid in methanol. After the addition of the acidified methanol, the cells were scraped from the surface of the culture dish. 50 μg of cold "carrier" PAF was added and the lipids were extracted by the method of Bligh and Dyer (37). The sample was dried under N_2, dissolved in a known volume of chloroform/methanol (9:1), and 10% was removed and used to determine the total radioactivity present. The remaining lipids were then separated by thin layer chromatography on precoated plates of silica gel 60 in $CHCl_3$/MeOH/glacial acetic acid/water (50:25:8:4). The silica was scraped, in fractions corresponding to authentic standards, from the entire lane of a TLC plate, and the radioactivity in each fraction was determined by liquid scintillation spectrometry. Calculation of the radioactivity incorporated into [^{3}H-*acetyl*]PAF was done as described (33). Identification of the radiolabeled product as PAF in EC incubated in this fashion was established by determining its mobility in TLC and HPLC systems, its pattern of degradation by phospholipases A_1 and A_2 and by a highly specific PAF acetylhydrolase isolated from human plasma (38), and by its biologic activity, which was characteristic of PAF (33). The specific activity of [^{3}H-*acetyl*]PAF varies little regardless of the vessel of origin of the EC or the agonist used (33).

The measurement of [^{3}H-*acetyl*]PAF synthesis in HUVEC was performed as described (22), and was similar to the method just outlined for BPAEC.

Measurement of Ca^{2+} uptake and release by EC

Assays in divalent cation-free buffer. Incubations of EC with tritiated acetate and H_2O_2 were done in nominally Ca^{2+} and Mg^{2+}-free HBSS with Hepes and 0.1% EDTA, or in control HBSS with Hepes.

Assay of $^{45}Ca^{2+}$ flux into endothelial cells. Culture medium was removed from confluent monolayers of EC and the monolayers washed twice with 1 ml of HBSS. The monolayers were then incubated at 25°C for the indicated times with 1 ml of HBSS ($[Ca^{2+}]$ = 1.3 mM, pH 7.4) containing 5 μCi/ml of $^{45}CaCl_2$ and the indicated concentrations of agonist. Control dishes were incubated identically, but without an agonist. At the indicated times, the incubation buffer was removed and the culture dish immersed in three sequential washes of ice-cold HBSS (without calcium) containing 0.1% EDTA to remove any remaining extracellular $^{45}Ca^{2+}$. The monolayers were solubilized in 1 ml of 1 M NH_4OH and placed in scintillation vials for determination of radioactivity.

Assay of $^{45}Ca^{2+}$ flux from EC. Culture medium was removed from EC monolayers and replaced with 1 ml of medium M199 containing 5 μCi/ml of $^{45}CaCl_2$. After 1 h of incubation at 37°C, the labeling medium was removed and the monolayers were washed twice with 1 ml of HBSS. The monolayers were stimulated with the indicated agonist concentration in 1 ml of calcium-free HBSS (pH 7.4) at 25°C. Control incubations were performed identically, but without an agonist. At the indicated times, the incubation buffer was removed and the monolayers were washed with 1 ml of HBSS. The monolayers were solubilized in 1 ml of 1 M NH_4OH and placed in scintillation vials for determination of radioactivity.

Measurements of EC injury and cytotoxicity

The morphology of EC and detachment of EC from the monolayers was assessed by phase-contrast microscopy. The uptake of trypan blue was measured by a minor modification of the method detailed by Patterson (39). Lactate dehydrogenase (LDH) release was measured as described (36).

Measurement of neutrophil adherence to EC and to cell-free surfaces

Human neutrophils were isolated, labeled with ^{111}indium, and their adherence to HUVEC monolayers was measured as described (40, 41). In most experiments EC monolayers were pretreated with H_2O_2 (diluted from a 30% stock solution with HBSS/0.5% human serum albumin) for various times at 37°C in an atmosphere of 5% CO_2, 95% air. In some experiments HUVEC were pretreated with H_2O_2 in Ca^{2+}- and Mg^{2+}-free HBSS with 0.5% human serum albumin or in Ca^{2+}- and Mg^{2+}-free HBSS with 0.5% human serum albumin, 20 mM Hepes, and 1 mM EGTA (pH 7.2–7.4) instead of control HBSS. Radiolabeled PMN were then added and incubated as indicated in Fig. 8. Calculation of the fraction of adherent PMN was done as described (40). In some experiments H_2O_2 or another agonist was added to suspensions of ^{111}In-labeled PMN overlying EC monolayers and adherence was determined after incubation at 37°C in 5% CO_2, 95% air ("coincubation" experiments, Table II).

Measurement of the adherence of ^{111}In-labeled PMN to cell-free surfaces was done by a minor modification of the method of Zimmerman et al. (42). The cell-free surfaces included tissue culture wells pretreated for 1 h at 37°C with 50 or 2000 μg/ml gelatin (Type A, Fischer Scientific Co.), 50 μg/ml human fibronectin (Collaborative Research, Inc., Waltham, MA) or whole human serum. Subendothelial matrices were prepared as described (42).

Results

H_2O_2 stimulates BPAEC to synthesize PAF. In a preliminary experiment, we found that [^{3}H-*acetyl*]PAF accumulation by HUVEC that were prelabeled with [^{3}H]acetate and then incubated with PMN and ionophore A23187 (IoA, 10 μM) for 5 min was greater than when incubated with IoA alone, suggesting the possibility that IoA-stimulated PMN released an activity that enhanced PAF synthesis (EC + IoA, 1,250±500 cpm; PMN + IoA, 310±125 cpm; EC + PMN + IoA, 6,400±700 cpm; buffer-treated EC, PMN, or EC + PMN, 100±30 cpm or less). In three subsequent experiments, we found that there was increased accumulation of [^{3}H-*acetyl*]PAF when EC were in-

cubated in the presence of PMA-treated neutrophils (two- to fivefold increase over control, depending on time of incubation, ratio of PMN to EC, and concentrations of phorbol myristate acetate [PMA]). Since PMA did not stimulate PAF synthesis in EC or PMNs when incubated alone under these conditions, this result also suggests that a product of the activated PMN induced PAF synthesis. While the specific molecular specie(s) involved was not identified in these experiments, and conditions that might indicate its nature by inference were not included (i.e., the presence of O_2 radical "scavengers" such as catalase, protease inhibitors, etc.), we considered the possibility that the activity was H_2O_2. H_2O_2 is generated by neutrophils activated by IoA or PMA, and can alter membrane phospholipids in EC (7, 43). To determine if H_2O_2 can stimulate PAF synthesis in endothelium, we first examined its effect on bovine endothelial cells and found that cultured BPAEC reproducibly synthesized PAF when they were treated with H_2O_2. In 20 experiments, EC treated with 10 mM H_2O_2 for 30 min incorporated 8,948±5,089 cpm of [^{3}H]acetate into [^{3}H-*acetyl*]PAF, compared with 386±221 cpm in buffer-treated EC. The response was dependent on the concentration of H_2O_2 and demonstrated a narrow concentration–response relationship. There was consistent accumulation of [^{3}H-*acetyl*]-PAF at concentrations of H_2O_2 of 1–20 mM with a maximal effect at 8–10 mM (Fig. 1). At concentrations of H_2O_2 above 10 mM, there was a concentration-dependent decrease in incorporation of labeled acetate (Fig. 1). In three experiments there were 1,033±513 cpm in [^{3}H-*acetyl*]PAF in BPAEC treated with 100 mM H_2O_2 compared with 10,333±3,177 cpm in EC stimulated with 10 mM H_2O_2. There was no incorporation of [^{3}H]acetate in BPAEC treated with 500 or 1,000 mM H_2O_2 (n = 2). The accumulation of [^{3}H-*acetyl*]PAF in H_2O_2-

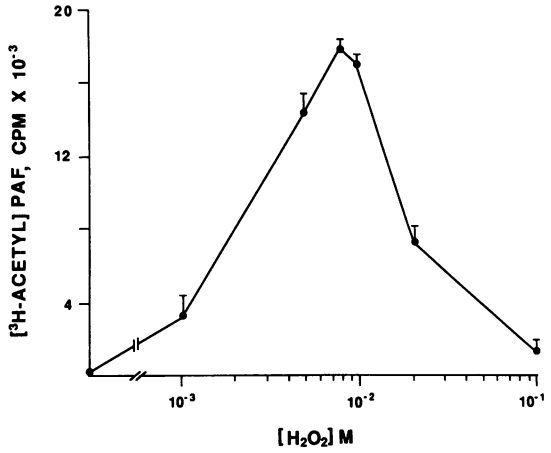

Figure 1. H_2O_2 stimulates the accumulation of [^{3}H-*acetyl*]PAF by BPAEC. Confluent primary BPAEC monolayers were incubated in buffer containing [^{3}H]acetate and various concentrations of H_2O_2 for 30 min at 37°C. The incorporation of [^{3}H]acetate into [^{3}H-*acetyl*]-PAF was measured as described in Methods. Each point represents the mean of duplicate determinations. Five additional experiments in which the effect of multiple concentrations of H_2O_2 were examined yielded similar results. In the experiment shown, the accumulation of [^{3}H-*acetyl*]PAF in BPAEC that were stimulated by 5 or 10 mM H_2O_2 was 1,200±300 and 100±50 cpm, respectively, when catalase (500 U/ml) was included in the incubation buffer, a > 90% reduction in each case.

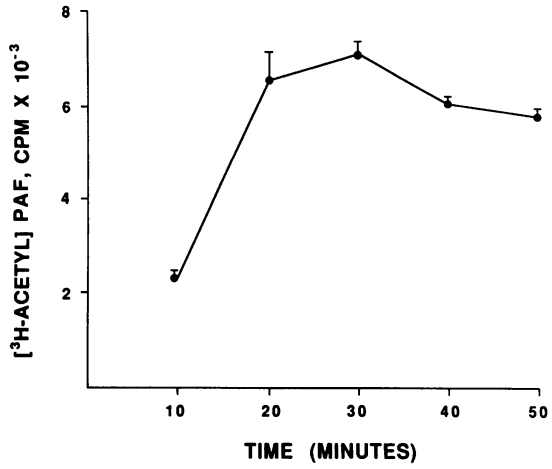

Figure 2. PAF synthesis by H_2O_2-stimulated BPAEC is dependent on the time of incubation. BPAEC were incubated in buffer containing 10 mM H_2O_2 and [^{3}H]acetate for various times, indicated on the horizontal axis, and incorporation of the label into [^{3}H-*acetyl*]PAF was determined as described in Methods. The points represent the means of duplicate determinations in a single experiment. At 30 min, there were 450±50 cpm of [^{3}H-*acetyl*]PAF in monolayers treated with control buffer.

simulated BPAEC was dependent on the time of incubation (Fig. 2). Maximal accumulation occurred at 20–30 min and then declined at a rate that varied from experiment to experiment (compare Fig. 2 with Figs. 5 and 6). However, significant amounts of [^{3}H-*acetyl*]PAF were still present at 50 or 60 min in each of three experiments in which incubations were done for these periods.

To establish that H_2O_2 was the stimulus responsible for PAF synthesis, catalase was added to incubation solutions containing H_2O_2. We found that PAF synthesis by BPAEC was inhibited by ~ 90% by coincubation with catalase (Fig. 1, see legend; Table I). Inactivation of catalase by boiling prevented the inhibition (Table I). Furthermore, superoxide dismutase was largely ineffective in preventing H_2O_2-stimulated PAF synthesis (Table I). Incubation of BPAEC for 30 min in buffer containing glucose (22 mM) and glucose-oxidase (1,600 mU/ml), a system that generates H_2O_2 (5, 8, 12), caused the accumulation of 4,800±400 cpm [^{3}H-*acetyl*]PAF in the absence of catalase and 32±10 cpm when catalase was present (duplicate determinations). Glucose/glucose oxidase also stimulated PAF accumulation in BPAEC in three additional experiments in which the concentration of glucose oxidase was varied between 100 and 1,600 mU/ml. In two experiments treatment of BPAEC with bleomycin (10^{-3} U/ml), a drug that may cause pulmonary vascular injury in part by the generation of oxygen radicals (2), or BCNU (1,3bis[chloroethyl]-nitrosourea; 50 mg/ml), which may enhance H_2O_2-induced EC injury by disrupting the glutathione redox cycle (8), caused a two- to fivefold potentiation of PAF accumulation in response to 10 mM H_2O_2. Neither drug potentiated PAF accumulation stimulated by bradykinin (33) in parallel incubations.

H_2O_2, and H_2O_2-generating systems, have previously been reported to stimulate EC to synthesize PGI_2 (43), and we have found that the synthesis of PGI_2 and PAF are concordant events in activated human and bovine endothelium (22, 23). In an experiment to test this association with H_2O_2 as the

Table I. H_2O_2-stimulated PAF Synthesis by BPAEC Is Inhibited by Catalase and Is Dependent on Extracellular Divalent Cations

Experiment	Condition	[^{3}H-*acetyl*]PAF	Change
		cpm	%
I	Control buffer	600±150	
	H_2O_2 (10 mM)	13,000±100	
	H_2O_2 (10 mM) + catalase	900±300	−98
	H_2O_2 (10 mM) + inactive catalase	11,700±200	−10
	H_2O_2 (10 mM) + SOD	8,700±150	−33
II	Control buffer	300±50	
	H_2O_2 (10 mM)	5,400	
	H_2O_2 (10 mM) + catalase	350±200	−99
	H_2O_2 (10 mM) + SOD	6,000	+11
III	Control buffer	400±150	
	H_2O_2 (7.5 mM)	9,800±800	
	H_2O_2 (7.5 mM), Ca^{2+}, Mg^{2+}-free buffer	350±75	−100
	H_2O_2 (10 mM)	14,000±2,000	
	H_2O_2 (10 mM), Ca^{2+}, Mg^{2+}-free buffer	100±50	−100
	H_2O_2 (25 mM)	1,200±75	
	H_2O_2 (25 mM), Ca^{2+}, Mg^{2+}-free buffer	75±10	−100

BPAEC were incubated in control buffer, buffer containing H_2O_2 with or without catalase (500 U/ml), or SOD (500 U/ml), or in control or Ca^{2+}- and Mg^{2+}-free buffer with H_2O_2, for 30 min. In one experiment catalase was inactivated by boiling. Incorporation of [^{3}H]-acetate into [^{3}H-*acetyl*]PAF was measured as described in Methods. Values indicate single determinations or mean±SD of duplicate determinations. Values for [^{3}H-*acetyl*]PAF in EC treated with control buffer were subtracted from PAF levels in EC stimulated with H_2O_2 when the "change" was calculated.

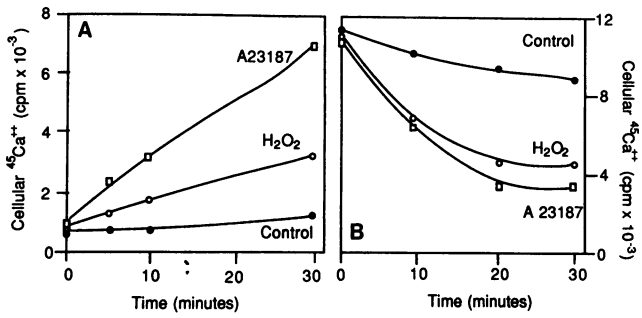

Figure 3. Endothelial cells stimulated with H_2O_2 have increased calcium permeability. (*A*) H_2O_2-stimulated $^{45}Ca^{2+}$ uptake by BPAEC. Bovine pulmonary artery endothelial cells (10^6 cells per dish) were incubated at 25°C in the presence of $^{45}Ca^{2+}$ (5 μCi/ml) in HBSS containing control buffer, H_2O_2 (10 mM), or calcium ionophore A23187 (10^{-5} M). At the indicated times, the incubation buffer was removed and the cellular $^{45}Ca^{2+}$ content determined as described in Methods. Each point represents a single determination in one dish. The results shown are representative of two separate experiments. (*B*) H_2O_2-stimulated $^{45}Ca^{2+}$ efflux from preloaded BPAEC. Bovine pulmonary artery cells were incubated in medium containing 5 μCi/ml $^{45}Ca^{2+}$ for 1 h at 37°C. The incubation medium was removed, the monolayers were washed with HBSS, and were then incubated at 25°C in calcium-free control buffer (HBSS, pH 7.4) or the same buffer containing H_2O_2 (10 mM) or calcium ionophore A23187 (10^{-5} M). At the indicated times, the incubation buffer was removed and the $^{45}Ca^{2+}$ content was determined as described in Methods.

agonist, we found that 10 mM H_2O_2 stimulated the release of 6-keto-$PGF_{1\alpha}$ (measured by radioimmunoassay; reference 22) and accumulation of [^{3}H-*acetyl*]PAF in parallel, with a maximal accumulation of each at 20 min.

H_2O_2 stimulates $^{45}Ca^{2+}$ uptake by BPAEC. PAF synthesis by BPAEC in response to H_2O_2 was dependent on extracellular divalent cations. When H_2O_2 was incubated in both submaximal and maximal concentrations with BPAEC monolayers in Ca^{2+}- and Mg^{2+}-free buffer, the accumulation of PAF was completely inhibited (Table I). PAF synthesis by endothelium stimulated by other agonists, including the receptor-mediated ligands bradykinin and ATP and the calcium IoA, is dependent on extracellular Ca^{2+}; furthermore, these agonists stimulate Ca^{2+} uptake by EC (Whatley et al., manuscript submitted for publication). To determine if H_2O_2 also induces calcium transfer, we stimulated BPAEC monolayers in buffer containing $^{45}Ca^{2+}$ and found a time-dependent increase in labeled calcium associated with the cells (Fig. 3 *A*). Ionophore A23187 (IoA) also stimulated $^{45}Ca^{2+}$ uptake in parallel incubations. A second experiment yielded qualitatively similar results. To further demonstrate that H_2O_2 increased the permeability of EC plasma membranes to Ca^{2+}, we preloaded the cells with $^{45}Ca^{2+}$ and then treated them with H_2O_2 or IoA in calcium-free buffer. This maneuver created a large concentration gradient between Ca^{2+} in the cytoplasm and the extracellular space; under these conditions increased plasma membrane permeability to calcium would result in efflux of Ca^{2+} from the cell. Stimulation of the EC with 10 mM H_2O_2 resulted in loss of labeled Ca^{2+} from the cells, as did treatment with the ionophore (Fig. 3 *B*). These experiments demonstrate that H_2O_2 induces increased permeability of EC membranes for Ca^{2+} and/or that it activates calcium transport mechanisms that cause both uptake and extrusion. The alterations in Ca^{2+} transport were temporally associated with PAF synthesis (Fig. 2).

H_2O_2-induced PAF synthesis by BPAEC is temporally dissociated from lytic cell injury. BPAEC, when subjected to concentrations of H_2O_2 that induced maximal synthesis of PAF (Fig. 1), exhibited no morphologic evidence of injury such as swelling, detachment from the monolayer, or lysis. Moreover, trypan blue was excluded by EC stimulated with H_2O_2 in this concentration range (Fig. 4). This finding indicates that the synthesis of PAF is not simply a consequence of plasma membrane disruption, and that the increased permeability to Ca^{2+} (previous section) was selective rather than nonspecific. There was a parallel increase in abnormal cell morphology and trypan blue uptake above H_2O_2 concentrations of 20 mM, accompanying the dramatic fall in PAF accumulation at these concentrations (Fig. 4). A time course of PAF accumulation compared to trypan blue uptake in response to 10 mM H_2O_2 demonstrated that there was minimal uptake of the dye at the time of maximal PAF accumulation, followed by a progressive increase in trypan blue uptake during the descending slope of the PAF accumulation curve (Fig. 5).

PAF remains associated with the cells in H_2O_2-stimulated BPAEC monolayers. Newly synthesized PAF remains associated with human (22, 23, 41) and bovine (33) EC stimulated

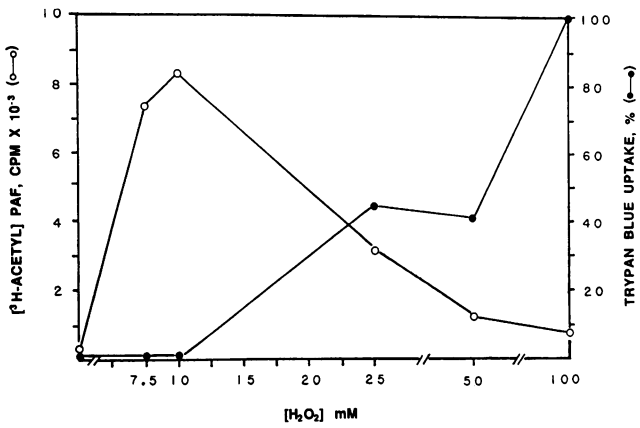

Figure 4. Maximal PAF synthesis by H_2O_2-treated BPAEC is dissociated from increased permeability to trypan blue. BPAEC monolayers were treated with buffer containing various concentrations of H_2O_2 and [^{3}H]acetate for 30 min at 37°C and [^{3}H-*acetyl*]PAF accumulation was measured as described in Methods. Trypan blue staining was done by a minor modification of a described technique (39); uptake was determined by phase-contrast microscopy (300–500 cells were counted in each monolayer) and is expressed as the percentage of cells with blue staining.

with a variety of agonists that interact with plasma membrane receptors. However, it is possible that it may be released from EC that are injured by oxidants or other pathologic stimuli (44). To determine if PAF synthesized by H_2O_2-stimulated BPAEC is released into the fluid phase, we incubated EC monolayers with H_2O_2 for various periods and measured the fractions of [^{3}H-*acetyl*]PAF in the incubation buffer and in the cells after they were extracted separately. In four experiments, 1% or less of the labeled PAF was found in the incubation medium after stimulation of the EC with 10 mM H_2O_2 for 30 min at 37°C. We found a similar result when 30 mg/ml fatty acid-free bovine serum albumin, to which PAF binds, was included in the incubation buffer. In an experiment to examine the time dependency of the distribution of newly synthesized PAF in H_2O_2-stimulated BPAEC, the phospholipid remained associated with the cell pellet at all time points and little, if any, was released into the incubation medium (Fig. 6). Even at 60 min, when as many as 40% of the EC treated with 10 mM H_2O_2 may be permeable to trypan blue (mol wt 961) (Fig. 5), PAF (mol wt = 524) was retained by the endothelium.

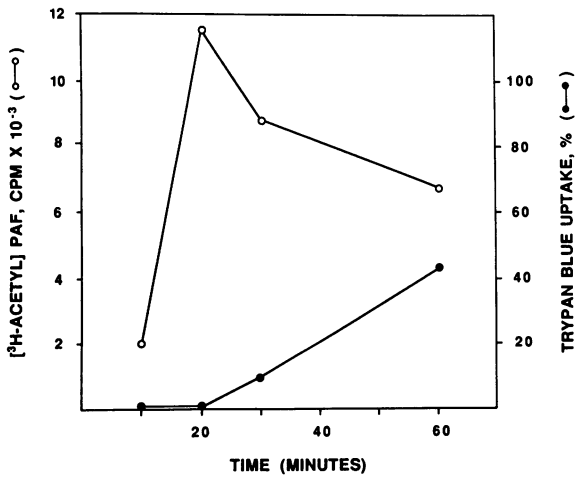

Figure 5. The time-dependent alterations in PAF accumulation and trypan blue uptake are dissociated in H_2O_2-stimulated BPAEC. [^{3}H-*acetyl*]PAF accumulation and trypan blue uptake were measured as described in Fig. 4 in replicate BPAEC monolayers that were stimulated with 10 mM H_2O_2.

H_2O_2 stimulates PAF synthesis by human endothelium. HUVEC were used as a readily available human cell type that shares many common features with in situ endothelium and with endothelium cultured from other human vessels (19). We found that H_2O_2 reproducibly stimulated [^{3}H]acetate incorporation into [^{3}H-*acetyl*]PAF in these cells. The response was concentration-dependent with a threshold at 0.5–1 mM H_2O_2, a maximal effect at 5–10 mM H_2O_2, and a sharp decrease at 100 mM H_2O_2. In contrast, H_2O_2 (1–100 mM) did not stimulate [^{3}H-*acetyl*]PAF accumulation in isolated human PMN, even though PMN synthesize PAF in response to several agonists (36), indicating a degree of specificity of the H_2O_2 effect. PAF accumulation by H_2O_2-stimulated HUVEC was also time-dependent. In each of five experiments maximal [^{3}H-*acetyl*]PAF occurred at 10–20 min in response to 10 mM H_2O_2, followed by a variable decline to basal, or near basal, levels by 60 min (Fig. 7). Thus the accumulation of PAF in HUVEC stimulated with a maximal concentration of H_2O_2 was usually more abbreviated than in BPAEC (Figs. 2, 5, and 6). In an experiment comparing submaximal and maximal concentrations of H_2O_2 (1 and 10 mM) in HUVEC, there was a broader and more prolonged time course in response to the lower concentration of H_2O_2 (Fig. 7).

HUVEC treated with 10 mM H_2O_2 excluded trypan blue during the first 60 min of incubation, the period of PAF accumulation, and the monolayers remained intact. The response thereafter was variable. In one experiment essentially all cells were permeable to trypan blue, and there was extensive detachment and lysis of the EC, by 120 min. In two additional

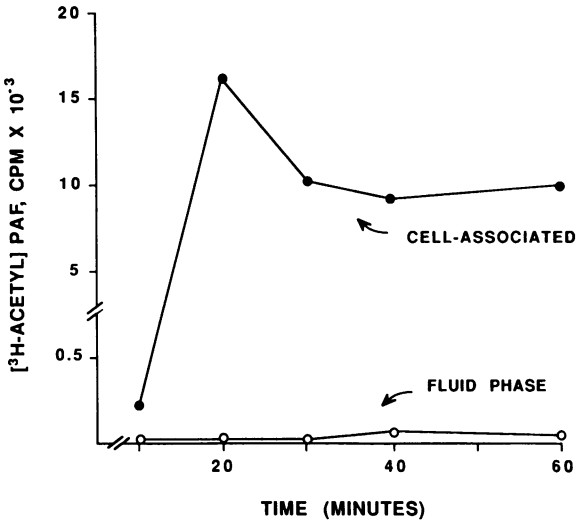

Figure 6. PAF synthesized by H_2O_2-stimulated BPAEC is cell-associated. EC monolayers were incubated with 10 mM H_2O_2 and [^{3}H]-acetate for various times at 37°C. At the end of the incubation periods the buffer was removed, the lipids in the buffer and EC monolayers were separately extracted, and the amount of [^{3}H-*acetyl*]PAF was determined in each. The points indicate the mean of duplicate determinations in a single experiment. A qualitatively similar result was found in a second experiment in which the incubations were stopped at 10, 30, and 50 min.

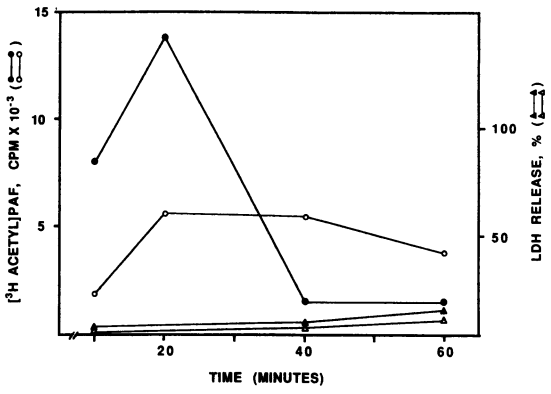

Figure 7. H_2O_2-induced [^{3}H-*acetyl*]PAF accumulation in human endothelium is dissociated from cytolytic injury. HUVEC were incubated with 1 mM (○, △) or 10 mM (●, ▲) H_2O_2 and [^{3}H]acetate, and the accumulation of [^{3}H-*acetyl*]PAF was measured as described in Methods. LDH was measured (36) in the cell fraction (after solubilization with Triton X-100) and in the incubation buffer of replicate monolayers and the fraction that was released into the incubation buffer was calculated. EC treated with control buffer in the absence of H_2O_2 released no LDH at 10 or 60 min.

experiments EC developed altered morphology (retracted, angular cells) after 2 h, but significant desquamation of EC and trypan blue uptake did not occur until 3–6 h after treatment of the EC with H_2O_2. In addition, in two experiments EC were treated with 10 mM H_2O_2 for 20 min, incubated with catalase (500 U/ml, to degrade any remaining extracellular H_2O_2) or control buffer for 10 min, and then covered with complete culture medium and returned to the incubator for 18 h. In both experiments there was an adherent monolayer of EC that excluded trypan blue at the end of this period, although individual cells had been lost from the monolayer and many cells had undergone shape change; there was no obvious difference in monolayers "rescued" with catalase and complete medium compared to complete medium alone. These experiments indicated that PAF synthesis was temporally dissociated from lethal injury to the EC assessed by trypan blue uptake and from complete morphologic disruption of the monolayer. Significant LDH release was a late event that was also dissociated from PAF synthesis. 8% and 12% of total cellular LDH was released from HUVEC treated with 1 mM or 10 mM H_2O_2 for 60 min (Fig. 7). In a second experiment there was no LDH release and maximal PAF accumulation when HUVEC monolayers were stimulated with 10 mM H_2O_2 for 10 min, whereas there was decreased PAF accumulation in association with increased LDH release in response to 100 and 500 mM H_2O_2 (90% LDH release from EC treated with 500 mM H_2O_2). Thus in HUVEC, as in BPAEC, PAF synthesis was temporally dissociated from indices of extensive plasma membrane damage and cytolytic injury. Also as in H_2O_2-stimulated BPAEC, the newly synthesized [^{3}H-*acetyl*]PAF was retained by the EC (not shown).

In an experiment to determine the effect of pretreatment of EC with H_2O_2 on subsequent PAF synthesis stimulated by receptor-mediated agonists, we pretreated HUVEC with 0.5 or 3 mM H_2O_2 for 10 min, removed the buffer, and added human α-thrombin (2.0 U/ml) (20, 42) for an additional 10-min incubation. Pretreatment with 0.5 or 3 mM H_2O_2 reduced PAF accumulation by 47% and 51%, respectively, compared to the thrombin response in replicate monolayers pretreated with control buffer.

H_2O_2 induces endothelial cell-dependent neutrophil adhesion. We have previously observed that the synthesis of PAF by HUVEC is tightly coupled with a functional alteration that results in the adhesion of neutrophils (PMN) to the EC (20, 40–42), suggesting that the two events are related and that they are components of a rapid EC activation response (23). To determine if H_2O_2 induces endothelial cell-dependent neutrophil adhesion, we pretreated HUVEC monolayers for 20 min with H_2O_2 (10 mM); the H_2O_2 was then removed and replaced with buffer containing ^{111}In-labeled PMN. Neutrophil adhesion was measured after a 5-min incubation. In eight experiments, this resulted in a two- to sixfold increase in the adhesion of labeled PMN when compared to adherence to EC pretreated with control buffer (Table II). Examination of the monolayers by phase-contrast microscopy demonstrated that single neutrophils adhered to the surface of the EC, with rare aggregates of PMN also adhering, and that the monolayers were intact at this time. The response was concentration-dependent (Fig. 8) with a threshold at 0.5–1.0 mM H_2O_2 and a maximal effect at 5–10 mM H_2O_2. Pretreatment of HUVEC with 100 mM H_2O_2 resulted in PMN adherence that was con-

Table II. H_2O_2 Induces EC-dependent PMN Adhesion

Condition	Adhesion (Mean±SD)	Experiments (*n*)
	%	
I. Pretreatment		
EC + buffer	7±3	8
EC + H_2O_2 (5 mM)	24±8	8
EC + H_2O_2 (10 mM)	25±7	8
EC + H_2O_2 (10 mM), wash	27±0.4	2
EC + H_2O_2 (10 mM), catalase	9±3	4
EC + H_2O_2 (10 mM), SOD	25±4	2
II. Cell-free surfaces		
CFS + PMN + buffer	6±3	8
CFS + PMN + H_2O_2 (1 mM)	6±2	5
CFS + PMN + H_2O_2 (5 mM)	8±3	5
CFS + PMN + H_2O_2 (10 mM)	8±4	8
III. Coincubation		
EC + PMN + buffer	8±4	2
EC + PMN + H_2O_2 (5 mM)	15±2	2
EC + PMN + H_2O_2 (10 mM)	22±0	2

In pretreatment protocols, EC monolayers were incubated with control buffer (HBSS/0.5% human serum albumin) or H_2O_2 in HBSS/0.5% human serum albumin for 20 min at 37°C; the incubation mixture was then removed, ^{111}In-labeled PMNs were added and incubated for 5 min, and adherence determined as in Methods and Fig. 8. In some experiments EC were washed with 1 or 2 vol of HBSS/0.5% human serum albumin after incubation with H_2O_2, before addition of PMN; in other experiments catalase (500 U/ml) or SOD (500 U/ml) was included in the incubation mixture with H_2O_2. In experiments with cell-free surfaces (CFS), ^{111}In-labeled PMN were added to gelatin-coated culture wells, buffer or H_2O_2 was added, and PMN adherence was measured after 5 min of incubation. In coincubation protocols, control buffer or H_2O_2 was added to ^{111}In-labeled PMN overlying EC monolayers and PMN adherence was determined after a 20-min incubation.

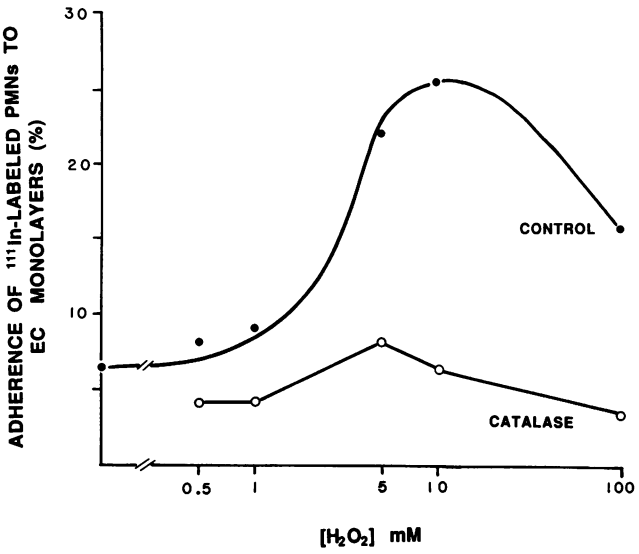

Figure 8. Endothelial cell-dependent PMN adherence is induced by H_2O_2 in a concentration-dependent fashion. HUVEC were pretreated with control buffer (HBSS/0.5% human serum albumin) or with various concentrations of H_2O_2 diluted in HBSS/0.5% human serum albumin for 20 min at 37°C. Catalase (500 U/ml) was included in some incubations. The incubation mixtures were then removed and ^{111}In-labeled PMNs (5.5×10^6/ml in HBSS/0.5% human serum albumin) were added and incubated for an additional 5 min. The fraction of adherent PMNs was determined as described (40) after confirming that the leukocytes had adhered to the surface of the EC by inspection (phase-contrast microscopy). The means of results from two experiments are shown.

siderably lower than adhesion induced by 10 mM H_2O_2 (12% vs. 25%, $n = 5$ and 8, respectively), a pattern similar to that seen when PAF accumulation was assayed. H_2O_2-induced PMN adhesion was inhibited by catalase (Fig. 8; Table II) but not by SOD (Table II). Incubation of EC with H_2O_2 in divalent cation-free buffer completely inhibited subsequent PMN adhesion; addition of Ca^{2+}, but not Mg^{2+}, returned the response to control levels. Direct addition of 10 mM H_2O_2 to PMN suspensions overlying HUVEC monolayers, followed by a 20-min incubation, also stimulated PMN adhesion, but the adherence under these conditions was less than in parallel incubations where EC were pretreated with H_2O_2 for 20 min before addition of the PMN (Table II). This may be in part due to the generation of PGI_2, which can blunt PMN adhesiveness (45), by the H_2O_2-stimulated EC, or to the ability of PMN to "scavenge" H_2O_2 (46).

Washing of the EC monolayers with buffer after pretreatment with H_2O_2 did not diminish PMN adhesion when the leukocytes were subsequently added and incubated for 5 min (Table II). In addition, H_2O_2 did not cause PMN to adhere to cell-free surfaces (gelatin-coated tissue culture wells) when it was added to the PMN and they were incubated for the same period as in incubations with EC (Table II); this excludes the possibility that "carryover" of H_2O_2 directly induced increased PMN adhesiveness. Pretreatment of subendothelial matrices or incubation wells coated with gelatin (50 mg/ml), Fn (50 mg/ml), or whole human serum (used as a source of vitronectin and thrombospondin as well as vWF and Fn) with 10 mM H_2O_2 for 20 min followed by the addition of PMN for 5 min did not cause increased PMN adhesion compared to surfaces pretreated with control buffer ($n = 2$). Thus, under the conditions of these experiments, the H_2O_2-stimulated PMN adhesion resulted from increased adhesiveness of the endothelium and was dependent on the EC.

The H_2O_2-induced neutrophil adhesion was a time-dependent event. Pretreatment of the HUVEC with 10 mM H_2O_2 for as little as 5 min resulted in enhanced leukocyte binding. Although there was variation from experiment to experiment, the maximal effect was at 20 min and PMN adhesion declined when the monolayers were pretreated for longer periods. This temporal pattern was similar to the time course of H_2O_2-stimulated PAF accumulation in HUVEC. Therefore, we measured PAF accumulation and neutrophil adhesion in parallel using replicate EC monolayers from the same culture. The two responses were concordant in these experiments (Fig. 9).

Pretreatment of HUVEC with the potent phospholipase inhibitor *p*-bromophenacyl bromide (pBPB; 25 μM for 15 min) (47) completely prevented enhanced PMN adhesion to H_2O_2-treated monolayers, reducing the adherence to the level seen in monolayers treated with control buffer rather than H_2O_2. PMN adhesion induced by thrombin, but not adhesion stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), was blocked by pBPB in parallel incubations. In two experiments PAF synthesis by HUVEC stimulated with H_2O_2 (5 or 10 mM), thrombin, or IoA was inhibited by greater than 95% by pretreatment of the EC with pBPB (25 μm) for 15 min. In two experiments to determine if RNA or protein synthesis was required for H_2O_2-induced PMN adhesion, pretreatment of HUVEC with cycloheximide (20 or 35 μM for 1 or 4 h) (48) or actinomycin D (5 μg/ml for 4 h) (49) caused no decrease in neutrophil adherence to EC monolayers treated with 5 or 10 mM H_2O_2. Inclusion of monensin (1 μM) (48) during the 20-min preincubation period of HUVEC with 10 mM H_2O_2 did not reduce PMN adhesion to the monolayers in two experiments, suggesting that redistribution of preformed pro-

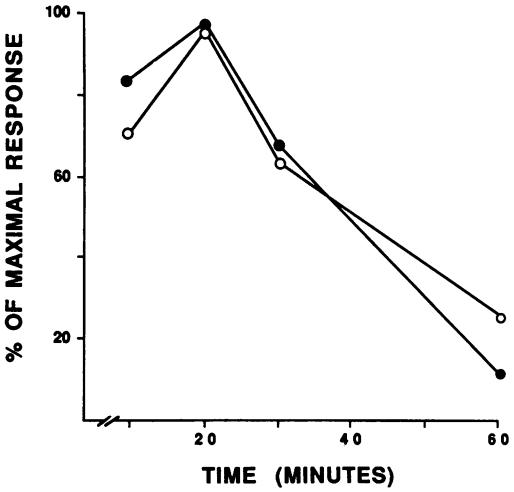

Figure 9. Endothelial cell-dependent PMN adhesion and PAF synthesis are temporally-associated events in H_2O_2-stimulated EC. HUVEC were grown in individual 35-mm culture dishes, and incubated with 10 mM H_2O_2 for the indicated times. [^{3}H-*acetyl*]PAF accumulation was determined as in Methods and PMN adhesion was measured as in Methods and Fig. 8. The figure indicates the mean data from three experiments for each measurement; in two experiments [^{3}H-*acetyl*]PAF accumulation and PMN adhesion were measured in parallel in replicates from the same EC culture.

teins was not involved (50). Furthermore, polyclonal antisera to human vWF and FN did not block PMN adhesion to H_2O_2-treated EC (n = 2). These results suggest that rapid expression of these adhesive glycoproteins on the EC surface (51), or alteration by H_2O_2 of vWF or FN that was constitutively associated with the EC plasma membrane or pericellular matrix (52), was not the mechanism of enhanced PMN adhesion. In an additional experiment we pretreated PMNs with buffer, 10^{-8} M PAF, or 10^{-8} M lyso-PAF (which is biologically inactive [24]) for 5 min at 37°C, added them to EC monolayers that had been pretreated with 5 mM H_2O_2 for 20 min, and then measured adherence after a 5-min incubation. Pretreatment with PAF reduced PMN adhesion to H_2O_2-activated monolayers by 65%, compared to 13% with lyso-PAF. Adherence of PMN induced by exogenous fMLP was not substantially reduced by either PAF or lyso-PAF (88% of the value for buffer-pretreated PMN in each case). These data are consistent with specific desensitization of PMN adhesion to H_2O_2-treated endothelium by pretreatment with PAF (40, 41).

Discussion

In this report we present evidence that BPAEC and HUVEC in primary culture synthesize and accumulate PAF when they are stimulated with hydrogen peroxide. Accumulation of PAF induced by H_2O_2 is concentration- and time-dependent, is associated with a calcium flux across the plasma membrane, and is temporally dissociated from lytic cell injury. H_2O_2 also induces rapid endothelial cell-dependent adherence of neutrophils that is temporally coupled with PAF accumulation in human endothelium. These observations document that H_2O_2 can directly activate EC and suggest a new mechanism by which oxidants may initiate or amplify inflammatory vascular injury. Although the concentrations of exogenously delivered reagent H_2O_2 required to induce these effects were high, and it is unknown if such concentrations are achieved in inflammed tissue in vivo, neutrophils generate and release sufficient H_2O_2 to cause high local concentrations (1, 12, 53)[2] and high local concentrations of endogenously generated oxidants may occur under hyperoxic conditions (3). Furthermore, the experiments that we have described may indicate a paradigm for responses of EC that are perturbed by oxidants, regardless of whether or not the concentrations of H_2O_2 that we utilized are achieved in vivo.

It has been documented previously that active oxygen species, including H_2O_2, can cause cytotoxic injury to EC (1–14). H_2O_2 causes lysis of cultured endothelium (4–11, 56) and, in concert with proteases generated by inflammatory cells (57), may cause desquamation of EC from the subcellular matrix. Similar alterations in vivo would result in wholesale destruction of the luminal surface. However, evidence for widespread EC lysis and extensive denudation of the intima is not commonly found in the acute phases of conditions that are thought to involve oxidant-induced vascular injury, even though there is concurrent evidence for abnormal vascular function (such as increased permeability, altered vasoreactivity, or thrombosis) (2, 3, 58, 59). This suggests that the EC are intact, but that their basal functions have been perturbed. Furthermore, some models of oxidant-induced vascular injury indicate that there are reversible alterations in endothelial permeability that do not involve cell death (14). The biochemical mechanisms involved in oxidant-induced, nonlytic EC alterations are largely unknown (1). Our studies demonstrate that H_2O_2 induces PAF synthesis by human and bovine endothelium, a biochemical response that may contribute to such functional alterations.

PAF synthesis requires specific enzymatic activities (60). Although more than one pathway for PAF synthesis exists in mammalian cells, in endothelium it occurs by the sequential phospholipase A_2-catalyzed hydrolysis of 1-0-alkyl-2-acyl-*sn*-glycero-3-phosphocholine to form 1-0-alkyl-*sn*-glycero-3-phosphocholine ("lyso-PAF") followed by acetylation of this molecule to form PAF (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); the latter step is catalyzed by an intracellular acetyl transferase (60–62). The mechanism by which H_2O_2 "switches on" this enzymatic pathway may involve a Ca^{2+} flux across the EC plasma membrane since H_2O_2 caused increased permeability of the EC for Ca^{2+} and accumulation of radiolabeled Ca^{2+} in the cells (Fig. 3). Furthermore, a sustained increase in intracellular Ca^{2+} induced by receptor-mediated and receptor-independent perturbation of EC results in PAF synthesis (Whatley et al., manuscript submitted for publication). Shasby et al. previously reported that a xanthine/xanthine oxidase system caused increased permeability of porcine EC for $^{45}Ca^{2+}$ (63); preliminary experiments indicate that linoleate peroxide causes $^{45}Ca^{2+}$ release from preloaded, cultured EC and that H_2O_2 induces the generation of linoleate peroxide by these cells (64). Lipid peroxides, including linoleate peroxide, may act as Ca^{2+} ionophores (65). Thus H_2O_2 may have induced increased calcium permeability and PAF synthesis by the EC by causing the generation of lipid peroxides. Increased intracellular Ca^{2+} also initiates the synthesis of PGI_2 by endothelium (66). Consistent with this, we found that H_2O_2 induced the coordinate accumulation of PAF and 6-keto-$PGF_{1\alpha}$ (see Results) confirming earlier observations that H_2O_2 can stimulate PGI_2 synthesis by EC (7, 43) as well as our previous finding that PAF and PGI_2 synthesis are initiated in concert in activated endothelium (22, 23, 33). These findings suggest that H_2O_2 induces Ca^{2+} flux, activation of phospholipase A_2 (thought to be a Ca^{2+}-dependent enzyme), and the hydrolysis of one or more membrane phospholipid precursors in endothelium, yielding free arachidonate and lyso-PAF for subsequent conversion to PGI_2 and PAF, respectively (Whatley et al., manuscript submitted for publication).

As in our experiments, previous studies with cultured EC from several sources demonstrate that enzymatic synthesis of biologically active molecules can be stimulated by H_2O_2 in the absence of lytic injury (7, 43). Additional biologic and biochemical alterations in EC induced by H_2O_2 or other active O_2 species at time points before cell lysis, or in cells treated with sublytic concentrations of H_2O_2, include potassium efflux and the release of cytoplasmic purines (7), reorganization of actin filaments (63), and reduction in cellular ATP levels (11).

We found that the concentration-response relationship for H_2O_2-induced PAF synthesis was quite narrow (Fig. 1), and that concentrations of H_2O_2 greater than 10 mM decreased or abolished PAF accumulation in both BPAEC and HUVEC. These results suggest that high concentrations of H_2O_2 may inactivate one or more of the enzymes involved in PAF syn-

2. Recently Nathan (54) has reported that PMN release "massive" quantities of H_2O_2 under certain conditions. Also, Bozeman et al. (55) estimated that millimolar concentrations of H_2O_2 may be achieved in inflammatory lesions containing neutrophils.

thesis. We also found that pretreatment of HUVEC with submaximal concentrations of H_2O_2 reduced PAF accumulation when the monolayers were subsequently stimulated with thrombin (see Results). A variable effect of oxidants on eicosanoid synthesis, depending on the concentration of the oxidant, has also been reported (7, 10, 43). Whorton et al. (10) observed that pretreatment of cultured porcine endothelium with concentrations of H_2O_2 (0.01–0.1 mM) that did not induce PGI_2 synthesis, or coincident cell lysis, impaired PGI_2 synthesis when the EC were subsequently treated with exogenous arachidonate or ionophore A23187, and that cyclooxygenase, but not PGI_2 synthase, was inhibited under these conditions. Taylor et al. found that t-butyl hydroperoxide stimulated the production of PGI_2 at low concentrations and inhibited it at high concentrations; the inhibition at high concentrations was reversed by addition of an oxygen radical scavenger (67). These reports, and our observations on PAF synthesis, are consistent with evidence that H_2O_2 and other oxidants can cause rapid, selective damage to enzymes and other intracellular proteins, and intracellular proteolysis of the altered molecules (68). Thus the effect of H_2O_2 on the production of lipid autacoids by EC may depend heavily on variables that include the concentrations of the oxidant (Figs. 1 and 7) and endogenous antioxidants (8, 12), time (Fig. 7), and the presence or absence of additional agonists.

We found that H_2O_2 induced neutrophil adherence that was dependent on the endothelium. Endothelial cell-dependent PMN adherence is a novel biologic response to H_2O_2 that has not been previously reported, although PMN adhesion to the intima of oxidant-injured vessels and to EC cultured under hyperoxic conditions has been described (69, 70). EC-dependent PMN adherence has been observed when cultured endothelium is stimulated by a variety of naturally occurring inflammatory mediators including thrombin (20, 40, 42), sulfidopeptide leukotrienes (41), and cytokines (49, 71), and may contribute to the accumulation of PMN in vessels in certain pathologic conditions such as the adult respiratory distress syndrome (72). In our experiments, H_2O_2-induced, EC-dependent neutrophil adherence was very rapid, with an onset within minutes and a peak that usually occurred within 20 min (Fig. 9), and was not dependent on *de novo* protein synthesis by the EC. These features clearly differentiate it from EC-dependent PMN adhesion stimulated by the cytokines tumor necrosis factor and interleukin 1, and by endotoxin, which require $\sim$ 1 h for onset, 4–6 h for the maximal effect, and the synthesis of new protein(s) (49, 71). The rapidity with which H_2O_2 stimulates EC-dependent PMN adhesion is similar to the onset induced by thrombin, LTC_4 and LTD_4 (20, 40–42).

There are several possible molecular events that may contribute to H_2O_2-induced EC-dependent adhesion. H_2O_2 may stimulate the translocation of a preformed adhesive protein from an intracellular compartment to the EC plasma membrane. The lack of an effect of 4-h preincubation of the EC with cycloheximide, or of treatment of EC with monensin (50), argue against this possibility, and polyclonal antibodies against two adhesive glycoproteins (vWF and fibronectin) that are associated with EC (51) did not inhibit PMN adhesion. A second possibility is that H_2O_2 induces a conformational change, or a change in the primary structure, of a constitutive surface or pericellular protein (68) causing it to become adhesive (52). However, treatment of matrices of relevant proteins or subendothelial matrices with H_2O_2 under the conditions of these experiments did not induce enhanced PMN adherence. A third possibility is that PAF that is synthesized by H_2O_2-stimulated EC mediates the adhesive interaction. We have previously reported evidence that PAF that is endogenously synthesized by activated EC, and that remains cell-associated, may cause PMN adhesion (23, 40–42, 61). In the current experiments the time courses for H_2O_2-stimulated PAF accumulation and for PMN adhesion to the EC were strikingly similar (Fig. 9) as were the concentration–response relationships. H_2O_2-induced PMN adhesion was inhibited by the potent (47, 73) but nonspecific (73) phospholipase inhibitor pBPB, which also inhibited PAF synthesis. Furthermore, there was reduced binding of PMN that were specifically "desensitized" by pretreatment with submaximal concentrations of PAF to H_2O_2-treated EC. These observations suggest that a portion of the PAF synthesized by EC in response to H_2O_2 is located in the plasma membrane or is otherwise available for interaction with adjacent neutrophils. In addition, competitive PAF receptor antagonists diminish PMN adherence to H_2O_2-treated HUVEC (Zimmerman et al., manuscript in preparation).

PAF synthesis, EC-dependent PMN adhesion, and PGI_2 production are rapid responses of endothelial cells that are activated by specific agonists (23). This report demonstrates that H_2O_2, like receptor-mediated agonists, can stimulate the first two of these, adding them to the repertoire of biochemical and biologic responses of vessels that are perturbed by active O_2 metabolites (1). Because of the vasoactive and prothrombotic effects of PAF (24–33, 44), and the ability of adherent PMNs to cause endothelial damage (1, 4–6, 56, 57), activation of EC by H_2O_2 has the potential to mediate pathologic events in oxidant-induced vascular injury states before the onset of lytic destruction of the intima, or in segments of the intima that are not destined for necrosis because they have been perturbed by sublytic concentrations of the toxic O_2 species. Since PMN release H_2O_2 into the fluid phase (1, 55), the activation of EC by H_2O_2, resulting in PAF synthesis, provides the basis for a reciprocal amplification loop between the two cells. We have observed that PMN that become adherent to activated EC develop morphologic evidence indicating that the leukocytes in turn become activated (membrane spreading and polarization), suggesting that signals received by the PMN from the EC can close such an amplification loop (40–42, 61). It is also possible that such a reciprocal interaction may occur in physiologic as well as pathologic inflammatory events, since the EC generate biologically active molecules (74), such as PGI_2, that may modulate PMN responses (45, 74, 75), and create a regulated, homeostatic interaction.

Acknowledgments

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References

1. Henson, P. M., and R. B. Johnston. 1987. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *J. Clin. Invest.* 79:669–674.

2. Frank, L. 1985. Oxidant injury to pulmonary endothelium. *In* The Pulmonary Circulation and Acute Lung Injury. S. I. Said, editor. Futura Publishing Co., Mt. Kisco, NY. 283–305.

3. Crapo, J. D. 1986. Morphologic changes in pulmonary oxygen toxicity. *Annu. Rev. Physiol.* 48:721–731.

4. Sacks, T., C. F. Moldow, P. R. Craddock, T. K. Bowers, and H. S. Jacob. 1978. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. *J. Clin. Invest.* 61:1161–1167.

5. Weiss, S. J., J. Young, A. F. LoBuglio, A. Slivka, and N. F. Nimeh. 1981. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J. Clin. Invest.* 68:714–721.

6. Suttorp, N., and L. M. Simon. 1982. Lung cell oxidant injury. Enhancement of polymorphonuclear leukocute-mediated cytotoxicity in lung cells exposed to sustained in vitro hyperoxia. *J. Clin. Invest.* 70:342–350.

7. Ager, A., and J. L. Gordon. 1984. Differential effects of hydrogen peroxide on indices of endothelial cell function. *J. Exp. Med.* 159:592–603.

8. Harlan, J. M., J. D. Levine, K. S. Callahan, B. R. Schwartz, and L. A. Harker. 1984. Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *J. Clin. Invest.* 73:706–713.

9. Martin, W. J. 1984. Neutrophils kill pulmonary endothelial cells by a hydrogen-peroxide-dependent pathway. An *in vitro* model of neutrophil-mediated lung injury. *Am. Rev. Respir. Dis.* 130:209–213.

10. Whorton, A. R., M. E. Montgomery, and R. S. Kent. 1985. Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured porcine aortic endothelial cells. *J. Clin. Invest.* 76:295–302.

11. Spragg, R. G., D. B. Hinshaw, P. A. Hyslop, I. U. Schraufstätter, and C. G. Cochrane. 1985. Alterations in adenosine triphosphate and energy charge in cultured endothelial cells and P388D_1 cells after oxidant injury. *J. Clin. Invest.* 76:1471–1476.

12. Dobrina, A., and P. Patricia. 1986. Neutrophil-endothelial cell interaction. Evidence for and mechanisms of the self-protection of bovine microvascular endothelial cells from hydrogen peroxide-induced oxidative stress. *J. Clin. Invest.* 78:462–471.

13. Starkebaum, G., and J. M. Harlan. 1986. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J. Clin. Invest.* 77:1370–1376.

14. Toth, K. M., D. P. Clifford, E. M. Berger, C. W. White, and J. E. Repine. 1984. Intact human erythrocytes prevent hydrogen peroxide-mediated damage to isolated perfused rat lungs and cultured bovine pulmonary artery endothelial cells. *J. Clin. Invest.* 74:292–295.

15. Till, G. O., K. J. Johnson, R. Kunkel, and P. A. Ward. 1982. Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. *J. Clin. Invest.* 69:1126–1135.

16. Tate, R. M., K. M. VanBenthuysen, D. M. Shasby, I. F. McMurtry, and J. E. Repine. 1982. Oxygen-radical-mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. *Am. Rev. Respir. Dis.* 126:802–806.

17. Burghuber, O., M. M. Matthias, I. F. McMurtry, J. T. Reeves, and N. F. Voelkel. 1984. Lung edema due to hydrogen peroxide is independent of cyclooxygenase products. *J. Appl. Physiol.* 56:900–905.

18. Weisfeldt, M. L. 1987. Reperfusion and reperfusion injury. *Clin. Res.* 35:13–20.

19. Jaffe, E. A. 1985. Physiologic functions of normal endothelial cells. *Ann. N.Y. Acad. Sci.* 454:279–291.

20. Prescott, S. M., G. A. Zimmerman, and T. M. McIntyre. 1984. Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) when stimulated with thrombin. *Proc. Natl. Acad. Sci. USA.* 81:3534–3538.

21. Camussi, G., M. Aglietta, F. Malavasi, C. Tetta, W. Piacibello, F. Sanavio, and F. Bussolino. 1983. The release of platelet-activating factor from human endothelial cells in culture. *J. Immunol.* 131:2397–2403.

22. McIntyre, T. M., G. A. Zimmerman, K. Satoh, and S. M. Prescott. 1985. Cultured endothelial cells synthesize both platelet-activating factor and prostacyclin in response to histamine, bradykinin, and adenosine triphosphate. *J. Clin. Invest.* 76:271–280.

23. Zimmerman, G. A., R. E. Whatley, T. M. McIntyre, and S. M. Prescott. 1987. Production of platelet-activating factor, a biologically active lipid, by vascular endothelial cells. *Am. Rev. Respir. Dis.* 136:204–207.

24. Hanahan, D. J. 1986. Platelet-activating factor: A biologically active phosphoglyceride. *Annu. Rev. Biochem.* 55:483–509.

25. Camussi, G., and J. R. Brentjens. 1987. The role of platelet activating factor in inflammation. *In* Platelet-activating Factor and Related Lipid Mediators. F. Snyder, editor. Plenum Publishing Corp., New York and London. 299–323.

26. Voelkel, N. F., S. W. Chang, T. J. McDonnell, J. Y. Westcott, and J. Haynes. 1987. Role of membrane lipids in control of normal vascular tone. *Am. Rev. Respir. Dis.* 136:214–217.

27. Lewis, J. C., J. T. O'Flaherty, C. E. McCall, R. L. Wykle, and M. G. Bond. 1983. Platelet activating factor effects on pulmonary ultrastructure in rabbits. *Exp. Mol. Pathol.* 38:100–108.

28. Chang, S-W., F. O. Fedderson, P. M. Henson, and N. F. Voelkel. 1987. Platelet-activating factor mediates hemodynamic changes and lung injury in endotoxin-treated rats. *J. Clin. Invest.* 79:1498–1509.

29. Heffner, J. E., S. A. Shoemaker, E. M. Canham, M. Patel, I. F. McMurtry, H. G. Morris, and J. E. Repine. 1983. Acetyl glyceryl ether phosphorylcholine-stimulated human platelets cause pulmonary hypertension and edema in isolated rabbit lungs: role of thromboxane A_2. *J. Clin. Invest.* 71:351–357.

30. Hamasaki, Y., M. Mojarad, T. Saga, H-H. Tai, and S. I. Said. 1984. Platelet-activating factor raises airway and vascular pressures and induces edema in lungs perfused with platelet-free solution. *Am. Rev. Respir. Dis.* 129:742–746.

31. Kenzora, J. L., J. E. Pérez, S. R. Bergmann, and L. G. Lange. 1984. Effects of acetyl glyceryl ether phosphorylcholine (platelet-activating factor) on ventricular preload, afterload, and contractility in dogs. *J. Clin. Invest.* 74:1193–1203.

32. Burhop, K. E., H. Van Der Zee, R. Bizios, J. E. Kaplan, and A. B. Malik. 1986. Pulmonary vascular response to platelet-activating factor in awake sheep and the role of cyclooxygenase metabolites. *Am. Rev. Respir. Dis.* 134:548–554.

33. Whatley, R. E., G. A. Zimmerman, T. M. McIntyre, S. M. Prescott. 1988. Endothelium from diverse vascular sources synthesizes platelet-activating factor when stimulated by specific agonists. *Arteriosclerosis.* 8:321–331.

34. Mueller, H. W., J. T. O'Flaherty, and R. L. Wykle. 1984. The molecular species distribution of platelet-activating factor synthesized by rabbit and human neutrophils. *J. Biol. Chem.* 259:14554–14559.

35. Ramesha, C. S., and W. C. Pickett. 1986. Platelet-activating factor and leukotriene biosynthesis is inhibited in polymorphonuclear leukocytes depleted of arachidonic acid. *J. Biol. Chem.* 259:7592–7595.

36. Sisson, J. H., S. M. Prescott, T. M. McIntyre, and G. A. Zimmerman. 1987. Production of platelet-activating factor in stimulated human polymorphonuclear leukocytes. Correlation of synthesis with release, functional events, and leukotriene B_4 metabolism. *J. Immunol.* 138:3918–3926.

37. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

38. Stafforini, D. M., S. M. Prescott, and T. M. McIntyre. 1987.

Human plasma platelet-activating factor acetylhydrolase. Purification and properties. *J. Biol. Chem.* 262:4223–4230.

39. Patterson, M. K. 1979. Measurement of growth and viability of cells in culture. *In* Cell Culture. *Methods Enzymol.* 58:141–152.

40. Zimmerman, G. A., T. M. McIntyre, and S. M. Prescott. 1985. Thrombin stimulates the adherence of neutrophils to human endothelial cells in vitro. *J. Clin. Invest.* 76:2235–2246.

41. McIntyre, T. M., G. A. Zimmerman, and S. M. Prescott. 1986. Leukotrienes C_4 and D_4 stimulate human endothelial cells to synthesize platelet-activating factor and bind neutrophils. *Proc. Natl. Acad. Sci. USA.* 83:2204–2208.

42. Zimmerman, G. A., T. M. McIntyre, and S. M. Prescott. 1986. Thrombin stimulates neutrophil adherence by an endothelial cell-dependent mechanism: Characterization of the response and relationship to platelet-activating factor synthesis. *Ann. N.Y. Acad. Sci.* 405:349–368.

43. Harlan, J. M., and K. S. Callahan. 1984. Role of hydrogen peroxide in the neutrophil-mediated release of prostacyclin from cultured endothelial cells. *J. Clin. Invest.* 74:442–448.

44. Camussi, G., I. Pawlowski, F. Bussolino, P. B. Caldwell, J. Brentjens, and G. Andres. 1983. Release of platelet-activating factor in rabbits with antibody-mediated injury to the lung: The role of leukocytes and of pulmonary endothelial cells. *J. Immunol.* 131:1802–1807.

45. Zimmerman, G. A., G. A. Wiseman, and H. R. Hill. 1985. Human endothelial cells modulate granulocyte adherence and chemotaxis. *J. Immunol.* 134:1866–1874.

46. Berger, E. M., C. J. Beehler, R. N. Harada, and J. E. Repine. 1987. Phagocytic cells as scavengers of hydrogen peroxide (H_2O_2). *Clin. Res.* 35:170A. (Abstr.)

47. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witzum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3000–3004.

48. Dejana, E., S. Colella, L. A. Languino, G. Balconi, G. C. Corbascio, and P. C. Marchisio. 1987. Fibrinogen induces adhesion, spreading, and microfilament organization of human endothelial cells in vitro. *J. Cell Biol.* 104:1403–1411.

49. Bevilacqua, M. P., J. S. Putzer, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* 76:2003–2011.

50. Uchida, N., H. Smilowitz, and M. L. Tanzer. 1979. Monovalent ionophores inhibit secretion of procollagen and fibronectin from cultured human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 76:1868–1872.

51. Reinders, J. H., P. G. de Groot, J. Dawes, N. R. Hunter, H. A. A. van Heugten, J. Zandbergen, M. D. Gonsalves, and J. A. van Mourik. 1985. Comparison of secretion and subcellular localization of von Willebrand protein with that of thrombospondin and fibronectin in cultured human vascular endothelial cells. *Biochim. Biophys. Acta.* 844:306–313.

52. Vercelloti, G. M., J. McCarthy, L. T. Furcht, H. S. Jacob, and C. T. Moldow. 1983. Inflamed fibronectin: an altered fibronectin enhances neutrophil adhesion. *Blood.* 62:1063–1069.

53. Test, S. T., and S. J. Weiss. 1984. Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *J. Biol. Chem.* 259:399–405.

54. Nathan, C. F. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J. Clin. Invest.* 80:1550–1560.

55. Bozeman, P. M., J. R. Hoidal, and V. L. Shepherd. 1988. Oxidant-mediated inhibition of ligand uptake by the macrophage mannose receptor. *J. Biol. Chem.* 263:1240–1247.

56. Smedley, L. A., M. G. Tonnensen, R. A. Sandhaus, C. Haslett, L. A. Guthrie, R. B. Johnston, P. M. Henson, and G. S. Worthen. 1986. Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *J. Clin. Invest.* 77:1233–1243.

57. Harlan, J. M., P. D. Killen, L. A. Harker, G. E. Striker, and D. G. Wright. 1981. Neutrophil-mediated endothelial injury in vitro. *J. Clin. Invest.* 68:1394–1403.

58. Bachofen, M., and E. R. Weibel. 1977. Alterations in the gas exchange apparatus in adult respiratory distress syndrome associated with septicemia. *Am. Rev. Respir. Dis.* 116:589–615.

59. Van Benthuysen, K. M., I. F. McMurtry, and L. D. Horwitz. 1987. Reperfusion after acute coronary occlusion in dogs impairs endothelium-derived relaxation to acetylcholine and augments contractile reactivity in vitro. *J. Clin. Invest.* 79:265–274.

60. Snyder, F. 1987. The significance of dual pathways for the biosynthesis of platelet activating factor: 1-alkyl-2-lyso-*sn*-glycero-3-phosphate as a branchpoint. *In* New Horizons in Platelet-activating Factor Research. C. M. Winslow and M. L. Lee, editors. John Wiley & Sons, Ltd., London. 13–25.

61. Prescott, S. M., G. A. Zimmerman, and T. M. McIntyre. 1987. The production of platelet-activating factor by cultured human endothelial cells: Regulation and function. *In* Platelet Activating Factor. F. Snyder, editor. Plenum Publishing Corp., New York. 323–339.

62. Bussolino F., F. Brevario, M. Aglietta, F. Sanavio, A. Bosia, and E. Dejana. 1987. Studies on the mechanism of interleukin 1 stimulation of platelet-activating factor synthesis in human endothelial cells in culture. *Biochim. Biophys. Acta.* 927:43–54.

63. Shasby, D. M., S. E. Lind, S. S. Shasby, J. C. Goldsmith, and G. W. Hunninghake. 1985. Reversible oxidant-induced increases in albumin transfer across cultured endothelium: alterations in cell shape and calcium homeostasis. *Blood.* 65:605–614.

64. Shasby, D. M. 1987. Lipid peroxidation in Ca^{2+} dependent, reversible oxidant injury to endothelium. *Clin. Res.* 35:539A. (Abstr.)

65. Serhan, C., P. Anderson, E. Goodman, P. Dunham, and G. Weissmann. 1981. Phosphatidate and oxidized fatty acids are calcium ionophores. Studies employing arsenazo III in liposomes. *J. Biol. Chem.* 256:2736–2741.

66. Jaffe, E. A., J. Grulich, B. B. Weksler, G. Hampel, and K. Watanabe. 1987. Correlation between thrombin-induced prostacyclin production and inositol triphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J. Biol. Chem.* 262:8557–8565.

67. Taylor, L., M. J. Menconi, and P. Polgar. 1983. The participation of hydrogen peroxides and oxygen radicals in the control of prostaglandin synthesis. *J. Biol. Chem.* 258:6855–6857.

68. Davies, K. J. A., and A. L. Goldberg. 1987. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* 262:8220–8226.

69. Del Maestro, R., H. H. Thaw, J. Björk, M. Planker, and K. E. Arfors. 1980. Free radicals as mediators of tissue injury. *Acta Physiol. Scand. (Suppl.).* 492:153–168.

70. Bowman, C. M., E. N. Butler, and J. E. Repine. 1983. Hyperoxia damages cultured endothelial cells causing increased neutrophil adherence. *Am. Rev. Respir. Dis.* 128:469–472.

71. Pohlman, T. H., K. A. Stanness, P. G. Beatty, H. D. Ochs, and J. M. Harlan. 1986. An endothelial cell surface factor(s) induced *in vitro* by lipopolysaccharide, interleukin-1, and tumor necrosis factor$_\alpha$ increases neutrophil adherence by a CDw18-dependent mechanism. *J. Immunol.* 136:4548–4553.

72. Zimmerman, G. A., A. D. Renzetti, and H. R. Hill. 1984. Granulocyte adherence in pulmonary and systemic arterial blood samples from patients with adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 129:798–804.

73. Hofmann, S. L., S. M. Prescott, and P. W. Majerus. 1982. The effects of mepacrine and p-bromophenacyl bromide on arachidonic acid release in human platelets. *Arch. Biochem. Biophys.* 215:237–244.

74. Zimmerman, G. A., and D. Klein-Knoeckel. 1986. Human endothelial cells inhibit granulocyte aggregation in vitro. *J. Immunol.* 136:3839–3847.

75. Rampart, M., and T. J. Williams. 1986. Polymorphonuclear leukocyte-dependent plasma leakage in the rabbit skin is enhanced or inhibited by prostacyclin, depending on the route of administration. *Am. J. Pathol.* 124:66–73.