Oxygen Radicals Induce Human Endothelial Cells to Express GMP-140 and Bind Neutrophils

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Abstract. The initial step in extravasation of neutrophils (polymorphonuclear leukocytes [PMNs]) to the extravascular space is adherence to the endothelium. We examined the effect of oxidants on this process by treating human endothelial cells with H_2O_2, t-butylhydroperoxide, or menadione. This resulted in a surface adhesive for PMN between 1 and 4 h after exposure. The oxidants needed to be present only for a brief period at the initiation of the assay. Adhesion was an endothelial cell–dependent process that did not require an active response from the PMN. The adhesive molecule was not platelet-activating factor, which mediates PMN adherence when endothelial cells are briefly exposed to higher concentrations of H_2O_2 (Lewis, M. S., R. E. Whatley, P. Cain, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1988. *J. Clin. Invest.* 82:2045–2055), nor was it ELAM-1, an adhesive glycoprotein induced by cytokines. Oxidant-induced adhesion did not require protein synthesis, was inhibited by antioxidants, and, when peroxides were the oxidants, was inhibited by intracellular iron chelators.

Granule membrane protein-140 (GMP-140) is a membrane-associated glycoprotein that can be translocated from its intracellular storage pool to the surface of endothelial cells where it acts as a ligand for PMN adhesion (Geng, J.-G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. *Nature (Lond).* 343:757–760). We found that endothelial cells exposed to oxidants expressed GMP-140 on their surface, and that an mAb against GMP-140 or solubilized GMP-140 completely blocked PMN adherence to oxidant-treated endothelial cells. Thus, exposure of endothelial cells to oxygen radicals induces the prolonged expression of GMP-140 on the cell surface, which results in enhanced PMN adherence.

L EUKOCYTES circulate in the blood, yet their function, mediation of inflammatory reactions and attenuation of infection, is manifested in the extravascular space. This results from the directed migration of polymorphonuclear leukocytes (PMNs)[1] and monocytes into infected or perturbed areas by an exquisitely regulated communication between the circulating leukocytes and the endothelial cells that form the interface between blood and the extravascular space. Activated endothelial cells express several molecules that induce unactivated PMN to bind to them. Stimulation of endothelial cells for several hours with tumor necrosis factor-α (TNF-α), interleukin-1, or endotoxin (3) causes endothelial cells to bind PMN. This process is dependent on new protein synthesis, and, depending on the time of stimulation, is mediated by the surface expression of endothelial leukocyte adhesion molecule-1 (ELAM-1) (3), a member of the recently identified selectin (7,20) family. There are also mechanisms that act more rapidly to induce endothelial cell–dependent PMN adhesion. One is unique in that the mediator, platelet-activating factor (PAF), is a phospholipid. Endothelial cells do not normally contain PAF, but synthesize appreciable amounts of it within minutes of appropriate stimulation (23, 24, 40). While all of this PAF remains associated with the endothelial cell (30), a portion of it is expressed on the endothelial cell surface where it is recognized by the PMN receptor for PAF (42). This endothelial cell–dependent adhesion process requires activation of the PMN by endothelial cell–associated PAF and the functional upregulation of the PMN CD11/CD18 adhesive glycoprotein[2] (42).

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1. *Abbreviations used in this paper*: ELAM-1, endothelial-leukocyte adhesion molecule-1; HBSS/A, HBSS containing 0.5% human serum albumin; PAF, platelet-activating factor; PMN, polymorphonuclear leukocyte; *t*-BuOOH, *tert*-butylhydroperoxide; TNF-α, tumor necrosis factor-α; vWF, von Willebrand factor.

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2. Zimmerman, G. A., K. D. Patel, S. M. Prescott, R. P. McEver, and T. M. McIntyre, manuscript submitted for publication.

The third known mechanism of endothelial cell–dependent PMN adhesion is demonstrated by endothelial cells stimulated with phorbol esters (7), or rapidly acting agonists like thrombin and histamine[2] through the expression of a second member of the selectin family of adhesive receptors for leukocytes. These rapidly acting agonists transiently induce translocation of the adhesive glycoprotein GMP-140 (20) (also known as PADGEM protein; 5, 3) from specialized intracellular granules, the Weibel-Palade bodies, to the surface of the stimulated cells (5, 9, 21). PMNs adhere to purified, immobilized GMP-140 and to COS cells transfected with its cDNA (7). Both PAF and GMP-140 are expressed by endothelial cells stimulated with rapidly acting agonists, where they act in concert to mediate maximal PMN adherence.[2]

PMN adherence and recruitment appear to play a critical role in the oxidant-induced tissue injury that accompanies a variety of pathologic processes (15, 19, 32). In many instances, PMNs are attracted into the affected area by the initial insult and then exacerbate the tissue damage by releasing a variety of injurious agents, including H_2O_2. The mechanisms by which PMNs infiltrate areas subjected to oxidant damage are not completely defined. However, this process is a major pathologic one as about half of the damage observed upon reperfusion of ischemic tissue is prevented by either removing circulating PMN or inhibiting their function, by treatment with a variety of antioxidants, or by chelation of transition metals such as ionic iron (8). We observed (14) that H_2O_2 itself is an agonist for PAF synthesis by endothelial cells, and that it also induced PMN adherence to these cells. These responses occurred within minutes of exposure to H_2O_2, providing that the concentration was at least 1 mM, and PMN adhesion was completely dependent on the PAF-mediated mechanism.

Recently Nathan (27, 28) observed that activated leukocytes, if they have spread onto a favorable surface, generate large amounts of oxygen radicals for prolonged periods of time. This raised the possibility that endothelial cells may be exposed to relatively high concentrations of oxidants for several hours. Indeed, activation of 10^7 PMN in the presence of endothelial cells generated H_2O_2 equivalent to 0.3 mM (34), while other studies (reviewed in 14) have shown that endothelial cells are exposed to oxidants for prolonged periods in hyperoxic pulmonary injury and other pathologic states. Furthermore, PMN accumulate at the intimal surface in these conditions (1, 6). We report that endothelial cells exposed for 1–4 h to submillimolar concentrations of H_2O_2, or other oxidants, bound significant numbers of PMN. Prolonged surface expression of GMP-140 was solely responsible for this process, and therefore was unlike PMN adhesion in response to receptor-mediated agonists where GMP-140 expression is only transient, and is only partially responsible for PMN adhesion. This is a new mechanism for PMN adhesion to endothelium in which a pathologic agonist induces unregulated expression of a proadhesive molecule.

Materials and Methods

Materials

HBSS and M199 were from Whittaker M. A. Bioproducts (Walkersville,

MD), and human serum albumin (25%) was from Miles Laboratories, Inc. (Elkhart, IN). Purified human thrombin was the kind gift of Dr. John Fenton (Albany, NY), and recombinant human TNF-α was provided by Genentech, Inc. (San Francisco, CA). GMP-140 was solubilized and purified as described (7). PAF was from Avanti Polar Lipids, Inc. (Birmingham, AL). [^{3}H]Acetate was from New England Nuclear (Boston, MA), and ^{111}Inoxine was purchased from the Radiopharmacy Service at the University of Utah. L659,989 was the kind gift of John Chabala of Merck, Sharp and Dohme Research (Rahway, NJ) and WEB 2086 was kindly provided by Peggy Ganong of Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). mAbs H18/7 and 60.3 were generously donated by Michael Bevilacqua (Boston, MA), and Patrick Beatty and John Harlan (Seattle, WA), respectively. 2'-7'-,Bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein, acetoxymethyl ester, and propidium iodide were from Molecular Probes (Eugene, OR). Ascorbate was from Eastman Kodak Co. (Rochester, NY). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation and Culture

Human umbilical vein endothelial cells were cultured in 24-mm multiwell plates (Costar Data Packaging Corp., Cambridge, MA) as described (40). Only monolayers of primary cultures that were tightly confluent were used for these studies. Human intestinal smooth muscle cells (CRL 1692) and HL60 cells were obtained from the American Type Culture Collection. HL-60 cells were cultured as described (42). PMN were isolated from fresh human blood and labeled with ^{111}In-oxine as described (40).

Cell Adhesion and Labeling Techniques

Endothelial cells were treated with HBSS containing 0.5% human serum albumin (HBSS/A) and various agonists for the stated period of time, washed twice with HBSS and the adherence of ^{111}In-PMN or ^{111}In-HL-60 cells to monolayers of endothelial cells was performed as described (40, 42). Synthesis of PAF was determined by following the incorporation of [^{3}H]acetate into a phospholipid that comigrated with PAF on thin-layer chromatography plates as described (23). This product previously has been shown to be authentic PAF (30). The effects of PAF receptor antagonists were examined following a 5-min preexposure of PMN to the antagonists as described (42). Endothelial cells were labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein, acetoxymethyl ester (CFAM) by incubating the monolayers with 2.8 μM CFAM for 5 min at 37°. The monolayers were then washed with HBSS and incubated in this buffer for an additional 2 min to allow hydrolysis of the ester bond. The monolayers were then washed twice with HBSS before agonist addition. Propidium iodide and trypan blue exclusion were determined by exposing control or oxidant-treated monolayers to 50 μM propidium iodide or 0.4% trypan blue for 5 min. The monolayers were washed twice with HBSS and then visualized by fluorescence microscopy using a Nikon DM580/61-A filter cube when propidium iodide was the dye. Endothelial cell monolayers were fixed with glutaraldehyde by exposing washed monolayers to 2.5% glutaraldehyde for 30 min. Microwave fixation (2) of monolayers was performed for 1 min in a Litton microwave set at maximum irradiation. PMNs were fixed by collecting freshly isolated PMN (5.5 × 10^6/ml in HBSS/A) by centrifugation at 1,350 g for 5 min, resuspending them in 5× the original volume of ice-cold 2% formaldehyde for 5 min, and then reisolating them by centrifugation. They were then resuspended at the original concentration in HBSS/A. Protein synthesis was inhibited in endothelial cell monolayers by incubating the monolayers with 5 μg/ml actinomycin D or 5 μM emetine for 15 min at 37° before addition of the experimental agent. These monolayers, in the continued presence of actinomycin D or emetine, were incubated for 2 h before the effect of the inhibitors on adhesion was determined.

The effect of the blocking anti-GMP-140 mAb G1 or the nonblocking monoclonal anti-GMP-140 antibody S12 (7) on PMN adherence was examined by exposing endothelial cell monolayers to buffer or experimental agents for the stated time, and then incubating the monolayer with 10 μg/ml of the purified antibody for 10 min. The antibody was removed, and fresh antibody and ^{111}In-PMN were added to determine adherence. The effect of purified GMP-140 or fibrinogen was examined by exposing ^{111}In-PMN to 5 μg/ml of either protein for 20 min before initiation of the adherence assay, which also contained these proteins at this concentration. All experiments, except that performed with limited amounts of H18/7, have been performed a minimum of two times with equivalent results.

Binding of S12 to endothelial cell monolayers was measured by incubating the monolayers with HBSS/A with or without agonist for the stated
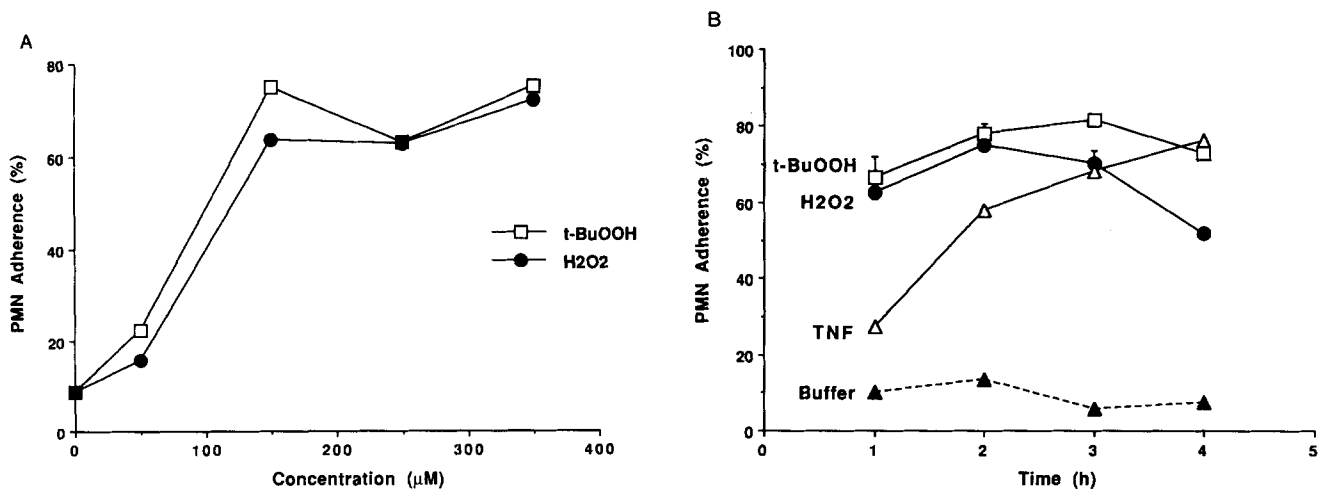
Figure 1. PMN adherence to human endothelial cells treated with t-BuOOH or H_2O_2 as a function of (A) concentration or (B) time. Primary cultures of human umbilical vein endothelial cells were treated (A) with HBSS/A, or the stated concentration of t-BuOOH or H_2O_2 in HBSS/A for 2 h at 37° or (B) HBSS/A without or with 250 μM t-BuOOH, 250 μM H_2O_2, or 500 U/ml TNF-α for the stated time. After incubation, the medium was removed, and the monolayers were washed twice. PMN adhesion to the endothelial cell monolayers was then determined by layering 2.5×10^6 ^{111}In-PMN over the endothelial cells and incubating them for 15 min at 37°. Nonadherent cells were removed and loosely adherent cells were collected with two washes. Adherent cells were solubilized with 1 M NH_4OH and ^{111}In-labeled material was recovered for determination of gamma radioactivity. The values presented are the percent of PMN that bound, compared to the total added to each well, and are given as a mean and range of duplicate values.

period of time before this medium was removed and the monolayer fixed with 1% formaldehyde (9). The fixed monolayers were washed thrice with HBSS, and then incubated with 2 μg/ml S12 or isotyped-matched T10 (anti-gpIIb/IIIa) (22) for 45 min at 25°. These antibodies were removed, the monolayer washed thrice with HBSS, and then the monolayer was incubated with ^{125}I-labeled sheep anti-mouse IgG (1/100 dilution; Amersham Corp., Arlington Heights, IL) for 20 min at 25°. Unbound antibody was removed with three washes of HBSS/A before the label was solubilized with 1N NH_4OH and quantitated by gamma counting. Adherence of PMN to monolayers incubated in parallel was performed as described above.

Release of von Willebrand Factor (vWF) was quantitated by ELISA by coating microtiter dishes with 10 μg/ml rabbit antihuman vWF (Behring Diagnostics, La Jolla, CA) during an overnight incubation at 4°. These wells were washed four times with PBS (pH 7.2) containing 0.05% Tween-20 (PBS/T), and then incubated with either 1/10 dilutions (into PBS containing 1% BSA) of supernatants of endothelial cells treated with HBSS/A with or without 250 μM t-BuOOH for the stated time, or with various concentrations of human vWF (the kind gift of Gerald Roth, University of Washington, Seattle, WA). After 90 min at 37°, the wells were washed six times with PBS/T, and then incubated for 90 min at 37° with a 1/1,000 dilution of goat antihuman vWF IgG (Atlantic Antibodies, Scarborough, ME). These wells were then washed six times with PBS/T, incubated for 90 min at 37° with a 1/1,000 dilution of rabbit anti-goat IgG conjugated to HRP (type VI; Sigma Chemical Co.). The wells were washed six times with PBS/T and incubated for 20 min at 37° with o-phenylenediamine (0.1 ml at 0.4 mg/ml) before the reaction was stopped with 8N H_2SO_4. Bound antibody was quantitated by determining the optical density at 492 nm.

Results

Peroxides Induce Human Endothelial Cells to Bind PMN

We previously observed (14) that exposure of endothelial cells to millimolar concentrations of H_2O_2 rapidly stimulates the synthesis of PAF, and induces the adherence of PMN. Adhesion of PMN under these circumstances is mediated by PAF synthesized and expressed by the endothelial cells (40). The data in Fig. 1 show that much lower concen-

trations of H_2O_2 also induced the adherence of PMN to monolayers of endothelial cells if the incubation was prolonged. The optimal concentration of H_2O_2 lay between 150 and 350 μM when the incubation time was 2 h (Fig. 1 a). Alteration of the endothelial cell surface in response to H_2O_2 developed rapidly with maximal PMN adherence occurring after 1–3 h of continuous incubation with H_2O_2 (Fig. 1 b). Adherence at these times was completely independent of PAF accumulation (vide infra), and therefore differs from the mechanism that we previously elucidated.

We next investigated the effect of a lipid-soluble peroxide, t-BuOOH, on human endothelial cells. We found that it too stimulated these cells to bind PMN with a concentration relationship like that of H_2O_2 (Fig. 1a). The magnitude of the endothelial cell–dependent adhesion in response to this peroxide was equivalent to that induced by TNF-α, which, in general, was greater than that in response to agents such as thrombin (41). The time relationship of PMN adhesion also was similar to that of H_2O_2 (Fig. 1b), except that, although not shown in this experiment, there was no adherence to endothelial cells treated with t-BuOOH for 30 min or less. The generation of an adhesive surface on the endothelial cells in response to t-BuOOH was considerably faster than that induced by TNF-α (Fig. 1), but was much slower than that induced by thrombin (40), histamine[2] or high concentrations of H_2O_2 (14).

The endothelial cell monolayer did not need to be exposed to H_2O_2 or t-BuOOH continuously in order to induce PMN adhesion. When endothelial cells were pulsed for 15 or 30 min with t-BuOOH and then incubated in the absence of exogenous peroxide for 105 or 90 min, for a total of 2 h of incubation, the amount of adherence was 36 and 116%, respectively, of that in response to a continuous 2-h exposure to t-BuOOH (Fig. 2). The same protocol using H_2O_2 as the oxidant showed that it, too, did not have to be present
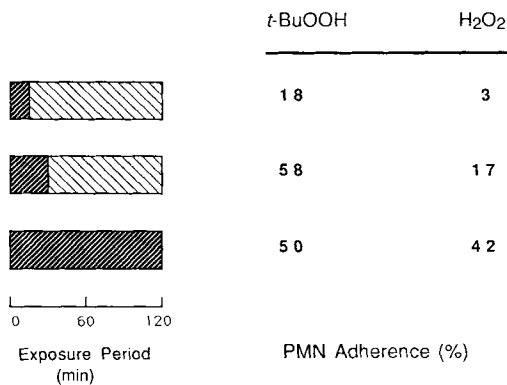
	t-BuOOH	H$_2$O$_2$
	18	3
	58	17
	50	42

0   60   120

Exposure Period (min)   PMN Adherence (%)

Figure 2. Effect of a single pulse of H$_2$O$_2$ or t-BuOOH on subsequent PMN adhesion to endothelial cells. Endothelial cell monolayers were either treated continuously with 250 μM t-BuOOH or H$_2$O$_2$, or were treated for 15 or 30 min at the initiation of the experiment. The peroxide was then removed, the monolayer washed, and the incubation continued for 105 or 90 min, respectively, in peroxide-free HBSS/A. At the end of the 2-h experiment, PMN adhesion to the pulsed or continuously treated monolayers was examined as described under Fig. 1. The shaded portion of the bar shows the period during which peroxides were present, and the striped bar shows the length of the subsequent peroxide-free incubation. The basal adhesion in this experiment was 13%, which was subtracted from the values shown.

throughout the experiment. However, the pulse with H$_2$O$_2$ had to extend for at least 30 min, and even then it was only 40% as effective as a continuous 2-h exposure of the monolayer to H$_2$O$_2$. This experiment showed that the process induced by treatment of endothelial cells with peroxide was self-sustaining once oxidation had been initiated.

The nature of the protocol that we employed, where the PMNs were not exposed to peroxide, suggested that oxidant-induced PMN adhesion described in Figs. 1 and 2 was an endothelial cell–dependent process. To confirm this and to determine if an active PMN response was required for adherence, we used several strategies. PMN-dependent adhesion requires that the adhesive glycoprotein complex CD11/CD18 be activated (39). We found that mAb 60.3, which blocks the function of this class of integrins (39), failed to prevent the adherence induced by either H$_2$O$_2$ or t-BuOOH (not shown). We also found that there was little diminution of adherence in response to oxidant treatment when the PMN were first fixed by treatment with formaldehyde (Fig. 3). That this effectively prevented PMN-dependent processes was shown by the lack of adherence in response to direct PMN agonists, 10^{-7} M phorbol myristate acetate or 10 μM A23187 (Fig. 3 legend). In addition, we found that direct treatment of PMN with peroxides under the conditions of these experiments did not induce them to become adhesive (not shown). Therefore, PMN adherence to oxidant-treated endothelial cells was an endothelial cell–dependent process that required no active response from the adherent PMN.

We asked whether the generation of an adhesive surface after treatment with the peroxides was a response specific to endothelial cells. We treated cultured human smooth muscle cells with either 250 μM H$_2$O$_2$ or t-BuOOH for 2 and 4 h. These cells normally do not bind, nor can they be induced to bind, PMN. This treatment did not alter the number of PMNs that adhered to these cells (5 vs 3% adherence to un-

treated smooth muscles cells: in the same experiment adherence to endothelial cells was 40%). We conclude that the generation of an adhesive surface was not a general response of cells to oxidant stress.

Induction of PMN binding required that the oxidant-treated endothelial cells be viable. We fixed the endothelial cell monolayer with glutaraldehyde or by microwave irradiation (2), and then exposed them to either H$_2$O$_2$ or t-BuOOH. There was no increased adhesion of PMN to endothelial cells that had been fixed before oxidant exposure (not shown). In contrast, endothelial cells that were first treated with TNF-α, H$_2$O$_2$ or t-BuOOH and then fixed by either method did demonstrate increased PMN adherence (not shown).

We used phase-contrast microscopy to examine endothelial cells that had been treated with H$_2$O$_2$ or t-BuOOH, and
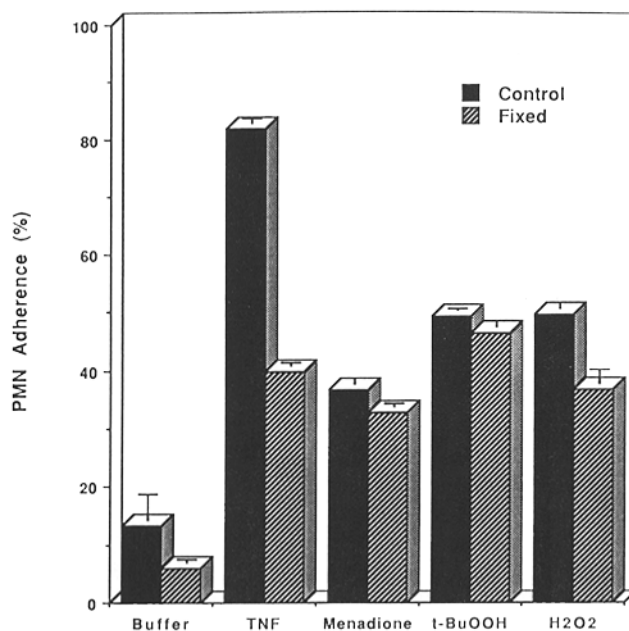

Figure 3. Adhesion of fresh or formaldehyde-fixed PMN to endothelial cells treated with menadione, H$_2$O$_2$, or t-BuOOH. Endothelial cells were treated with HBSS/A, or 300 μM t-BuOOH, 1 mM H$_2$O$_2$, 250 μM menadione, or 1,000 U TNF-α in HBSS/A. After 4 h of incubation at 37°, the incubation buffer was removed and the monolayer washed twice. The ability of the incubated endothelial cell monolayers to bind PMN was determined as in Fig. 1. PMNs were either freshly isolated, or had been fixed with formaldehyde (Materials and Methods). Not shown is the effect of fixation on PMN adherence in response to direct stimulation of PMN with PMA, or stimulation of both cell types with A23187. Endothelial cell monolayers were incubated with HBSS/A for 3 h 55 min, washed with HBSS/A, A23187 (10 μM) in HBSS/A was added, and then 5 min later fresh or fixed PMNs were added. This measured adherence in response to both PMN-dependent and endothelial cell–dependent mechanisms. When PMA (10^{-7} M) was the agonist, it was added directly to PMN overlying endothelial cell monolayers at the initiation of the adherence assay, so that only PMN-dependent adherence was measured. Fresh PMN adhered to the monolayer (buffer, 4 ± 1%; A23187, 75 ± 2%; PMA, 81 ± 6%), but fixed PMN did not (buffer, 3 ± 0%; A23187, 10 ± 2%; PMA, 3 ± 1%). Data are expressed as the percent of adherent cells compared to total cells added to each well, and are presented as a mean and range of duplicate values.
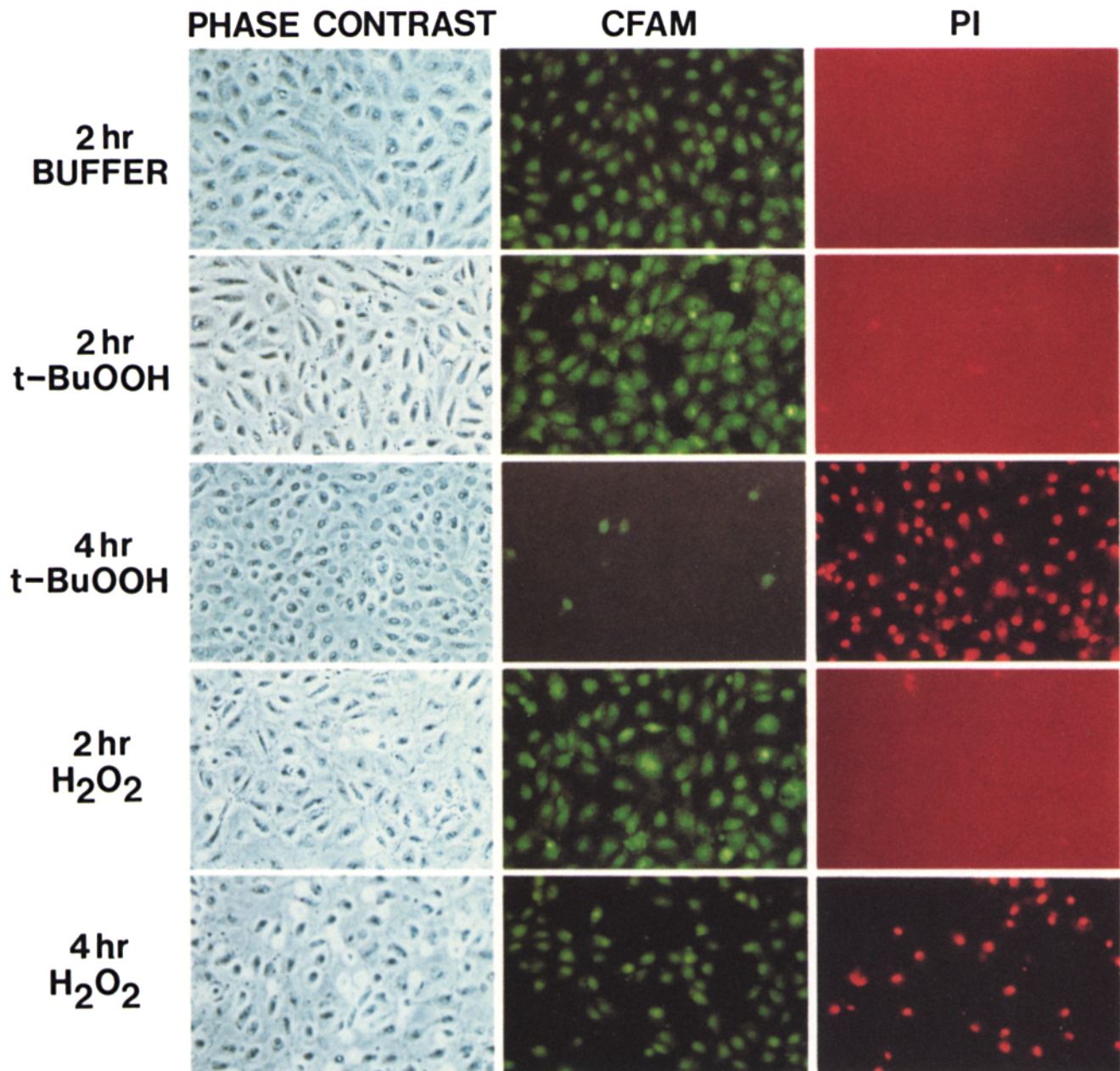
Figure 4. Morphology and integrity of endothelial cells treated with *t*-BuOOH or H_2O_2. Growth medium was removed from confluent monolayers of endothelial cells, they were washed twice with HBSS/A, and then exposed to 2.8 μM carboxyfluorescein acetoxymethyl ester or control buffer for 5 min. These monolayers were washed, incubated in HBSS/A for 2 min to allow the methyl ester to be hydrolyzed, and then washed twice with HBSS/A before addition of oxidants. Fresh HBSS/A with or without 250 μM H_2O_2 or *t*-BuOOH was layered over the cells, and after 2 and 4 h of incubation at 37° the cells were examined by phase-contrast microscopy. The anuclear, uniformly stained objects found after 4 h of *t*-BuOOH treatment are blebs that are still attached to the monolayer and are not in this focal plane. Monolayers labeled with carboxyfluorescein were examined by fluorescence microscopy using a Nikon B1 filter cube. Exclusion of propidium iodide was determined using monolayers exposed to HBSS/A or peroxides for 0, 115, or 235 min. These monolayers were washed, incubated with 50 μM propidium iodide for 5 min, and then washed twice before examination by fluorescence microscopy.

found that the morphology of these monolayers differed from control monolayers and from each other (Fig. 4). A typical H_2O_2-treated monolayer appeared pyknotic and desiccated. Additionally, the cells often had retracted so that the monolayer developed large gaps. This, however, did not account for the increased PMN adherence as visual inspection showed that the majority of the PMN was adherent to endothelial cells, and not the exposed substrate. Furthermore, endothelial cells exposed to 2.5 μg/ml cytochalasin B displayed extreme cellular retraction, but no increase in PMN adherence (not shown). In contrast to the appearance of H_2O_2-treated monolayers, the nuclei of *t*-BuOOH-treated endothelial cells were distorted and swollen (Fig. 4). Additionally, within several hours the cells developed blebs that subsequently pinched off from the cell surface. The marked differences in the appearance of monolayers treated with H_2O_2 or *t*-BuOOH suggested that the mechanisms of action of the two peroxides also might be different.

Table I. Inhibition of PMN Adherence to Peroxide-treated Endothelial Cells by Antioxidants and Iron Chelators

	Agonist		
	t-BuOOH	H_2O_2	TNF-α
	% Inhibition		
Butylated hydroxytoluene*	91 ± 1	70 ± 1	14
NDGA	63 ± 3	23 ± 4	31
Ascorbate	88 ± 4	92 ± 1	0
α,α-dipyridyl	94 ± 1	44 ± 0	0
Desferal	100 ± 0	88 ± 3	10

Endothelial cell monolayers were incubated for 2 h with HBSS/A, 250 μM t-BuOOH or H_2O_2, or 500 U/ml TNF-α. PMN adherence was determined as described under Fig. 1. The data represent five separate experiments. These effectors did not affect PMN-dependent adherence, as determined by adherence to a gelatin matrix in response to 1 nM PAF.
* Effector concentrations were: 50 μM butylated hydroxytoluene; 0.1 mM nordihydroguaiaretic acid (NDGA); 0.1 mM ascorbate; 2 mM α,α-dipyridyl; 20 mM Desferal.

The effect that oxidant exposure had on the viability of the endothelial cell monolayer was assessed by examining the retention of carboxyfluorescein, a negatively charged, water-soluble fluorescent dye, that had been introduced into the cells before their exposure to peroxide. Fig. 4 shows that endothelial cells were highly fluorescent after labeling, and that they retained the carboxyfluorescein dye after 2 h of exposure to either H_2O_2 or t-BuOOH. We also found (Fig. 4) that the cells excluded propidium iodide, which stains the nucleus of dead cells, and trypan blue (not shown) after 2 h of treatment with the oxidants. Half of the cells in monolayers exposed to H_2O_2 for 4 h still retained carboxyfluorescein and excluded propidium iodide, but few of the cells treated with t-BuOOH maintained a functional permeability barrier. Lysis of the monolayer by a brief exposure to digitonin or Triton X-100 immediately decreased cellular fluorescence to background levels, showing that the dye was not retained by the oxidant-treated cells by interaction with cellular components. Viability, as measured by dye retention, correlated with the ability of monolayers to recover their normal morphology after exposure to the peroxides: monolayers that were treated for 4 h with H_2O_2, washed, and returned to growth medium for 16 h appeared healthy, while those exposed to t-BuOOH for the same period did not recover their usual morphology. These results show that expression of the pro-adhesive surface was a function of viable cells, but that lethally injured cells still remained adhesive for PMN.

Generation of the Endothelial Cell Pro-adhesive Surface Is a Response to Oxygen Radicals

The appearance of the adhesive endothelial cell surface was the result of oxidative reactions carried out in or near the endothelial cells. First, PMN adherence was dependent on the peroxide function of t-BuOOH, as treatment of the endothelial cell monolayers with equivalent concentrations of t-butanol did not promote PMN adherence (not shown). Second, PMN adherence was also induced by intracellularly generated oxygen radicals. Endothelial cell monolayers treated with menadione, a quinone that undergoes reductive cycling with the production of O_2^- and H_2O_2 (29, 31), also

became adhesive for PMN (Fig. 3). Adherence in response to menadione usually was less than that induced by maximally effective concentrations of t-BuOOH ($n = 8$).

The third line of evidence that the peroxide induction of endothelial cell–dependent PMN adhesion required intracellular metabolism was that this process required intracellular iron. Transition metals, such as iron, have peroxidase activity and catalyze radical formation during peroxide breakdown (25). We found that adherence in response to either H_2O_2 or t-BuOOH was inhibited by coincubation with the Fe^{+3} chelator desferal (Table I). The essential Fe^{+3} was localized within the endothelial cells because conalbumin, a chicken transferrin not transported by human cells, was unable to inhibit the adhesion induced by either H_2O_2 or t-BuOOH (not shown). In contrast to the results obtained with t-BuOOH or H_2O_2, PMN adhesion induced by endothelial cell metabolism of menadione was not blocked by desferal (28 ± 2% adherence with 20 mM desferal, 26 ± 2% without desferal; unstimulated adherence was 5 ± 1%). The impression that there were differences in the mechanism of action of these oxidants was strengthened when the effect of the hydrophobic transition metal chelator, α,α-dipyridyl, on endothelial cell–dependent adhesion was examined. α,α-Dipyridyl blocked almost all of the adhesion due to t-BuOOH, while it inhibited only 44% of the adhesion induced by H_2O_2. These chelators did not affect TNF-α–induced adherence. These results suggest that the exogenous peroxides had to be metabolized to more potent oxidizing radicals in an intracellular, iron-dependent reaction, while direct intracellular generation of oxygen free radicals did not require free iron to exert their effects.

Finally, we used free radical scavengers to directly test whether induction of the pro-adhesive state by peroxides was due to radical formation. Butylated hydroxytoluene, a lipid-soluble antioxidant, effectively inhibited adherence induced by either H_2O_2 or t-BuOOH (Table I). Nordihydroguaiartic acid, an inhibitor of lipoxygenase-catalyzed reactions and a nonspecific inhibitor of radical reactions, also inhibited PMN adhesion, but not as effectively as butylated hydroxytoluene. Ascorbate, a water-soluble antioxidant, inhibited 90% of the adherence induced by either H_2O_2 and t-BuOOH. Thus, termination of oxidative radical chain reactions by lipid- or water-soluble antioxidants prevented the appearance of the adhesive endothelial cell surface that resulted from peroxide exposure.

PAF Does Not Mediate Adherence after Prolonged Oxidant Exposure

Our initial experiments to identify the molecular mechanism of adherence in response to prolonged oxidant exposure examined the potential role of endothelial cell PAF in this event. We first examined the time course of PAF synthesis after oxidant exposure by pulse labeling the cultures with [³H]acetate for 10 min throughout the incubation (24). We found (Fig. 5 *a*) that 250 μM H_2O_2 induced PAF synthesis immediately after exposure to H_2O_2 and during the subsequent half-hour of exposure. This synthesis had fallen to undetectable levels after 60 min of exposure. In a separate experiment (not shown), there was no detectable synthesis of [³H]PAF at either 100 or 240 min of stimulation with H_2O_2. The effect of t-BuOOH on the endothelial cells differed from that of H_2O_2 in that there was no detectable synthesis of
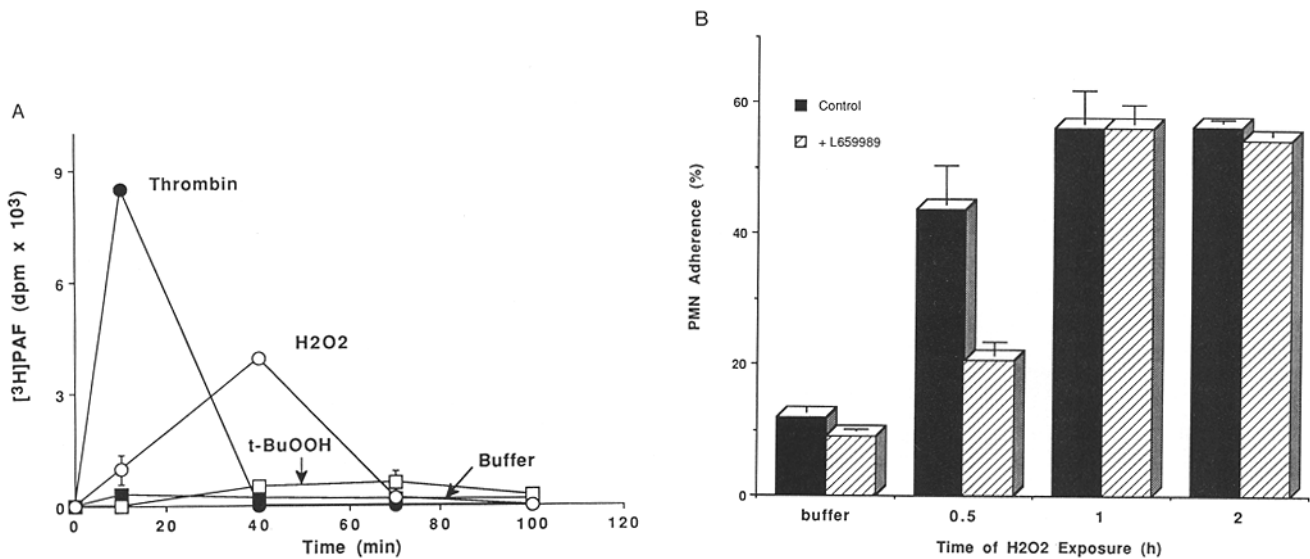
Figure 5. (A) Rate of synthesis of [³H-acetyl]PAF by endothelial cells at various times after stimulation with H_2O_2, t-BuOOH, or thrombin; (B) effect as a function of time of a PAF receptor antagonist on PMN adherence. Endothelial cells were washed with HBSS/A and at time = 0 the buffer was switched to 37° HBSS/A with or without 250 μM t-BuOOH, 250 μM H_2O_2 or 2 U/ml human thrombin. 10 min before the stated time, 25 μCi of [³H]acetate was added to the incubation medium, and the incubation with the radiolabel was allowed to continue for 10 min at 37°. Incorporation of [³H]acetate was stopped by removing the incubation medium (<1% of the [³H]PAF is released into the overlaying medium) and adding 1 ml methanol to the monolayer. [³H]PAF was quantitated using total radioactivity in the lipid extract and the ratio of [³H]PAF to recovered radioactivity from the chromatography plate. In B, endothelial cell monolayers were washed and treated with HBSS/A with or without 350 μM H_2O_2 for the stated period of time. This was then removed, the monolayers washed, and PMN that had been preincubated for 5 min with 100 μM L659,989, or an equivalent amount of DMSO vehicle, were layered over the monolayer. This allowed the concentration of L659,989 to be maintained at 100 μM throughout the coincubation. Nonadherent PMNs were removed and the tightly adherent PMNs were quantitated as in Fig. 1. When the effect of a second PAF receptor antagonist, WEB 2086, was examined (not shown) its concentration was 10 μM, and the concentration of H_2O_2 or t-BuOOH was 250 μM. The incubation time was 2 h. In parallel experiments, both L659,989 and WEB 2086 inhibited PAF-induced PMN adherence to a gelatin matrix in a fashion that depended on the concentration of PAF. Data are expressed as a mean and range of duplicate values.
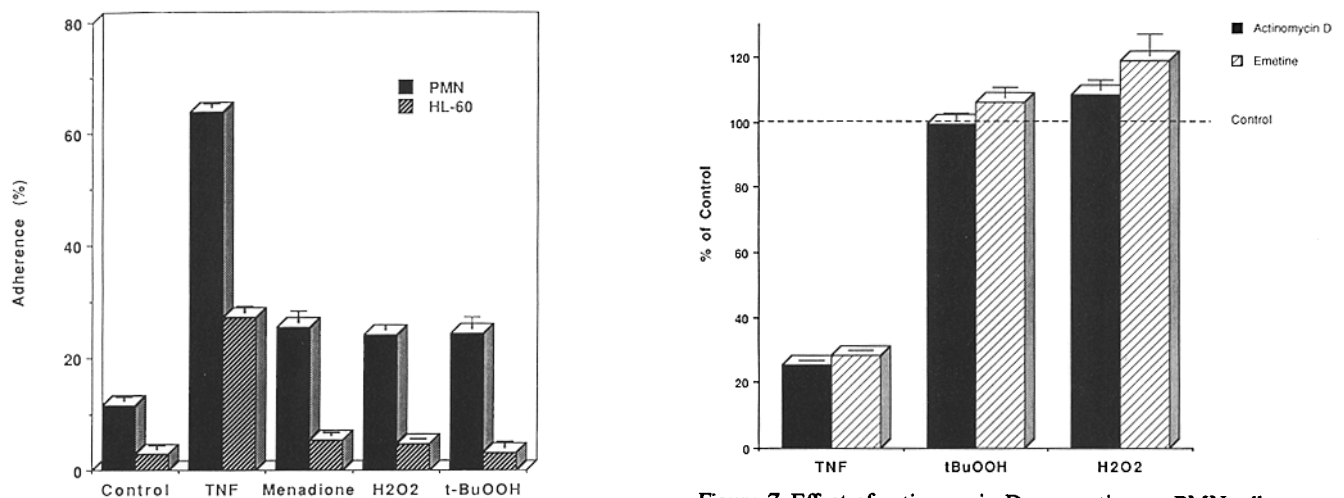

Figure 6. Adherence of PMN or undifferentiated HL-60 cells to endothelial cells stimulated with menadione, t-BuOOH, or H_2O_2. Endothelial cells grown to confluence were treated with HBSS/A, or 300 μM t-BuOOH, 1 mM H_2O_2, 300 μM menadione, or 1,000 U TNF-α in HBSS/A. After 4 h of incubation at 37°, the incubation medium was removed, and the monolayer washed twice before the ability of the incubated endothelial cell monolayers to bind ¹¹¹In-PMN or ¹¹¹In-HL-60 cells was determined as described under Fig. 1 using a 5-min incubation period. The values presented are the percent of cells that bound compared to the total added to each well, and are given as a mean and range of duplicate values.

Figure 7. Effect of actinomycin D or emetine on PMN adherence to endothelial cells treated with t-BuOOH, H_2O_2, or TNF-α. Endothelial cells were treated for 15 min at 37° with or without 5 μg/ml of actinomycin D or 5 μM emetine in HBSS/A. The cells were washed, and then treated with HBSS/A buffer, or 250 μM t-BuOOH, 250 μM H_2O_2 or 1,000 U TNF-α in HBSS/A for 2 h at 37° in the continued presence of the protein synthesis inhibitors. The incubation buffer was removed, the monolayers were washed twice, and PMN adherence was measured as described under Fig. 1. Data are expressed as the percent of adherent cells compared to total cells added to each well, and are presented as a mean and range of duplicate values.
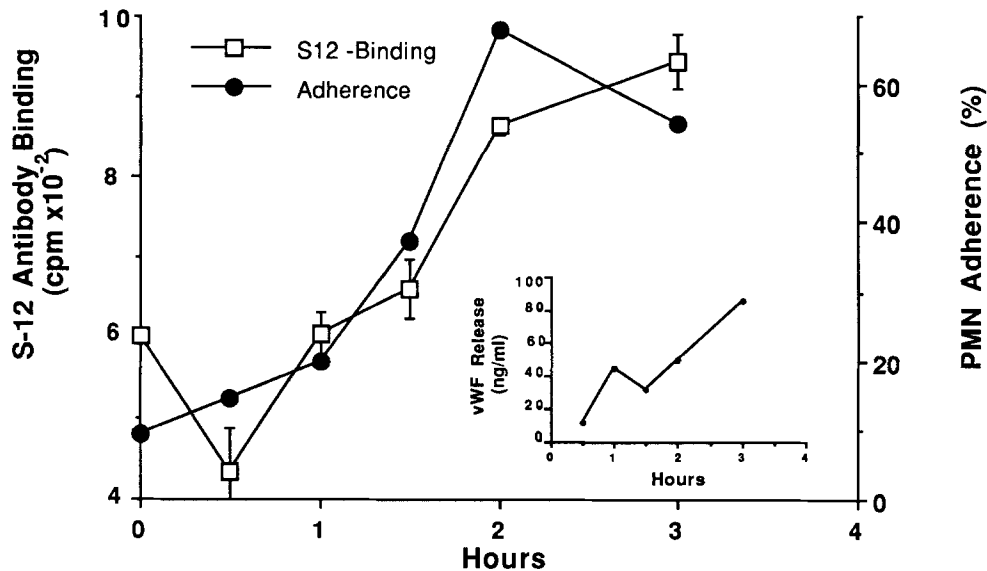
Figure 8. Effect of *t*-BuOOH on GMP-140 surface expression, von Willebrand Factor release, and PMN adherence as a function of time. Endothelial cells were treated at 37° with HBSS/A with or without 250 μM *t*-BuOOH for the stated time. Supernatants were removed to determine vWF release, while the monolayers were fixed and stained with the anti-GMP-140 mAb S12 as described under Materials and Methods. Determination of PMN adherence was performed in parallel as described under Fig. 1. There was no detectable increase in S12 binding to monolayers incubated with HBSS/A alone, nor was there an increase in binding of the isotype-matched irrelevant antibody T10 to *t*-BuOOH-treated monolayers. Data are expressed as the amount of ^{125}I-labeled second antibody associated with the monolayer, and are presented as a mean and range of duplicate values.

[3H]PAF at any of the times examined. We next examined the ability of endothelial cells to retain for prolonged times the PAF synthesized immediately upon exposure to H_2O_2. We continuously exposed endothelial cells to H_2O_2 and [3H]acetate (four times the amount of [3H]acetate used in the pulse labeling experiment) and then extracted and purified cellular PAF. There was no detectable [3H]PAF remaining in the exposed cells after 90 or 120 min of stimulation (not shown).

We employed L659,989, a competitive antagonist of the PAF-receptor (10), to directly assess the role of endothelial cell–associated PAF as a signal for the adherence of PMN. As we previously observed, exposure of endothelial cells to H_2O_2 for short periods of time induced PMN adherence (14) (Fig. 5 *b*). L659,989 inhibited 64% of the PMN adherence induced by exposure of endothelial cell monolayers to H_2O_2 for 30 min. However, after 1 h of exposure, L659,989 had no measurable effect on H_2O_2-induced PMN adherence. We confirmed this result with a second, structurally unrelated receptor antagonist, WEB 2086, which also had no effect on *t*-BuOOH- or TNF-α-induced adherence (not shown). These results show that while H_2O_2 induces endothelial cells to bind PMN by a PAF-dependent mechanism soon after exposure to it, this mechanism does not account for PMN adherence after exposure to H_2O_2 for 60 min or longer, and plays no role in *t*-BuOOH-induced adhesion at any time.

ELAM-1 Does Not Mediate Adherence after Oxidant Exposure

ELAM-1 directly mediates neutrophil, monocyte, and HL-60 cell adherence to endothelial cells activated with cytokines such as TNF-α (3), and is the sole known mechanism by which PMNs adhere to endothelial cells with a prolonged time course. We investigated the role of ELAM-1 in PMN adhesion to oxidant-treated endothelial cells using HL-60 cells as a probe for the expression of ELAM-1. The data in

Fig. 6 show that although the oxidants induced PMN adherence in this experiment, they uniformly failed to stimulate the adherence of undifferentiated HL-60 cells. We also examined the effect of protein synthesis inhibitors on the development of the pro-adhesive surface. This approach relied on the observation that endothelial cells do not normally express either ELAM-1 or its messenger RNA (3, 4). Treatment of endothelial cell monolayers with actinomycin D, an inhibitor of transcription, failed to inhibit adherence in response to 250 μM H_2O_2 or *t*-BuOOH even though it blocked 70% of the adherence induced by TNF-α (Fig. 7). A similar effect was obtained when emetine was used to inhibit translation. Finally, as a direct and independent assessment of the role of ELAM-1, we employed H18/7, an mAb against ELAM-1 that blocks about one-half of the adherence induced by TNF-α (3). In this one experiment, H18/7 blocked 50% of the adherence induced by TNF-α, as expected, but failed to inhibit adherence in response to *t*-BuOOH (not shown). These results exclude ELAM-1, or any newly synthesized protein, as the mechanism for the adherence of PMN to oxidant-treated endothelial cells.

GMP-140 Mediates Adhesion after Prolonged Exposure of Endothelial Cells to Peroxides

GMP-140 is a recently characterized glycoprotein that has a predicted structure similar to that of ELAM-1 (11). It is a membrane-bound component of specialized granules in endothelial cells, the Weibel-Palade bodies, that are rapidly translocated to the cell surface in response to agonist stimulation (5, 9). GMP-140 mediates PMN adhesion to appropriately stimulated endothelial cells, GMP-140-coated surfaces, and COS cells transfected with its cDNA (7). Therefore, we asked whether treatment of endothelial cell monolayers with peroxide induced the translocation of GMP-140 to the cell surface. We found (Fig. 8) increased surface expression of GMP-140 as determined by increased binding of S12, an mAb against GMP-140, on endothelial cells treated with
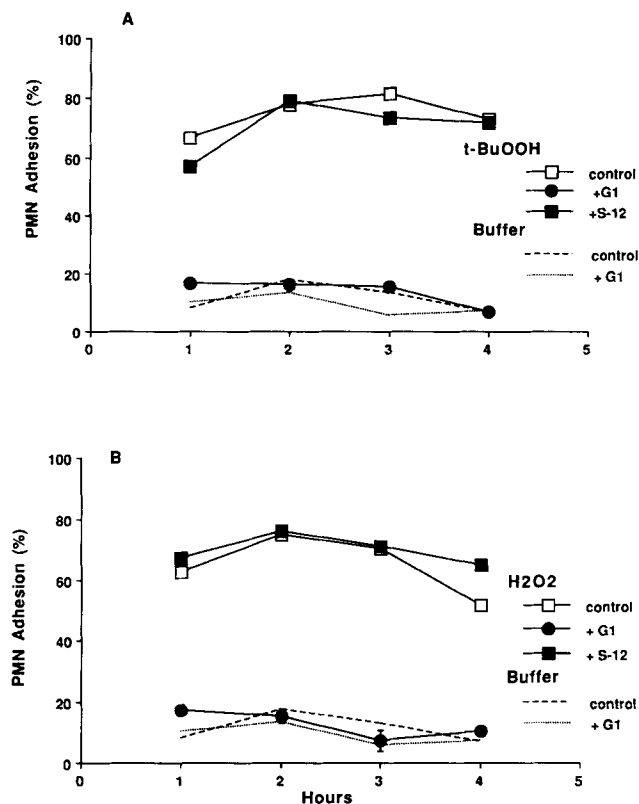
Figure 9. Effect of an mAb, G1, that blocks GMP-140 function on PMN adherence to endothelial cells treated with (*a*) *t*-BuOOH or (*b*) H_2O_2. Endothelial cells were treated at 37° for the stated time with HBSS/A, 250 μM *t*-BuOOH, or 250 μM H_2O_2 in HBSS/A. The incubation buffer was removed and the monolayer was incubated for 10 min at 37° in HBSS/A with 10 μg/ml G1 or the isotype-matched mAb S12. S12 also recognizes GMP-140 but does not block its function (7). ^{111}In-PMN adherence was measured as described under Fig. 1. Data are expressed as the percent of adherent cells compared to total cells added to each well, and are presented as a mean and range of duplicate values.

t-BuOOH. The time-dependent increase in surface expression of GMP-140 paralleled the increase in PMN adhesion. A soluble component of Weibel-Palade bodies, vWF, was secreted into the culture supernatant in response to treatment with *t*-BuOOH, confirming that Weibel-Palade bodies had fused with the plasma membrane. Parallel experiments showed that monolayers treated with buffer alone did not demonstrate increased S12 binding, that there was no corresponding increase in binding of T10 (a platelet-specific isotype-matched antibody), and that in the absence of the primary antibody, there was no increase in binding of the ^{125}I-labeled second antibody (not shown). Since H_2O_2-treatment also induced increased binding of S12 mAb (not shown), prolonged exposure to peroxides induced the translocation of Weibel-Palade bodies containing GMP-140 to the cell surface.

We next used G1, an mAb against GMP-140 that blocks PMN adhesion to GMP-140-coated surfaces and blocks PMN adhesion to endothelial cells expressing GMP-140 (7), to determine if the newly expressed GMP-140 was responsible for the adhesion of PMN to peroxide-treated endothelial cells. We found (Fig. 9) that G1 completely inhibited PMN adher-

ence to endothelial cells that had been exposed to H_2O_2 or *t*-BuOOH for 1–4 h. The isotype-matched mAb S12, which binds to a different epitope on GMP-140 and does not block its function (7), did not affect PMN adherence.

We also found that PMN adherence to peroxide-treated endothelial cells could be inhibited by pretreating the PMN with solubilized, purified GMP-140 (Fig. 10). Excess GMP-140 completely suppressed adhesion to both H_2O_2- and *t*-BuOOH-treated endothelial cell monolayers, and, since the PMNs were not aggregated, this effect was not due to an artifactual decrease in available PMN. Control incubations showed that the interaction between PMN and oxidant-treated endothelial cells was not affected by an irrelevant protein, fibrinogen, and that excess GMP-140 did not inhibit PMN adherence to endothelial cells expressing ELAM-1 in response to TNF-α stimulation. Our experiments demonstrated that, in response to oxidant treatment, human endothelial cells express GMP-140 for prolonged periods of time, and that it is the sole mechanism for the increased adhesion of PMN to oxidant-perturbed endothelial cell monolayers.

Discussion

We demonstrated that primary cultures of endothelial cells became adhesive for PMN after treatment either with a water-soluble or a lipid-soluble peroxide, or after intracellular generation of oxygen radicals. The increase in adhesion was equivalent to that induced by cytokine stimulation, up to 10-fold over basal values, but was not due to the same adhesion mechanism. Formation of the adhesive surface could be initiated by a short exposure of the endothelial cells to the oxidants, and clearly preceded irreversible oxidative damage to the cells. Adherence of PMN to oxidant-treated endothelial cells was due to an alteration of the endothelial cells themselves, rather than the activation of the adhesive response of PMN. Although H_2O_2 can directly stimulate
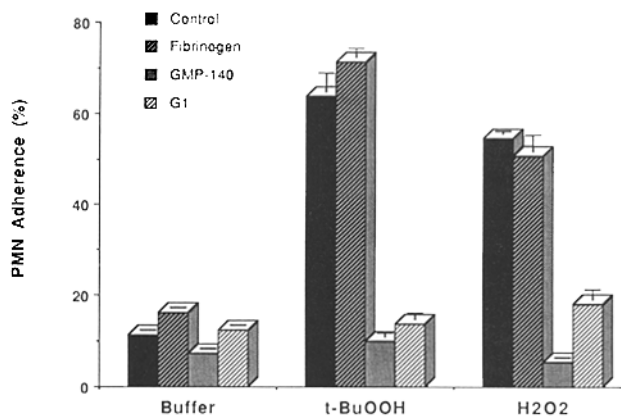

Figure 10. Effect of soluble GMP-140 on PMN adherence to peroxide-treated endothelial cells. Endothelial cells were treated for 2 h at 37° with HBSS/A, 250 μM *t*-BuOOH, or 350 μM H_2O_2 in HBSS/A. The incubation buffer was removed, the monolayers washed, and PMNs that had been pretreated for 20 min at 25° with 5 μg/ml of either soluble GMP-140 or fibrinogen were then added to the endothelial cells. PMN adherence was measured as described under Fig. 1. Data are expressed as the percent of adherent cells compared to total cells added to each well, and are presented as a mean and range of duplicate values.

the adhesive response of the U937 monocytic cell line (33), and our preliminary data showed a similar direct activation of PMN adhesion by high concentrations of peroxides (not shown), there were insufficient residual peroxides remaining in the system to induce this response. This was verified by demonstrating that PMN did not adhere to a human smooth muscle cell line, and that the mAb 60.3 did not block the adherence of PMN to oxidant-treated endothelial cells. Additional evidence that this was an endothelial cell–dependent process was the ability of formaldehyde-fixed PMN to adhere to oxidant-treated endothelial cells. This observation also demonstrated that the adhesive mechanism required no active response from the PMN.

The pro-adhesive molecule expressed by the oxidatively stressed endothelial cells was not the phospholipid PAF, although this lipid does mediate PMN adherence to endothelial cells treated with H_2O_2 for shorter periods of time (14) (Fig. 6). The surface expression of this potent phospholipid mediates PMN-endothelial cell adherence under other circumstances (14, 23, 24, 40, 42), but our data showed that there was little or no PAF present at the times examined here. Furthermore, we were unable to detect significant synthesis and accumulation of PAF in response to t-BuOOH treatment, even though it induced as much adherence as H_2O_2. As expected from these observations, PAF receptor antagonists failed to antagonize PMN adherence in response to prolonged peroxide treatment.

We found that PMN adherence to oxidant-treated endothelial cells did not require de novo protein synthesis. This suggested a role for a preformed mediator, and eliminated a role for ELAM-1 in this process. The lack of an effect by the mAb H18/7, directed against ELAM-1, supported this conclusion. Further evidence that ELAM-1 was not involved was the complete lack of adherence of the myelocytic cell line HL-60 to the oxidant-treated cells. The failure of HL-60 cells to bind was not expected as HL-60 cells, and monocytes, also bind to GMP-140 (7, 13). Indeed, treatment of human umbilical vein endothelial cells with a different lipid-soluble peroxide, cumene hydroperoxide, induces monocyte adherence to the exposed monolayer (26). One potential explanation is that the recloned line of HL-60 cells that we employed recognized ELAM-1 (since they bound to TNF-α-treated cells), but not GMP-140. If true, this would suggest that HL-60 cells possess different receptors for these selections that can be independently expressed.

The sole molecular mechanism responsible for the adherence of PMN to endothelial cell monolayers treated with low concentrations of oxidants for prolonged periods of time was the expression of GMP-140 on the cell surface. This was demonstrated by the complete inhibition of adherence by an mAb that blocks GMP-140-mediated PMN adherence, and by competitively blocking GMP-140 receptors on PMN with solubilized GMP-140. There was a marked difference between the expression of GMP-140 after oxidant treatment and its expression after stimulation of the endothelial cells with the rapidly acting agonists histamine or thrombin[2] (9). We found that GMP-140 was expressed on the peroxide-treated endothelial cells over a period of several hours, which is in sharp contrast to its transient expression after stimulation with the rapidly acting agonists where its presence on the cell surface is measured in just minutes (9). We have not identified the reason for this difference, but it is pos-

sible that the mechanisms responsible for its reinternalization (9) were not induced by the oxidizing agents, or that this mechanism was impeded by the oxidants. Also, peroxide-treated endothelial cells bound more PMN than did thrombin-treated monolayers, even though thrombin is a potent agonist for GMP-140 expression (9). Again, this may relate to the inability of peroxide-treated cells to reinternalize surface GMP-140, resulting in a higher steady-state abundance of GMP-140. Alternatively, it may reflect the lack of synthesis of PMN inhibitory compounds that decrease PMN adhesion to activated endothelial cells (38). The overall effect, however, is to produce an endothelial surface with very high adhesivity for prolonged times that is completely due to expression of GMP-140. This is in contrast to rapidly acting agonists where adhesion results from both GMP-140 and PAF expression[2], and in contrast to adhesion induced by short exposures to higher concentrations of H_2O_2, which is solely due to the expression of PAF (14).

The mechanism by which oxidants induced the translocation of GMP-140 from the Weibel-Palade bodies to the cell surface is not known, but free radicals were essential components. Free radicals have previously been shown to trigger exocytosis of histamine-containing granules from mast cells (18). In our experiments, there were differences between radical generation by H_2O_2 and t-BuOOH: t-BuOOH-mediated adherence was more sensitive to the actions of a hydrophobic iron chelator and lipid-soluble antioxidants. This suggests that the effects of both peroxides were mediated by the iron-catalyzed propagation of free radical oxidation reactions, but that lipid-soluble radicals (perhaps t-BuOO radicals) were essential intermediates in t-BuOOH-mediated GMP-140 surface expression. The lack of effect of conalbumin, a transferrin that is not translocated by human cells, suggests that the iron-catalyzed reactions occurred within the intracellular compartment. This is consistent with our observation that menadione, an intracellular source of O_2^- and H_2O_2 (29, 31), also resulted in the formation of a pro-adhesive endothelial cell surface. However the effect of menadione, unlike peroxide-stimulated GMP-140 expression, did not depend on free iron, a result also observed during the study of menadione-induced toxicity (17). Since the formation of radicals by alternative methods circumvents the requirement for iron, the essential role for ionic iron shows that H_2O_2 and t-BuOOH must be metabolized to free radicals in order to induce GMP-140 expression.

Extracellular generation of peroxides and oxidizing radicals might also induce GMP-140 translocation, and a potential source of these could be the radicals produced by activated neutrophils. Such radicals damage endothelial cells (12, 16, 35, 36), and do so in an iron-dependent fashion (12, 35). Thus extravascular PMN, when stimulated by appropriate agonists to produce oxidizing radicals, would be able to recruit other, circulating PMN by alteration of the intravascular surface. Endothelial cells have been widely employed as a target for oxidizing reagents because of the relevance of this model to a large number of diverse and important disease processes including adult respiratory distress syndrome, reperfusion injury, and atherosclerosis (15, 19, 32, 37). Recruitment of PMN to the sites of oxidative damage may serve to enhance the fundamental pathologic events and thereby exacerbate the disease process. Induction of Weibel-Palade body translocation to the cell surface and the attendant

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expression of GMP-140 on the surface of these endothelial cells may be one mechanism involved in the etiology of these diseases.

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