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Research Article

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Role of Hydrogen Peroxide in the Neutrophil-mediated Release of Prostacyclin from Cultured Endothelial Cells

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Abstract. We have examined the effect of activated neutrophils on the release of prostacyclin (PGI₂) from cultured endothelial cells by radioimmunoassay and thin layer chromatography of its stable metabolite, 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}). Phorbol myristate acetate-activated neutrophils induced a time- and dose-dependent release of 6-keto-PGF_{1α} from human and bovine endothelial cell monolayers, whereas phorbol myristate acetate alone and neutrophils alone did not. Pretreatment of the endothelial cells with aspirin prevented neutrophil-mediated 6-keto-PGF_{1α} release, indicating that it did not depend upon neutrophil-generated endoperoxides. Phorbol myristate acetate-activated neutrophils from a patient with chronic granulomatous disease failed to induce endothelial 6-keto-PGF_{1α} release. Addition of catalase but not of superoxide dismutase significantly reduced human and bovine endothelial 6-keto-PGF_{1α} release by phorbol myristate acetate-activated neutrophils. Catalase-inhibitable endothelial 6-keto-PGF_{1α} release was also observed after the addition of the hydrogen peroxide-generating system, glucose-glucose oxidase, to bovine and human endothelial cell monolayers. Bovine endothelial 6-keto-PGF_{1α} release induced by exogenously generated hydrogen peroxide was attenuated by the phospholipase inhibitor mepacrine, suggesting that hydrogen peroxide may act by triggering endothelial membrane phospholipase activation. The release of 6-keto-PGF_{1α} by enzymatically or neutrophil-generated hydrogen peroxide was not associated with endothelial cell lysis as assessed by ⁵¹Cr release. We conclude that exogenously generated

hydrogen peroxide or a hydrogen peroxide-derived product mediates rapid nonlytic release of PGI₂ from cultured endothelial cells.

Introduction

Since augmented neutrophil adherence to vascular endothelium is an early response to inflammation (1), neutrophils have been implicated in the pathogenesis of the altered vascular permeability associated with acute inflammation (2). The observation that neutrophil depletion attenuates permeability edema formation in several experimental models supports this hypothesis (3–5). Activated neutrophils have been shown to produce endothelial injury in vitro through the generation of toxic oxygen products (6, 7) and the release of granule proteases (8). Since prostacyclin (PGI₂)¹ has been demonstrated to have proinflammatory effects and to enhance edema formation (2, 9–16), we have examined the effect of activated neutrophils on endothelial PGI₂ release by the use of purified human peripheral blood neutrophils, cultured bovine and human endothelial cells (ECs), and radioimmunoassay (RIA) and thin-layer chromatography (TLC) of 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}), the stable breakdown product of PGI₂.

Methods

ECs. Bovine aortic and pulmonary artery and human umbilical vein ECs were prepared by collagenase treatment of vessels as previously described (17, 18) and maintained in 10% newborn calf serum (NBCS) (Gibco Laboratories, Gibco Div., Grand Island, NY) in Waymouth's medium (Gibco Laboratories, Gibco Div.). Bovine ECs were used in the 6th through the 15th passage. Individual experiments were performed with bovine ECs of the same strain and passage number. Human umbilical vein ECs were used in the first passage.

Preparation of neutrophils. Purified human peripheral blood neutrophils were prepared from heparinized blood from normal healthy donors by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ)

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1. *Abbreviations used in this paper:* EC, endothelial cell; NBCS, newborn calf serum; PGE₁, PGE₂, PGF_{1α}, PGF_{2α}, prostaglandins E₁, E₂, F_{1α}, F_{2α}; PGI₂, prostacyclin; PMA, phorbol myristate acetate; RIA, radioimmunoassay; SBTI, soybean trypsin inhibitor; SOD, superoxide dismutase; TLC, thin-layer chromatography.

gradient centrifugation and dextran sedimentation with hypotonic lysis of contaminating erythrocytes (19). Peripheral blood neutrophils were similarly prepared from blood obtained from a patient with chronic granulomatous disease and a patient with myeloperoxidase deficiency (courtesy of Dr. Henry Rosen, University of Washington, Seattle, WA). Neutrophils were suspended in Neuman-Tytell medium (Gibco Laboratories, Gibco Div.) at a concentration of 10^7 cells/ml.

RIA of EC 6-keto-PGF_{1α} release. ECs were plated at confluent density (10^5 cells/cm²) in 10% NBCS in Waymouth's medium in 16-mm or 23-mm wells (Cluster 3524 and 3512; Costar, Cambridge, MA) and allowed to adhere overnight. Before the experiment, monolayers were washed twice with serum-free Neuman-Tytell medium. The wash medium was then gently decanted and control or test agents or neutrophils in serum-free Neuman-Tytell medium were added. The monolayers were incubated at 37°C in 5% CO₂, and aliquots of cell-free supernatant medium were removed at intervals for determination of 6-keto-PGF_{1α}. Cell counts were performed on replicate wells by electronic counter (Particle Data, Inc., Elmhurst, IL).

The measurement of unextracted, cell-free supernatant medium from the endothelial cultures for 6-keto-PGF_{1α} was performed in some samples by the determination of competitive inhibition of ³H-6-keto-PGF_{1α} to anti-6-keto-PGF_{1α} binding by the use of labeled tracers, standards, and antiserum from New England Nuclear (Boston, MA) (New England Nuclear Technical Bulletin NEK-008) (20). The standard curve range was 10 to 500 pg added per 100 μl, with a normalized percent bound at 50% of 100–130 pg. Cross-reactivity of the 6-keto-PGF_{1α} antibody at a normalized percent bound of 50% was PGF_{1α}, 7.8%; prostaglandin E₁ (PGE₁), 3%; prostaglandin F_{2α} (PGF_{2α}), 2.7%; prostaglandin E₂ (PGE₂), 2%; prostaglandin A₁, <0.3%; prostaglandin A₂, <0.1%; thromboxane B₂, <0.1%; and 13,14-dihydro-15 keto-PGF_{2α}, <0.02% (New England Nuclear Technical Bulletin NEK-008). In other samples, 6-keto-PGF_{1α} levels were determined by RIA with ³H-6-keto-PGF_{1α} (Amersham Corp., Arlington Heights, IL), authentic 6-keto-PGF_{1α} standard kindly provided by Douglas McCarter and Dr. John Pike (Upjohn Co., Kalamazoo, MI), and antiserum generously supplied by Dr. William B. Campbell, University of Texas Health Science Center at Dallas. This anti-6-keto-PGF_{1α} antibody cross-reacted less than 0.9% with PGE₁, PGE₂, PGF_{1α}, PGF_{2α}, prostaglandin D₂, prostaglandin A₂, and thromboxane B₂, 3.5% with 6,15-diketo-PGF_{1α}, and 70% with 6-keto-PGE₁. The level of sensitivity was 10 pg/300 μl. Assays were run in duplicate and at two dilutions when necessary. Results were confirmed in selected samples by independent RIA of 6-keto-PGF_{1α} kindly performed by Dr. William B. Campbell (21). Results obtained in the RIAs are expressed as picograms of 6-keto-PGF_{1α} released per 10⁵ endothelial cells. For statistical calculations, samples with undetectable levels of 6-keto-PGF_{1α} were assumed to be equal to the lower limit of detection in the assay.

TLC of bovine EC 6-keto-PGF_{1α} release. In some experiments, EC prostaglandin release was also assessed by TLC (22). EC lipids were labeled by the incubating of the cells for 6 h with 1.0 μCi [³H]arachidonic acid (87.4 Ci/mmol, New England Nuclear) in Neuman-Tytell media. The cell monolayers were then washed three times with 10 mM Hepes buffer (Gibco Laboratories, Gibco Div.) containing 0.15 M NaCl, 0.005 M KCl, 0.0018 M CaCl₂, 0.001 M MgCl₂, glucose 1 g/liter, and fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to remove the unincorporated [³H]arachidonic acid. The monolayers were then incubated with control or test medium or agents. Cell-free supernatant medium was collected from each well after 30–60 min of incubation. The monolayers were then washed once with the Hepes buffer solution and the wash was pooled with the original supernatant medium.

The supernatant medium was acidified to pH 3.5 with glacial acetic acid and extracted twice with five times volume of ethyl acetate/cyclohexane (1:1). The organic layer was evaporated to dryness with nitrogen. Unlabeled 6-keto-PGF_{1α} and PGE₂ (Sigma Chemical Co.) were added before extraction to localize these prostaglandins during chromatography. A small aliquot was also removed before and after extraction to correct for recovery. The dried residue was resuspended in 100 μl of ethyl acetate and applied to a 20 × 20 cm silica gel 60 TLC plate (Merck and Co., Darmstadt, Germany). The plates were developed twice in a water-saturated mixture of ethyl acetate/glacial acetic acid/isooctane (110:20:50), and dried in air, and the spots were located with iodine vapor. Areas corresponding to 6-keto-PGF_{1α}, PGE₂, and arachidonic acid were then removed by scraping, extracted with chloroform/methanol (9:1), and dried under nitrogen, and scintillate was added for counting.

⁵¹Cr-release assay. ECs were plated on Microtest III plates (Falcon Labware, Div. Becton-Dickinson and Co., Oxnard, CA) and labeled with sodium (⁵¹Cr) chromate (New England Nuclear) as previously described (8). After an overnight incubation, cells were washed three times with 1% NBCS in phosphate buffered saline (Gibco Laboratories, Gibco Div.) and then incubated with either test or control medium or cells. 50–100 μl of cell-free supernatant medium were removed at 4–6 h for determination of specific ⁵¹Cr release by the formula $(A - B)/(C - B) \times 100\%$. *A* represents the mean test ⁵¹Cr-counts per minute released, *B* represents the mean spontaneous ⁵¹Cr-counts per minute released, and *C* represents the mean maximum ⁵¹Cr-counts per minute released. Maximum ⁵¹Cr release was determined by incubation in 1% Triton X-100 (New England Nuclear). Spontaneous ⁵¹Cr release was determined in control monolayers incubated in Neuman-Tytell medium only and was 5–10% of maximum ⁵¹Cr release after 4 h of incubation. Statistical significance was determined by comparison of mean test and mean spontaneous ⁵¹Cr-counts per minute released by two-tailed, unpaired *t* statistic.

Reagents. Acetylsalicylic acid, A23187, phorbol myristate acetate (PMA), soybean trypsin inhibitor (SBTI), mepacrine (quinacrine dihydrochloride), fatty acid-free albumin, catalase (bovine liver, 11,800 U/mg), superoxide dismutase (SOD) (2,700 U/mg), and glucose oxidase (grade VII) were obtained from Sigma Chemical Co. Sodium arachidonate was obtained from Bio/Data Corp. (Horsham, PA).

Results

Neutrophil-mediated bovine endothelial 6-keto-PGF_{1α} release. Incubation of bovine EC monolayers with PMA alone or neutrophils alone did not induce significant release of 6-keto-PGF_{1α}. When neutrophils were activated by PMA, however, a marked increase in 6-keto-PGF_{1α} release was noted. Because baseline (medium alone) and stimulated (arachidonic acid) release of 6-keto-PGF_{1α} from bovine EC monolayers varied considerably among strains and passage number in the same strain, results from individual experiments were expressed as the fold-increase in 6-keto-PGF_{1α} release (Table I). The increase in 6-keto-PGF_{1α} was due to release from ECs and not from neutrophils, since 6-keto-PGF_{1α} could not be detected when neutrophils were activated by PMA in the absence of ECs (<100 pg/10⁷ neutrophils in nine experiments).

Time course and dose response of neutrophil-mediated bovine EC 6-keto-PGF_{1α} release. Significant 6-keto-PGF_{1α} release was observed after a 15-min incubation of bovine EC monolayers

Table I. Effect of PMA-activated Neutrophils on Bovine EC 6-keto-PGF_{1α} Release

Addition to endothelial monolayer	Fold-increase in 6-keto-PGF _{1α} release
PMA (4)	1.1±0.7 (<i>P</i> > 0.05)
Neutrophils (4)	1.1±0.5 (<i>P</i> > 0.05)
Neutrophils and PMA (18)	12.2±1.5 (<i>P</i> < 0.001)
Arachidonic acid (6)	35.1±3.8 (<i>P</i> < 0.001)

Control medium, PMA (10 ng/ml), neutrophils (neutrophil-to-endothelial ratio of 10 to 1), neutrophils and PMA, or arachidonic acid (50 μg/ml) were added to bovine aortic or pulmonary artery EC monolayers. Aliquots of cell-free supernatant medium were removed after 30 min of incubation for determination of 6-keto-PGF_{1α} release by RIA. Values represent the mean±1 SE of (*n*) separate experiments. In each individual experiment the fold-increase in 6-keto-PGF_{1α} release was determined from the mean of 2–4 replicate wells as (test – control)/control, where control represents mean 6-keto-PGF_{1α} (picograms per 10⁵ cells) released with medium alone and test represents mean 6-keto-PGF_{1α} (picograms per 10⁵ cells) released after incubation with test agent or cell (i.e., PMA, neutrophils, PMA and neutrophils, or arachidonic acid). The *P* value was determined by comparison of the mean value of 6-keto-PGF_{1α} released in control and test in each experiment by paired *t* statistic.

with PMA-activated neutrophils (Figure 1 A). Neutrophil-to-endothelial ratios as low as 2 to 1 produced a significant increase in bovine endothelial 6-keto-PGF_{1α} release (Figure 1 B).

Effect of inhibitors on neutrophil-mediated bovine endothelial 6-keto-PGF_{1α} release. The effect of inhibitors on bovine endothelial 6-keto-PGF_{1α} release induced by PMA-activated neutrophils is summarized in Table II. Aspirin pretreatment of the bovine EC monolayers prevented neutrophil-mediated 6-keto-PGF_{1α} release. SBTI, a potent inhibitor of neutrophil serine proteases, had no significant inhibitory effect. Superoxide dis-

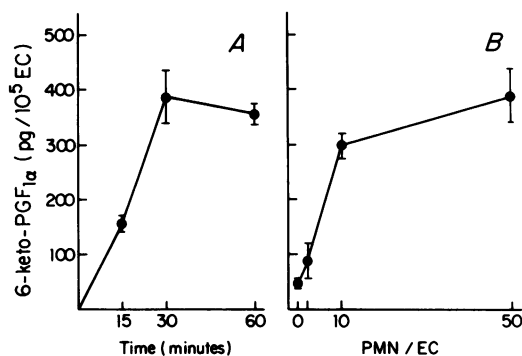


Figure 1. Time- and dose-response of neutrophil-mediated bovine EC 6-keto-PGF_{1α} release. PMA (10 ng/ml) and neutrophils (PMN) were added to bovine aortic EC monolayers. In A, the neutrophil-to-endothelial ratio was 50 to 1. In B, the incubation was 30 min. Values represent mean±1 SE of three replicate wells.

Table II. Effect of Inhibitors on Neutrophil-mediated Bovine EC 6-keto-PGF_{1α} Release

Addition to endothelial monolayer	Inhibitor	% Inhibition of endothelial 6-keto-PGF _{1α} release
PMA + neutrophils	Aspirin pretreatment (2)	100
	SBTI (2)	0
	SOD (6)	6±2 (<i>P</i> > 0.05)
	Catalase (11)	52±12 (<i>P</i> = 0.01)
	Catalase boiled (2)	0
A23187	Catalase (1)	0
Arachidonic acid	Catalase (1)	0

Bovine endothelial 6-keto-PGF_{1α} release was determined by RIA of aliquots of cell-free supernatant medium after a 60-min incubation with PMA (10 ng/ml)-activated neutrophils (neutrophil-to-endothelial ratio of 10 to 1) or a 30-min incubation with A23187 (20 μM) or arachidonic acid (50 μg/ml). Aspirin pretreatment of EC monolayers was performed by incubating monolayers with aspirin (1 mM) for 30 min and then washing with medium before the addition of PMA-activated neutrophils. Catalase (3,000 U/ml), catalase boiled for 10 min, SOD (270 U/ml), and SBTI (500 μg/ml) were added simultaneously with the PMA-activated neutrophils. The percent inhibition (±1 SE) of endothelial 6-keto-PGF_{1α} release was calculated as the increase in endothelial 6-keto-PGF_{1α} release induced by PMA-activated neutrophils: ((without inhibitor – with inhibitor)/without inhibitor) × 100%, where (*n*) represents the number of individual experiments. In each individual experiment, values of 6-keto-PGF_{1α} were determined from the means of from two to four replicate wells. The *P* value was determined by comparison of the mean fold-increase in 6-keto-PGF_{1α} levels induced by PMA-activated neutrophils with inhibitor and without inhibitor in each experiment by paired *t* statistic.

mutase also failed to prevent endothelial 6-keto-PGF_{1α} release. Catalase significantly reduced bovine endothelial 6-keto-PGF_{1α} release induced by PMA-activated neutrophils, although the degree of inhibition varied somewhat between experiments (range, 30–95%). Catalase did not inhibit endothelial 6-keto-PGF_{1α} release induced by arachidonic acid or calcium ionophore.

Effect of PMA-activated chronic granulomatous disease neutrophils on bovine EC 6-keto-PGF_{1α} release. The inhibition of endothelial 6-keto-PGF_{1α} release after the addition of catalase to PMA-activated neutrophils suggested that hydrogen peroxide or a hydrogen peroxide-derived product was a critical mediator. To assess further the role of hydrogen peroxide, we examined the effect of PMA-activated neutrophils from a patient with chronic granulomatous disease and a patient with myeloperoxidase deficiency on endothelial 6-keto-PGF_{1α} release. Chronic granulomatous disease neutrophils, which do not generate hydrogen peroxide, failed to induce 6-keto-PGF_{1α} release when activated by PMA (Table III). Note that PMA-activated myeloperoxidase-deficient neutrophils, which produce hydrogen peroxide but not hypochlorous acid, induced 6-keto-PGF_{1α} re-

Table III. Role of Oxygen Radicals in Bovine EC 6-keto-PGF_{1α} Release Induced by PMA-activated Neutrophils

Addition to endothelial monolayer	6-keto-PGF _{1α} pg/10 ⁵ EC
Experiment 1	
Normal neutrophils + PMA (3)	247
CGD neutrophils + PMA (3)	<30
Experiment 2	
Normal neutrophils + PMA (2)	227
CGD neutrophils + PMA (2)	<30

In two separate experiments bovine aortic EC monolayers were incubated with normal or chronic granulomatous disease neutrophils (neutrophil-to-endothelial ratio of 10 to 1) and PMA (10 ng/ml). After 60 min of incubation, an aliquot of cell-free supernatant medium was removed for determination of 6-keto-PGF_{1α} by RIA. Values represent means of (n) replicate wells.

lease similar to that induced by normal neutrophils (720±100 pg/10⁵ cells with normal PMA-activated neutrophils; 780±33 pg/10⁵ cells with myeloperoxidase-deficient PMA-activated neutrophils, mean±1 SE of triplicate wells).

Effect of glucose-glucose oxidase on bovine endothelial 6-keto-PGF_{1α} release. After the observations that endothelial 6-keto-PGF_{1α} release required a normal neutrophil oxidative burst and was catalase inhibitable, we examined the effect of a cell-free hydrogen peroxide-generating system, glucose-glucose oxidase, on bovine endothelial 6-keto-PGF_{1α} release. The addition of glucose-glucose oxidase to bovine EC monolayers produced significant catalase-inhibitable 6-keto-PGF_{1α} release (Table IV A). The boiling of glucose oxidase before its addition to the monolayer prevented 6-keto-PGF_{1α} release, and the boiling of catalase abolished its inhibitory effect, indicating that for both, enzymatic activity was required (Table IV A). Bovine EC 6-keto-PGF_{1α} release induced by glucose-glucose oxidase was inhibited by the phospholipase inhibitor mepacrine (23) (Table IV B).

Effect of hydrogen peroxide on human EC 6-keto-PGF_{1α} release. Because the bovine EC strains employed in these studies were multiply passaged, we were concerned that the effect of hydrogen peroxide on PGI₂ release might be due to some alteration in arachidonic acid metabolism that occurred with prolonged passage. It is known, for example, that the amount of 6-keto-PGF_{1α} released from bovine aortic and pulmonary artery ECs after stimulation with ionophore or arachidonic acid decreases markedly with passage and that there is a shift from production of PGI₂ to production of other prostanoids (24, 25). For this reason, we also examined the effect of hydrogen peroxide on first passage human umbilical vein ECs.

Both PMA-activated neutrophils and glucose-glucose oxidase induced dose-dependent release of 6-keto-PGF_{1α} from human umbilical vein ECs (Fig. 2). The amount of 6-keto-PGF_{1α} released

Table IV. Effect of Glucose-Glucose Oxidase on Bovine EC 6-keto-PGF_{1α} Release

Addition to endothelial monolayer	6-keto-PGF _{1α} pg/10 ⁵ EC
A	
Control medium (4)	523±142
Glucose oxidase (4)	2,452±140
Boiled glucose oxidase (4)	404±95
Glucose oxidase + catalase (4)	380±48
Glucose oxidase + boiled catalase (4)	1,714±333
B	
Control medium (3)	105±7
Glucose oxidase (3)	2,410±540
Glucose oxidase + mepacrine (3)	580±171

In A, glucose oxidase (25 mU/ml) or glucose oxidase boiled for 10 min with and without catalase (3,000 U/ml) or catalase boiled for 10 min were added to bovine aortic EC monolayers in Neuman-Tytell medium (22 mM glucose). In B, monolayers were incubated with mepacrine (10⁻⁴ M) for 30 min before the addition of glucose oxidase (10 mU/ml). After 30 min of incubation, aliquots of cell-free supernatant medium were removed for determination of 6-keto-PGF_{1α} by RIA. Values represent mean±1 SE of (n) replicate wells.

after incubation of the first passage human umbilical vein ECs with PMA-activated neutrophils or the hydrogen peroxide-generating system were considerably greater than that observed

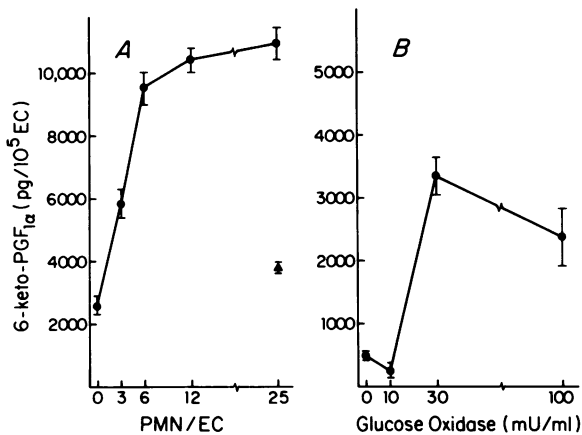


Figure 2. Hydrogen peroxide-mediated human umbilical vein EC 6-keto-PGF_{1α} release. In A, human EC monolayers were incubated in Neuman-Tytell medium (22 mM glucose) with or without PMA-activated neutrophils. Catalase (3,000 U/ml) (closed triangle) was added to the neutrophils before the addition of PMA. Aliquots of cell-free supernatant medium were removed at 30 min for determination of 6-keto-PGF_{1α}. Values represent mean±1 SE of four replicate wells. In B, human EC monolayers were incubated in Neuman-Tytell medium with or without glucose oxidase. At 30 min aliquots of cell-free supernatant medium were removed for determination of 6-keto-PGF_{1α} by RIA. Values represent mean±1 SE of three replicate wells.

with the bovine cells. However, the levels of 6-keto-PGF_{1α} released by A23187 or arachidonic acid were likewise five- to tenfold greater in human cells than in bovine cells (data not shown). As with bovine ECs, the release of 6-keto-PGF_{1α} from the human ECs incubated with PMA-activated neutrophils (Fig. 2 A) and with glucose-glucose oxidase (data not shown) was inhibited by catalase.

TLC of hydrogen peroxide-mediated EC 6-keto-PGF_{1α} release. The release of 6-keto-PGF_{1α} from ECs induced by neutrophil-derived and enzymatically generated hydrogen peroxide was confirmed by TLC. Table V A demonstrates the catalase-inhibitable release of labeled 6-keto-PGF_{1α}, PGE₂, and arachidonic acid from prelabeled human EC monolayers incubated with PMA-activated neutrophils. Table V B demonstrates the catalase-inhibitable release of these labeled products from prelabeled human EC monolayers incubated with glucose-glucose oxidase.

Relation of hydrogen peroxide-mediated endothelial 6-keto-PGF_{1α} release to cell lysis. The rapid release of 6-keto-PGF_{1α} from bovine EC monolayers during incubation with PMA-activated neutrophils was not correlated with EC lysis, as determined by ⁵¹Cr release. Specific endothelial ⁵¹Cr release was not observed even after a 4-h incubation of ⁵¹Cr-labeled EC monolayers with PMA-activated neutrophils (Table VI). PMA-activated neutrophils also failed to induce significant specific ⁵¹Cr release from human EC monolayers at 4 h (data not shown).

Table V. TLC of H₂O₂-mediated Human EC 6-Keto-PGF_{1α} Release

Addition to endothelial monolayers	³ H-counts per minute released		
	6-keto-PGF _{1α}	PGE ₂	Arachidonic acid
A			
Control medium	38±4	47±11	1,112±340
PMA + neutrophils	267±85	136±2	5,772±248
PMA + neutrophils + catalase	91±29	80±14	4,338±582
B			
Control medium	93±9	157±25	6,293±481
Glucose oxidase	292±69	385±108	48,491±12,536
Glucose oxidase + catalase	102±28	201±28	4,597±284

In A, human umbilical vein EC monolayers were labeled with [³H]arachidonic acid, washed, and then incubated for 60 min with control medium, or PMA (10 ng/ml) and neutrophils (neutrophil-to-endothelial cell ratio of 10 to 1) with and without catalase (3000 U/ml). In B, control medium or glucose oxidase (50 mU/ml) with and without catalase (3,000 U/ml) were incubated for 30 min with [³H]arachidonic acid-labeled human umbilical vein EC monolayers. After incubation the cell-free supernatant medium was removed for determination of labeled 6-keto-PGF_{1α}, PGE₂, and arachidonic acid release by TLC. Values represent mean±1 SD of duplicate wells.

Table VI. Effect of PMA-activated Neutrophils on Bovine EC Lysis

Addition to endothelial monolayers	⁵¹ Cr release	
	cpm released	% Specific release
Control medium (8)	234±15	
Neutrophils (8)	195±8	0
PMA (8)	236±20	0
PMA and neutrophils (8)	249±18	<1%
Triton X-100 (4)	2,202±11	

Bovine aortic EC monolayers were labeled overnight with ⁵¹Cr, washed, and then incubated with control medium, neutrophils (neutrophil to endothelial ratio of 10 to 1), PMA (10 ng/ml), neutrophils and PMA, or Triton X-100 (0.1%). After 4 h an aliquot of cell-free supernatant medium was removed for determination of ⁵¹Cr release. Values represent mean±1 SE of (n) replicates.

Discussion

The acute inflammatory response is characterized by neutrophil adherence to endothelium and neutrophil accumulation at the inflammatory site (1). The vascular response to neutrophil infiltration includes an increase in local blood flow and altered vascular permeability, resulting in edema formation. Numerous studies have implicated prostaglandins, particularly PGE₂ and PGI₂, as critical mediators of the hyperemia and increased permeability that accompany the neutrophil infiltration (2, 9–16). Because the vascular endothelium releases PGI₂ and PGE₂ when stimulated (26), ECs are a potential source for the local production of these prostaglandins during the acute inflammatory response.

Our studies demonstrate that PMA-activated human peripheral blood neutrophils induce a time- and dose-dependent release of PGI₂ from cultured ECs. Since PMA does not induce the release of arachidonic acid metabolites from human neutrophils (27), and aspirin pretreatment of the endothelium prevented 6-keto-PGF_{1α} release, it is unlikely that endothelial PGI₂ release occurs as a result of the provision of a substrate for endothelial cyclooxygenase by neutrophil-generated endoperoxides, as has been described for platelet-endothelial interactions (28). The failure of PMA to induce arachidonic acid release in human neutrophils also suggests that neutrophil lipoxygenase products are not involved in EC PGI₂ release by neutrophils stimulated with PMA. Since leukotrienes C₄ and D₄ have been demonstrated to induce endothelial PGI₂ synthesis (29), it is possible that neutrophil-activating agents that result in leukotriene production could provoke endothelial PGI₂ release through generation of leukotriene C₄ or D₄.

Because neutral proteases such as trypsin and thrombin have been demonstrated to stimulate endothelial PGI₂ release (30), neutrophil-derived neutral proteases such as elastase and cathepsin G might be expected to mediate endothelial 6-keto-

PGF_{1α} release. Several factors, however, suggest that neutrophil-derived neutral proteases are not involved in the 6-keto-PGF_{1α} release induced by PMA-activated neutrophils. First, at the concentration of PMA (10 ng/ml) and time course (15–30 min) used in these studies, there is minimal release of the neutrophil azurophilic granules that contain the neutral proteases (31). Second, SBTI, a potent serine protease inhibitor, did not prevent 6-keto-PGF_{1α} release induced by PMA-activated neutrophils. Finally, purified elastase and cathepsin G do not directly affect baseline endothelial PGI_{1α} release (32).

The failure of PMA-activated neutrophils from a patient with chronic granulomatous disease to induce endothelial PGI₂ release indicates that an oxygen radical is involved. Since catalase, but not superoxide dismutase, significantly reduces 6-keto-PGF_{1α} release from ECs incubated with PMA-activated neutrophils, it appears that hydrogen peroxide or a hydrogen peroxide-derived product is the critical neutrophil-generated mediator. This is supported by the observation that the hydrogen peroxide-generating system, glucose-glucose oxidase, can also induce catalase-inhibitable PGI₂ release from cultured ECs over the same time course. Catalase did not always completely inhibit neutrophil-mediated PGI₂ release, whereas it totally prevented glucose-glucose oxidase-mediated release; this may be due to exclusion of this enzyme at sites where neutrophils and ECs adhere closely.

Our observation that peroxide-stimulated prostaglandin generation by ECs is not associated with cellular lysis has also been recently reported by Ager and Gordon (33). These investigators found that porcine aortic endothelium synthesized PGI₂ and PGE₂ in response to enzymatically generated hydrogen peroxide at a concentration significantly lower than that which was lethal to the cells.

The results obtained by TLC confirm those obtained by RIA. The TLC results demonstrate that enzymatically or neutrophil-generated hydrogen peroxide induces the release of labeled arachidonic acid, 6-keto-PGF_{1α}, and PGE₂ from prelabeled endothelial cells. Hydrogen peroxide has been observed to induce PGE₂ release from fibroblasts, and an organic peroxide has been reported to induce PGI₂ release from ECs (34, 35). The inhibition of hydrogen peroxide-induced endothelial PGI₂ release by the phospholipase inhibitor mepacrine (23) suggests that hydrogen peroxide may act by triggering membrane phospholipase A₂, thus liberating arachidonic acid as a substrate for cyclooxygenase.

The conversion of the arachidonic acid released by the exogenously generated hydrogen peroxide to PGI₂ is a cyclooxygenase-catalyzed event, since it is inhibited by aspirin. The reported effects of peroxides on the cyclooxygenase-dependent conversion are variable. Peroxides have been observed to both activate (36, 37) and inactivate cyclooxygenase (38). Since hydroperoxides can inactivate endothelial PGI₂ synthetase (39), peroxides may also affect the subsequent conversion of the endoperoxides generated by cyclooxygenase. If cyclooxygenase is stimulated while PGI₂ synthetase is inhibited, enhanced synthesis of PGE₂ may occur (40).

The effects of exogenously generated peroxides on endothelial arachidonic acid metabolism may be critically dose dependent.

Taylor et al. noted that high concentrations of hydroperoxides inhibited 6-keto-PGF_{1α} production in bovine ECs and PGE₂ in fibroblasts, whereas low concentrations stimulated prostaglandin release (35). Hydrogen peroxide-induced endothelial prostaglandin production may thus be a net result of complex stimulatory and inhibitory effects on the various enzyme systems involved.

The observation that exogenously generated hydrogen peroxide can induce the release of PGI₂ from cultured ECs is of particular interest in acute inflammation where the endothelium may be exposed to hydrogen peroxide generated by inflammatory cells such as neutrophils and monocytes. The release of a potent vasodilator such as PGI₂ may augment local edema formation (2, 9–16). Alternatively, the release of PGI₂ could prevent the adherence and activation of additional inflammatory cells by elevating intracellular cyclic AMP (41–44). The demonstration that a toxic oxygen radical generated by one cell can trigger the release of arachidonic acid metabolites by another cell further illustrates the possibilities for interactions between inflammatory mediators.

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