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Research Article

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Cultured Endothelial Cells Synthesize Both Platelet-activating Factor and Prostacyclin in Response to Histamine, Bradykinin, and Adenosine Triphosphate

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Abstract

Cultured human endothelial cells synthesize prostacyclin (PGI_2), a potent inhibitor of platelet function, when stimulated with histamine, bradykinin, or ATP. Paradoxically, we report that these agonists also induced the rapid and sustained synthesis of platelet-activating factor (PAF) by endothelial cells. In fact, the synthesis of this potent activator of platelets and neutrophils was induced by stimulation of the same receptor subtype that induced PGI_2 synthesis: stimulation of a histamine H_1 or a bradykinin B_2 receptor induced both PAF and PGI_2 synthesis. However, two physiologically important differences exist between the production of PAF and PGI_2 by endothelial cells. The synthesis of PGI_2 proceeded for only 7.5 min before the abrupt termination of synthesis, whereas the synthesis of PAF was clearly detectable even 45 min after stimulation. Although maximal accumulation of PAF occurred after 10–15 min of stimulation, the prolonged synthesis resulted in the presence of PAF for up to 1 h after stimulation. Secondly, whereas PGI_2 was released from the cell monolayer, PAF remained cell-associated without significant release to the external medium. Endothelial cell-generated PAF, therefore, does not function as a hormone. The prolonged association of this potent activator of platelets and neutrophils with endothelial cells may mediate some of the inflammatory properties of histamine and bradykinin. It may also be a factor in the formation of a thrombogenic vascular surface, an event suggested to play a primary role in the pathogenesis of thrombosis and atherosclerosis.

Introduction

Vascular endothelium is composed of a single layer of endothelial cells that mediates the interaction of the blood with the rest of the organism. Among the functions demonstrated by endothelial cells is the presentation of a surface that does not activate the hemostatic system and thereby induce the formation of thrombi. This property is not shared by either the substratum or the smooth muscle cells and fibroblasts that underlie the endothelium. The difference in the endothelial cell surface that results in thromboresistance is not defined, but it is known that endothelium (1) and cultured endothelial cells (2) synthesize prostacyclin (PGI_2)[1] in response to a number of physiologic stimuli (3–7). Initially, the synthesis of this potent inhibitor of platelet aggregation and activation was postulated to participate in the maintenance of a nonthrombogenic surface (1). However, it is now apparent that endothelial cells secrete little, if any, PGI_2 in the absence of appropriate agonists, and inhibition of the cyclooxygenase activity with aspirin does not alter the adhesion of platelets to unstimulated endothelium (8). The role of PGI_2 is further obscured by the observation that stimulation of the endothelium with thrombin, a potent agonist for PGI_2 secretion, actually results in the loss of thromboresistance (8, 9).

The recent observation (10, 11) that endothelial cells make platelet-activating factor (PAF) in response to physiologic concentrations of thrombin may explain the generation of a thrombogenic surface in response to this agent. PAF, identified as 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (12), has the unusual property of being a potent agonist for the activation of both platelets and granulocytes (13), and treatment of endothelial cells with thrombin has been shown to increase the adhesion of granulocytes (10) and platelets (8) to the cell monolayer.

The synthesis of both PAF and PGI_2 in response to thrombin was unanticipated due to the potent, but opposing actions of these two agents on platelet and neutrophil function. Inasmuch as the nature of the endothelial cell recognition system for thrombin has not yet been explicitly defined, it is possible that thrombin induces two separate signals, and that the co-stimulation of PAF and PGI_2 synthesis is coincidental. In order to understand better the relationship between the synthesis of PAF and PGI_2, agonists that prompt endothelial cells to synthesize PGI_2 by a receptor-mediated mechanism were examined for their potential to also induce the synthesis of PAF. We found that agonists that promoted endothelial cell synthesis of PGI_2, namely, histamine (4), bradykinin (5), and ATP (7), induced the synthesis of PAF as well. The inflammatory mediators histamine and bradykinin have well characterized receptors, which has allowed us to demonstrate that the stimulation of a single receptor subtype, a histamine H_1 or a bradykinin B_2 receptor, resulted in the simultaneous induction of PAF and PGI_2 synthesis. Although the induction of synthesis of PAF and PGI_2 was tightly correlated, the synthesis and accumulation of PAF by endothelial cells extended well beyond

A preliminary report of portions of this work has been presented to the 57th Scientific Session of the American Heart Association at Miami Beach, FL, on 12 November 1984, and has been published as an abstract (1984. *Circulation.* 70[Suppl.]:A211).

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1. *Abbreviations used in this paper:* EDRF, endothelium-derived relaxing factor; HPLC, high-pressure liquid chromatography; PAF, platelet-activating factor; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGI_2, prostacyclin; TLC, thin-layer chromatography.

the abrupt end of PGI_2 synthesis. The endothelial cell-generated PAF was not released from the cells and remained associated with the cells even in the presence of physiological albumin concentrations. Because this PAF would not circulate in vivo, it is not likely to function as a hormone. Endothelial cell-associated PAF, however, may mediate some of the interactions of the endothelium with blood-borne cells. Such interactions may account for some of the effects of two of the classic mediators of inflammation, histamine and bradykinin, and also may play a role in vascular thrombogenesis.

Methods

1-O-[1′,2′-^{3}H]Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (45 Ci/mmol), sodium [^{3}H]acetate (2.8 Ci/mmol), and 6-[5,8,9,11,12,14,15-^{3}H(*N*)]-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were purchased from New England Nuclear (Boston, MA). Platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF), bovine serum albumin (essentially fatty acid-free), histamine, pyrilamine, cimetidine, bradykinin and its analogues were from Sigma Chemical Co. (St. Louis, MO). Sigma Chemical Co. also supplied adenosine, AMP, ADP, ATP, 2′-deoxy-ATP, inosine, inosine monophosphate, inosine diphosphate, and inosine triphosphate. The methylene and imido substituted analogues of ATP were from Pharmacia Fine Chemicals/P-L Biochemicals Inc. (Piscataway, NJ). Histamine analogs were the kind gift of Dr. Nancy L. Baenziger (Washington University, St. Louis), and purified human thrombin was generously provided by Dr. George J. Broze (Washington University, St. Louis).

Human endothelial cells were harvested from umbilical veins and grown to confluence in gelatin-coated 35-mm tissue culture dishes as previously described (10). Agonist-stimulated PAF accumulation was determined by using a slight modification of our previous protocol (10). The assay was initiated by replacing the growth medium with 1 ml of Hanks' buffered saline–10 mM *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4, containing 25 μCi of carrier-free [^{3}H]acetate. After incubation at room temperature for the stated time, the assay was terminated by the addition of 0.5 ml of 50 mM acetic acid in methanol. Labeling at this temperature resulted in only marginally less radioactivity incorporated into PAF than when the cells were labeled at 37° (13a). The cells remained viable and responsive in this serum-free buffer for 15 h, a period of time much longer than any incubation used in this study.

The material from all terminated assays was collected as follows: cells in acidified medium were scraped from the plate; the plate was washed twice with 1 ml of methanol; 7 nmol carrier PAF were added; and 1.25 ml of chloroform was added to the pooled fluid to form a monophase (14). The monophase was split with $CHCl_3$ and 0.1 M NaAc, and the lower phase was washed three times with preequilibrated upper phase. This was dried under N_2 and the lipids were resuspended in $CHCl_3$/MeOH (9:1). An aliquot of the recovered lipids was used to determine the total amount of radioactivity present, while the remainder was fractionated by thin-layer chromatography (TLC) that used silica gel 60 (Merck & Co., Darmstadt, Federal Republic of Germany) and solvent system II of Mueller et al. (15). Areas containing PAF, identified by H_2O misting, and the remainder of the lane were separately scraped into scintillation vials for quantitation. Duplicate assays show that the mean variation of PAF production by cultures derived from the same vein was ±5%. Chromatography in a second TLC system (16) showed that [^{3}H]acetate-labeled lipids elicited by histamine, bradykinin, or ATP migrated with authentic 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. Treatment of each of these radiolabeled lipids with *Crotalus adamanteus* phospholipase A_2 (10) showed that at least 90% of the radioactivity was incorporated into the *sn*-2 position. Treatment with *Rhizopus arrhizus* phospholipase A_1 showed that <10% of the radioactive PAF contained an *sn*-1 fatty acyl residue.

The partitioning of the newly synthesized PAF between the cell monolayer and the overlying fluid was examined using assay buffer or assay buffer supplemented with bovine serum albumin (essentially fatty acid-free). In these experiments, the assay was terminated by removing the assay medium and adding 50 mM acetic acid in 1.5 ml methanol/water (0.5:1) to the cell monolayer. The fluid removed from the plate was acidified with methanolic acetic acid and, excepting that the Bligh-Dyer monophase was centrifuged to remove precipitated albumin, these fractions were processed as before. Six separate experiments have shown that only a small percentage of the PAF is released from endothelial cells, with or without the addition of albumin to the assay buffer.

The rate of synthesis of PAF during agonist stimulation was estimated in two separate experiments by quantitating the amount of [^{3}H]PAF generated during a 3-min pulse of [^{3}H]acetate. The endothelial cell growth medium was exchanged for buffer containing an agonist, but no [^{3}H]acetate. At the specified time, this buffer was exchanged for 1 ml of buffer supplemented with 50 μCi of [^{3}H]acetate in addition to the agonist. The labeling was allowed to proceed for 3 min before the assay was terminated and processed as described above. The 3-min pulse protocol should reflect the rate of PAF synthesis as the degradation of exogenously added PAF was slow. The rate of hydrolysis of 1 nmol (in 1 ml) of [^{3}H-alkyl]PAF by endothelial cells was measured at 2-min intervals over a 10-min period and the accumulation of 1-[^{3}H]alkyl-*sn*-glycero-3-phosphocholine was linear with time and equal to 0.0028 nmol/min per (8×10^5 cells = one dish). Even with this amount of PAF, which was 5–10-fold higher than the maximal amount accumulated by 8×10^5 endothelial cells, the hydrolytic rate was 10% of the maximal rate of accumulation (10). The rate of PAF hydrolysis was not altered by agonist stimulation of the cells. The pulse-labeling protocol was also verified by pulse-labeling endothelial cells for various 3-min intervals over the first 12 min of stimulation and comparing the sum of this pulse-labeled PAF to the amount of PAF generated in the same experiment when measured by continuous [^{3}H]acetate labeling. The sum of the [^{3}H]PAF generated by the pulse-labeled plates was within 10% of the amount of [^{3}H]PAF accumulated by the continuously labeled endothelial cells, so pulse labeling accurately reflects the process which results in the accumulation of [^{3}H]acetate-labeled PAF.

The amount of PAF bioactivity present in high-pressure liquid chromatography (HPLC) fractions derived from two pooled agonist-stimulated or control monolayers was determined by monitoring the aggregation of isolated human polymorphonuclear leukocytes as described (10). These samples did not have carrier PAF added to the extraction step. The HPLC system employed a Varian 4.6 × 300-mm Micropak Si-5 column (Varian Associates, Inc., Palo Alto, CA) and the solvent system of Blank and Snyder (17).

The production of prostacyclin was determined by quantitation of the stable breakdown product, 6-keto-$PGF_{1\alpha}$, by a radioimmunoassay that used antibody generously provided again by Dr. Nancy Baenziger (Washington University, St. Louis, MO). Confluent cell monolayers were overlaid with 1 ml of buffer with or without agonist, and at the indicated times 100-μl aliquots were removed for 6-keto-$PGF_{1\alpha}$ quantitation. The volume of the assay was maintained by the addition of an equal volume of appropriate buffer, and the data presented have been corrected for the resulting dilution.

The effect of PAF on the tension of isolated canine femoral arterial rings was determined (18) by using a Grass Instrument Co. (Quincy, MA) FT-03 force transducer. The mounted ring was continuously perfused with Tyrodes solution equilibrated at 37°C with O_2/CO_2 (95:5) while the resting tension was stabilized at 2 *g*. Agents were tested by stopping this flow, adding the agent to the 10-ml organ bath that was continuously bubbled with the O_2/CO_2 mixture, and recording the strain gauge output with a strip-chart recorder. The effector compounds were removed by reinitiating buffer flow.

Results

Human endothelial cells make PAF in response to histamine. When human endothelial cells were stimulated with histamine,

they rapidly incorporated [^{3}H]acetate into a polar lipid that co-migrated in a HPLC system with authentic PAF. A standard of semisynthetic 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) eluted as a peak centered at 32–33 min (Fig. 1 *A*). The [^{3}H]acetate-labeled polar lipid from histamine-stimulated cells eluted as a peak 32 min after injection onto the column (Fig. 1 *B*). This material was not present in cells incubated in the absence of histamine, and was the only change in acetate-labeling after histamine stimulation. The lipid from histamine-stimulated cells that eluted from the HPLC column at 30 min possessed biologic activity characteristic of PAF. This material was able to induce the aggregation of cytochalasin B-treated human polymorphonuclear leukocytes (Fig. 1 *C*). Bioactive material was not present in the corresponding fractions from unstimulated endothelial cells.

The amount of PAF synthesized by endothelial cells was dependent upon the concentration of histamine employed as a stimulus (Fig. 2 *A*). A response was detectable at 10^{-7} M histamine and, in this experiment, was maximal at $\sim 5 \times 10^{-5}$ M. Although the response of cells isolated from a single umbilical vein was quite uniform, the concentration of histamine required for maximal stimulation varied up to fivefold among the cell isolates from different veins.

The addition of histamine to the cultured cells resulted in the immediate induction of cellular PAF synthesis (Fig. 2 *B*). Accumulation of PAF by endothelial cells increased with time during the first 10 min of histamine stimulation. Half of this accumulated PAF was then lost in the succeeding 5 min of incubation, followed by a slower rate of loss over the next 45 min. This pattern resulted in measurable amounts of cell-associated PAF for up to 1 h after the initiation of histamine stimulation.

The duration of the accumulation of PAF by endothelial cells in response to histamine was markedly different from the time course of histamine-elicited PGI_2 release from endothelial cells. The quantitation of 6-keto-$PGF_{1\alpha}$, the stable degradation product of PGI_2, by radioimmunoassay showed that unstimulated monolayers released little PGI_2 to the medium (Fig. 3). Histamine stimulation induced the linear accumulation of 6-keto-$PGF_{1\alpha}$ in the medium for 7.5 min before the abrupt termination of this accumulation. This pattern of 6-keto-$PGF_{1\alpha}$ accumulation was identical (Fig. 3) to that produced by thrombin, another agonist for PAF synthesis (10). The cessation of 6-keto-$PGF_{1\alpha}$ accumulation at 7.5 min shows that PGI_2 synthesis was terminated or sharply reduced by this time. Thus, whereas endothelial cells synthesize both PAF and PGI_2 in response to histamine and thrombin (10), the presence of endothelial cell PAF persisted well beyond the burst of PGI_2 synthesis and release from the cell monolayer.

Histamine stimulates both PAF and PGI_2 synthesis via an H_1 receptor. Stimulation of PGI_2 synthesis by human endothelial cells in response to histamine has been shown to be mediated through an H_1-type receptor (4). The data in Table I show that the histamine stimulation of PAF synthesis by endothelial cells is also mediated by an H_1-type receptor. The classic H_1 antagonist, pyrilamine, reduced the histamine response by ~95%; cimetidine, an H_2 antagonist, reduced this response by only 2%. In addition, PAF synthesis was induced by both of the H_1 receptor-specific agonists (19) tested. The magnitude of this stimulation was equivalent to or slightly greater than the histamine response itself. H_2-type agonists (19, 20) did not stimulate the synthesis of PAF by endothelial cells.

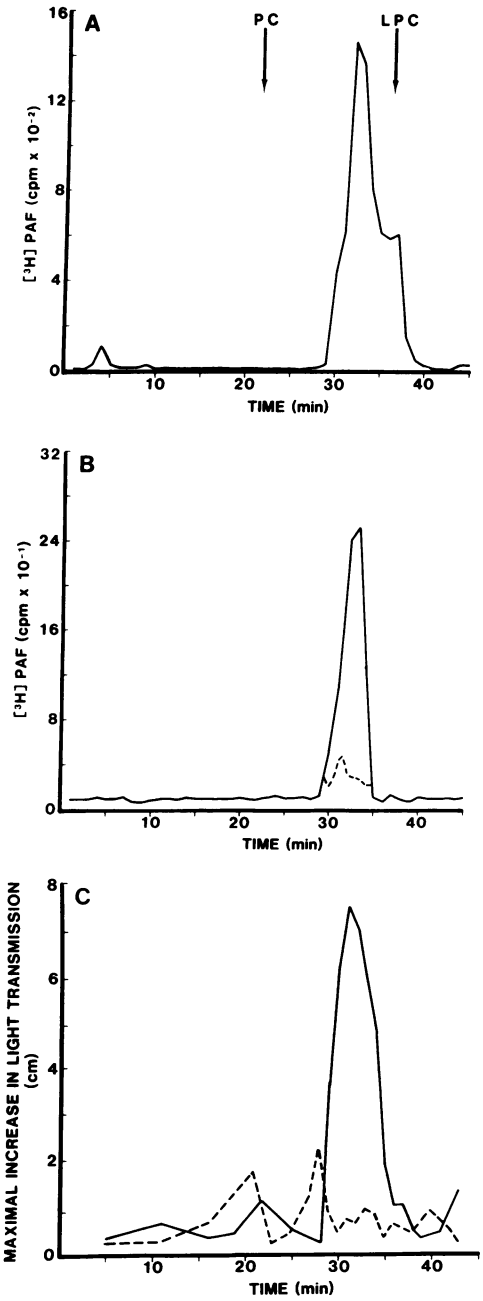

Figure 1. Histamine stimulates human endothelial cells to synthesize PAF. The medium overlaying confluent monolayers of human endothelial cells was exchanged for buffer with or without 10^{-5} M histamine. After 5 min at 24°C, the incubation was stopped with acidic methanol as described in Methods. The material to be tested for bioactivity was derived from two pooled culture plates and carrier PAF was not added to this extraction. Extracted lipids were injected onto a 4.6 × 300-mm Micropak Si-5 HPLC column and eluted at 1 ml/min as described (17). Fractions were collected at 0.5-min intervals and the radioactivity in the entire fraction was quantitated. Fractions from unlabeled cells were prepared for bioassay as described in Methods. This chromatogram is representative of the results obtained from three separate experiments. (*A*) 1-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine standard. The elution position of phosphatidylcholine (*PC*) and 1-O-alkyl-*sn*-glycero-3-phosphocholine (*LPC*) standards is shown by an arrow. (*B*) [^{3}H]Acetate-labeled lipid from histamine-stimulated endothelial cells (——); and unstimulated cells (- - -). (*C*) Granulocyte aggregation activity from histamine-stimulated endothelial cells (——); and unstimulated cells (- - -).

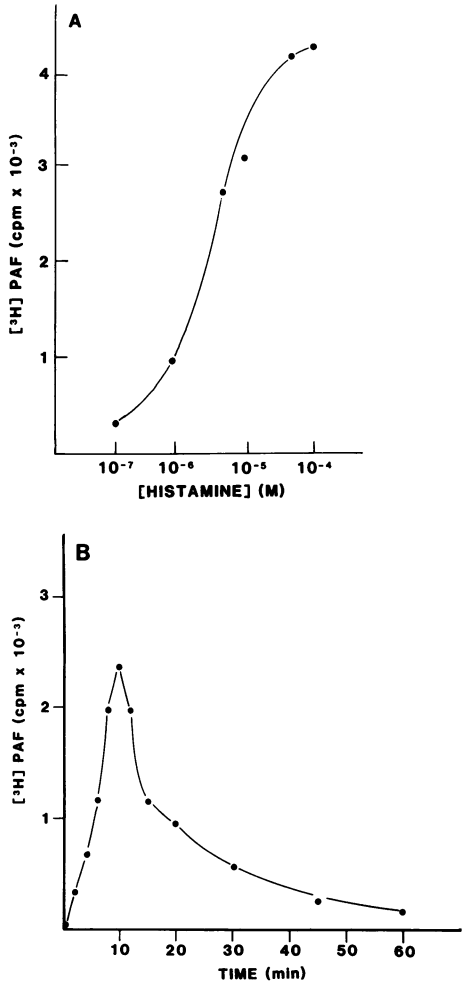

Figure 2. Accumulation of PAF by human endothelial cells as a function of histamine concentration or time. (*A*) The concentration of histamine employed to stimulate confluent monolayers of endothelial cells was varied. Cells were stimulated for 10 min in medium containing [^{3}H]acetate and the amount of radioactivity co-migrating with authentic PAF was determined as described in Methods. Five such experiments indicate that the response to histamine is concentration-dependent, but that the half-maximal concentration and the peak value can vary in different endothelial cell isolates. (*B*) Accumulation of PAF after stimulation with 10^{-5} M histamine for various periods of time was determined as described in Methods. These results are typical of four separate experiments.

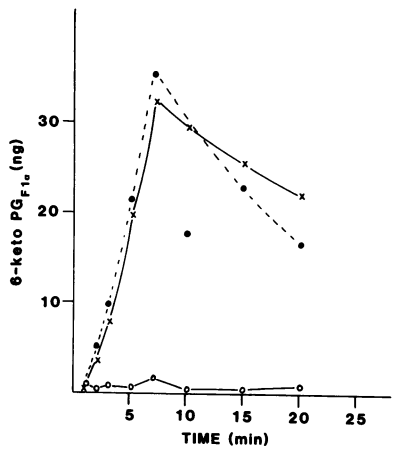

Figure 3. Effect of time of stimulation on PGI_2 production. Accumulation of the 6-keto-$PGF_{1\alpha}$ breakdown product of PGI_2 in the medium of histamine-stimulated or thrombin-stimulated cells. Aliquots of the assay medium were taken at the designated times and the amount of 6-keto-$PGF_{1\alpha}$ was determined by radioimmunoassay as described under Methods. The brief burst of 6-keto-$PGF_{1\alpha}$ release was confirmed in five additional experiments. The medium contained 10^{-5} M histamine (●); 2 U/ml human thrombin (×); or no additions (○).

Bradykinin stimulates endothelial cell PAF synthesis. Human endothelial cells, and those derived from other species, have been shown to respond to bradykinin by synthesizing PGI_2 (5). To determine whether or not PAF synthesis would also be initiated in response to this PGI_2 agonist, confluent monolayers of human endothelial cells were exposed to Met-Lys-bradykinin, a natural analogue of bradykinin that effectively stimulates both B_1 and B_2 receptors (21). The resulting data (Fig. 4) show that Met-Lys-bradykinin does indeed stimulate the synthesis of PAF in a time- and concentration-dependent manner. Synthesis and accumulation of PAF began immediately upon Met-Lys-bradykinin addition and the accumulation of PAF reached its maximum value 8–10 min later. The amount of PAF then gradually declined throughout the remainder of the experiment. This pattern resulted in the persistence of at least half of the cellular PAF even 30 min after the addition of Met-Lys-bradykinin to the cells. The inset shows that maximal stimulation was achieved with $\sim 10^{-6}$ M Met-Lys-bradykinin.

The presence of PAF at times well after Met-Lys-bradykinin stimulation of the cultured cells implied that the synthesis of PAF might be a protracted process. Therefore, the rate of synthesis of PAF was approximated by adding [^{3}H]acetate for a 3-min labeling period at various times after stimulation in medium initially lacking labeled acetate. The rate of synthesis was found to be greatest during the initial 3 min of stimulation, and then decreased with time (Fig. 5). However, 20 min after Met-Lys-bradykinin addition, the rate of synthesis was still over one-third of the initial value, and even 45 min after stimulation, the rate of PAF synthesis was three times the background rate.

Stimulation of bradykinin B_2 receptors induces both PAF and PGI_2 synthesis. The stimulation of both PAF and PGI_2 synthesis by activation of an H_1 receptor raised the possibility that a single type of bradykinin receptor might also be responsible for the stimulation of both PAF and PGI_2 synthesis. The data shown in Table II indicates that the induction of PAF and PGI_2 synthesis results from the stimulation of a B_2-type receptor: the order of potency (bradykinin ≧ Lys-bradykinin ≧ Met-Lys-bradykinin) was that expected of a B_2-type receptor (21); the amount of PAF resulting from a 5-min stimulation with bradykinin or Met-Lys-bradykinin (a potent B_1- and B_2-receptor agonist) was not affected by a selective (21) B_1-receptor antagonist (des-Arg^9-[Leu^8]bradykinin); and a selective B_1-receptor agonist (des-Arg^9-bradykinin) did not stimulate PAF synthesis by endothelial cells.

Examination of PGI_2 production in parallel with PAF determinations gave the same results: the order of potency was that expected for a B_2 receptor; a B_1-receptor antagonist was unable to inhibit stimulation; and a B_1-receptor agonist was an ineffective stimulus. These data indicate that PGI_2 synthesis

Table I. Histamine Stimulates Endothelial Cell PAF Synthesis via an H_1-type Receptor

Agonist	Receptor type	Antagonist	Receptor type	PAF	Agonist	Receptor type	PAF
				%			%
Histamine	H_1, H_2	—	—	100	Histamine	H_1, H_2	100
		Pyrilamine	H_1	4.6	2-(2-Pyridyl)ethylamine	H_1	131
		Cimetidine	H_2	98.6	2-Methylhistamine	H_1	117
		Pyrilamine	H_1		4-Methylhistamine	H_2	0
		+		7.8	Dimaprit	H_2	0
		Cimetidine	H_2		Impromidine	H_2	0

Confluent monolayers of human endothelial cells from different donors in two separate experiments were stimulated for 5 min with 10^{-5} M histamine in the presence or absence of receptor-selective antagonists, also at 10^{-5} M. Alternatively, cell monolayers were stimulated for 5 min with 10^{-5} M receptor-selective agonists, except that impromidine was present at 10^{-6} M. PAF was quantitated as described in Methods and is presented as percent of the amount of PAF elicited by histamine.

by human endothelial cells is also a consequence of the stimulation of a bradykinin B_2 receptor.

ATP stimulates endothelial cell PAF and PGI_2 synthesis. The induction of both PAF and PGI_2 synthesis by three endothelial cell agonists suggested that this coupled synthesis might be of a general nature. Examination of a number of biologic compounds (13a) revealed that exogenous ATP was able to stimulate endothelial cells to synthesize PAF (Table III). This stimulation of PAF synthesis was time-dependent (Fig. 6), with the maximal accumulation occurring ~15 min after ATP addition to the cells. The subsequent loss of the accumulated PAF proceeded linearly with the result that a little over 25% of the total PAF remained 30 min after ATP stimulation. The stimulation of PAF synthesis was detectable at 1 μM ATP, but there was little increased stimulation with ATP concentration until 300 μM ATP (not shown). This probably resulted from the rapid hydrolysis of extracellular

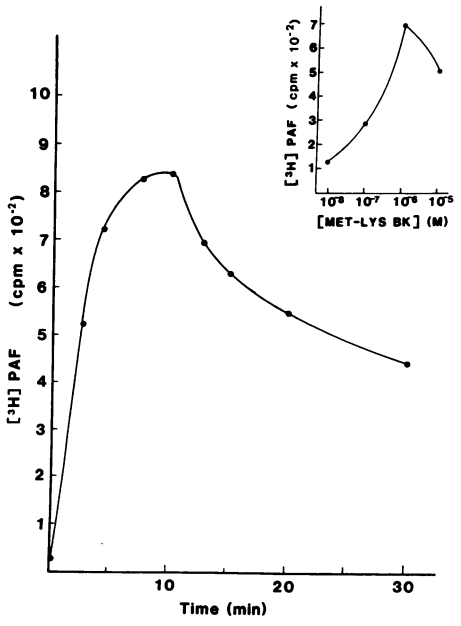

Figure 4. Met-Lys-bradykinin stimulation of PAF accumulation by human endothelial cells is time-dependent and concentration-dependent. Endothelial cells were stimulated with 10^{-6} M Met-Lys-bradykinin in the presence of [^{3}H]acetate for the designated periods of time and the amount of labeled PAF produced was quantitated as described in Methods. (*Inset*) The effect of varied concentrations of Met-Lys-bradykinin on the amount of PAF produced during a 10-min stimulation. The data from this experiment are identical to those obtained in another experiment using cultures derived from a different donor.

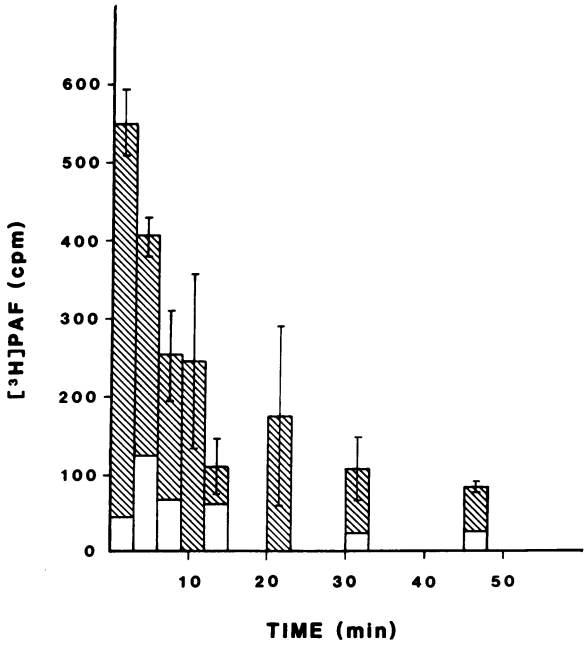

Figure 5. Synthesis of PAF by human endothelial cells as a function of time of Met-Lys-bradykinin stimulation. Confluent monolayers of endothelial cells were stimulated with 10^{-6} M Met-Lys-bradykinin in buffer lacking [^{3}H]acetate (time 0). At the designated time, the unlabeled medium was exchanged for 1 ml of buffer containing 50 μCi of [^{3}H]acetate in addition to 10^{-6} M Met-Lys-bradykinin. The cells were incubated for 3 min before the assay was terminated with acidic methanol, and the amount of radiolabeled PAF synthesized during the 3-min period was quantitated as described in Methods. The amount of [^{3}H]PAF produced by this protocol in the absence of Met-Lys-bradykinin is shown by the open bars. These data are the result of two separate experiments using cells derived from different donors.

Table II. Bradykinin Stimulates Endothelial Cell PAF and PGI_2 Synthesis via a B_2-type Receptor

Addition	[^{3}H]PAF	PGI_2
	cpm/dish	*ng of 6-keto-$PGF_{1\alpha}$/dish*
Bradykinin	399	5.0
+ des-Arg^9-[leu^8]Bradykinin	371	7.3
Lys-Bradykinin	337	5.6
Met-Lys-Bradykinin	293	6.8
+ des-Arg^9-[leu^8]Bradykinin	401	6.0
des-Arg^9-Bradykinin	108	2.3
None	80	2.6

Confluent monolayers of human endothelial cells were stimulated with the designated bradykinin homologues at 10^{-6} M in the presence or absence of the B_1 receptor-specific antagonist des-Arg^9-[leu^8]bradykinin, also present at 1 μM. The amount of PAF generated in 5 min or the amount of prostacyclin (measured as 6-keto-$PGF_{1\alpha}$) released in 10 min was determined as described in Methods. These results showing the presence of bradykinin B_2-receptors on human endothelial cells are supported by the results of three other separate experiments.

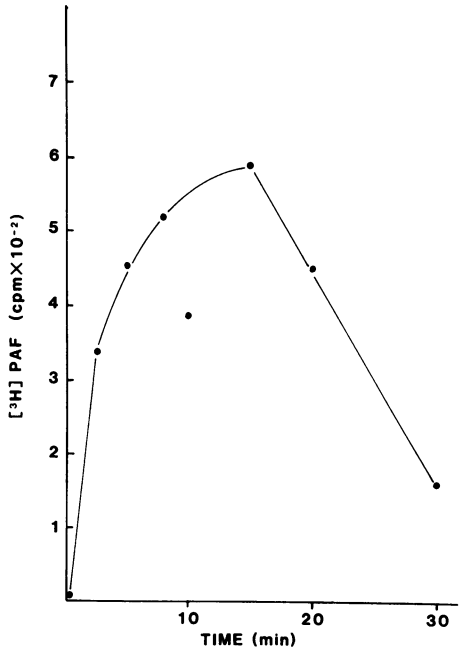

Figure 6. Adenosine triphosphate stimulation of PAF synthesis is time-dependent. Confluent monolayers of human endothelial cells were stimulated with 10^{-3} M ATP in the presence of [^{3}H]acetate, and the amount of PAF generated was determined as described in Methods. A second experiment with endothelial cells from a different donor gave similar results.

ATP by cultured endothelial cells (22). However, the presence of the hydrolysis products ADP, AMP, or adenosine was not responsible for ATP bioactivity as these compounds were unable to induce PAF synthesis (Table III). In addition, inosine and its phosphorylated derivatives, possible metabolites of ATP, were not able to stimulate PAF production (not shown). Deoxy-ATP was ~80% as effective as ATP, but the nonhydrolyzable ATP analogues containing an α,β-methylene or β,γ-methylene bridge were not agonists for PAF production. The slowly hydrolyzable β,γ-imido ATP derivative demonstrated ~15% of the activity of ATP.

Exogenous ATP also stimulated human umbilical vein endothelial cells to release PGI_2 (Table III). This is consistent with the previous demonstration (7) that porcine aortic endothelial cells synthesize small amounts of PGI_2 in response to extracellular ATP. The release of PGI_2 by human cells was time-dependent (not shown) and amounted to about twice the amount induced by exchanging growth medium for assay buffer ("control"). Other phosphorylated adenosine derivatives, the β,γ-methylene-ATP analogue, or adenosine itself did not induce PGI_2 synthesis. Thus, ATP induced the synthesis of both PAF and PGI_2 by stimulation of what appeared to be a common, or very similar, mechanism.

Table III. ATP Stimulates PAF and PGI_2 Synthesis by Endothelial Cells

Addition	[^{3}H]PAF	PGI_2
	cpm/dish	*ng of 6-keto-$PGF_{1\alpha}$/dish*
ATP	594	4.8
ADP	120	3.2
AMP	104	—
Adenosine	63	—
None	80	2.5

Endothelial cells were stimulated with 10^{-3} M ATP or other adenoside derivatives at 10^{-3} M, and the amount of PGI_2, as 6-keto-$PGF_{1\alpha}$, present after 7 min or the amount of PAF present after 15 min, was determined as described in Methods. The results are typical of three independent experiments.

PAF remains cell-associated. We have previously observed that the PAF elicited upon thrombin stimulation of endothelial cells was not released into the medium, but instead remained associated with the cell monolayer (10). Fig. 7 shows that the PAF synthesized in response to histamine, Met-Lys-bradykinin or ATP was also localized to the endothelial cells. Separation of the assay medium from the cell monolayer showed that 99% of the radiolabeled PAF synthesized in response to any agonist was associated with the cells, and that release of PAF was increased to no more than 2% of the total [^{3}H]PAF even when physiologic concentrations of albumin were included in the assay buffer. For comparison, the relative amount of PAF excreted from thrombin-stimulated cells in this experiment in the presence of 50 mg/ml bovine serum albumin (BSA) was 0.6%. Such data show that endothelial cell-generated PAF is not likely to be secreted from the endothelium and this PAF cannot function as a hormone.

PAF is not an endothelium-derived relaxing factor. The agonists thrombin, histamine, bradykinin, and ATP have been shown to induce endothelium to elaborate an unidentified vasodilator (18, 23), termed endothelial-derived relaxing factor, or EDRF, that mediates the vasodilation induced by these agonists. In that PAF is a potent hypotensive agent (24), and is made by endothelial cells in response to the same agonists that induce EDRF (23), it was of interest to determine if PAF

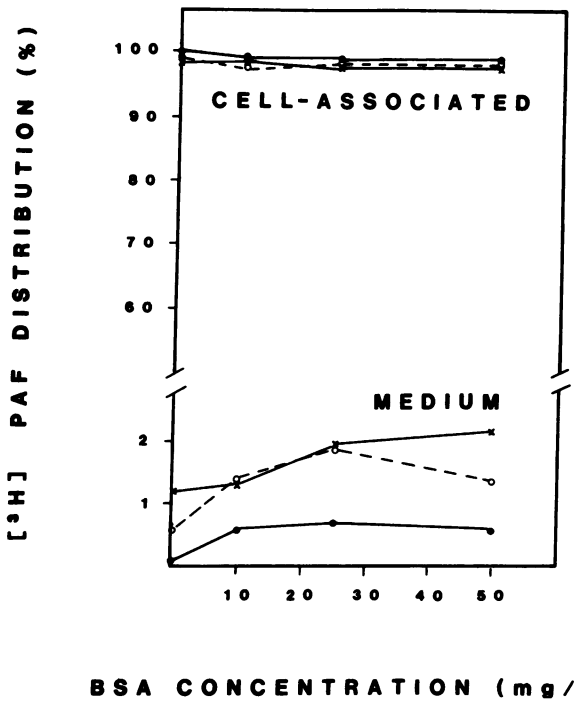

Figure 7. PAF remains associated with endothelial cells. The localization of histamine-, bradykinin-, or ATP-elicited PAF was examined in the absence or in the presence of increasing concentrations of essentially fatty acid-free BSA in the assay medium. After a 10-min stimulation, the medium was removed, the cell monolayer and its supernatant were acidified with HOAc in methanol, and these fractions were separately processed as described in Methods. The data is presented as the percentage of total counts associated with the fraction. The total amount of [^{3}H]PAF generated in BSA-free assay medium was: thrombin, 11,430 cpm; histamine (×), 3,515 cpm; Met-Lys-bradykinin (•), 407 cpm; ATP (○), 1,844 cpm. The association of PAF with the cell monolayer, rather than release of the newly synthesized PAF into the bathing medium, has been observed in six separate experiments.

is an EDRF. The assay for EDRF activity employed an ex vivo arterial ring preparation, derived from a canine femoral artery, equilibrated at a stable resting tension of 2 *g*. Addition of norepinephrine to the ring (Fig. 8) resulted in a strong contraction, indicating that the arterial smooth muscle was viable. The addition of acetylcholine to the contracted ring caused a relaxation to 60% of the maximal tension. Reinitiation of the flow of perfusate removed these effectors and allowed the ring to return to its resting tension. The addition of PAF to this relaxed ring did not induce a contractile response, nor did it interfere with the subsequent norepinephrine-induced contraction. The ability of the ring to contract had not been altered by the previous manipulations inasmuch as the tension generated during the second contraction was identical to that of the initial response. The addition of PAF to the contracted arterial ring did not induce the relaxation of this tissue. The small increase in tension upon the addition of PAF also occurred when the washout of the preparation was initiated. From these results, we conclude that PAF does not directly affect arterial vascular tone and is not an EDRF.

Discussion

Human endothelial cells in culture are stimulated by thrombin, histamine, bradykinin, and extracellular ATP. These compounds are vasoactive agonists that mediate reactions in the coagulation pathway and in the acute inflammatory response. The endothelial cell response to these agonists previously has been characterized as the induction of PGI_2 synthesis (3–7). We now show that these same agonists also simultaneously induce the synthesis of PAF. Further, where more than one type of receptor is known to exist, as for histamine and bradykinin, the induction of PAF and PGI_2 synthesis occurred in response to activation of the same receptor subtype. That PAF is also synthesized in response to these PGI_2 agonists is paradoxical in that PAF is an equally potent lipid autocoid that opposes the inhibitory actions of PGI_2 on platelets and

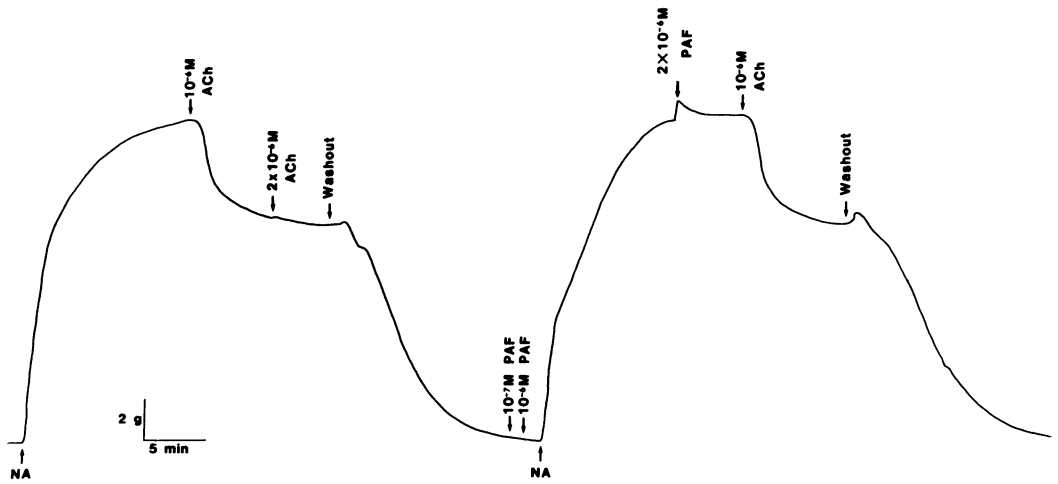

Figure 8. Effect of PAF on the tension of an ex vivo arterial preparation. A 5-mm canine femoral arterial ring was mounted between an immobilized hook in an organ bath and a hook attached to a strain gauge. The ring was perfused and a continuous record of tension was obtained as described in Methods. Similar results were obtained with arterial preparations from two other dogs, and when the agents were added in different sequences. Abbreviations are: *NA*, 10^{-6} M noradrenaline; *ACh*, acetylcholine; *PAF*, platelet-activating factor; WASHOUT, reinitiation of buffer flow.

neutrophils. Although agonist stimulation results in the co-induction of PAF and PGI_2 synthesis, we have shown here that temporal and spatial differences exist in the elaboration of PAF and PGI_2 by endothelial cells: the production of PAF is a protracted process in contrast to the brief period of PGI_2 synthesis; and endothelial cell-generated PAF remains associated with the cells rather than being released into the medium like PGI_2.

The [^{3}H]acetate-labeled lipid synthesized in response to histamine, bradykinin, exogenous ATP, and thrombin has been shown to be PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by (*a*) chromatography in two TLC systems, (*b*) elution with authentic PAF in the HPLC system of Blank and Snyder (17), (*c*) loss of acetate label and biologic activity by phospholipase A_2 treatment or saponification, (*d*) resistance to phospholipase A_1, (*e*) incorporation of label from 1-[^{3}H]alkyl-*sn*-glycero-3-phosphocholine (10), and (*f*) the ability to aggregate both platelets and neutrophils, an unusual property of PAF. The amount of PAF produced by endothelial cells is large enough to suggest that it is biologically significant as 10^6 cells synthesized 0.1–0.2 nmol within 10 min when stimulated with thrombin (10). If this PAF was released, this would be sufficient to produce up to 1 liter of bioactive fluid because the stimulation of platelet and neutrophil function (10, 25) can be detected at PAF concentrations as low as 10^{-10} M. The retention of PAF by endothelial cells may serve to further concentrate this mediator and potentiate its effect. The data in Table IV show that, although histamine, bradykinin, and ATP were less potent than thrombin, these agonists still cause a substantial, and presumably physiologically significant, accumulation of PAF. The amount of PAF and PGI_2 synthesized by endothelial cells was variable, which was a reflection of the variation among primary cultures derived from different umbilical veins. Duplicate assays using cells from the same source were within 5% of the mean value. Such variability of primary cultures of human endothelial cells has also been observed for histamine stimulation of prostacyclin production (Baenziger, N. L., personal communication).

Accumulation of PAF as a result of agonist stimulation of endothelial cell receptors was an immediate response. This accumulation proceeded linearly during the initial 5 min of stimulation and reached its maximal value in 10–15 min. Although the subsequent decline proceeded at a variable rate, the rate was sufficiently slow so that the amount of PAF remaining was significantly above background even 30–60 min after the addition of agonist. At least part of the persistence of endothelial cell PAF reflected the continued synthesis of PAF throughout this period. The apparent synthetic rate was greatest during the initial 3 min, with a subsequent progressive decline in this rate. However, 30 min after stimulation, synthesis was still estimated to be 25% of the initial rate, and the synthesis of PAF was clearly detectable even 45 min after agonist stimulation. In contrast to the prolonged period of PAF synthesis, the period of active PGI_2 synthesis was short-lived; the burst of PGI_2 synthesis terminated in less than 7.5 min after its initiation. This ephemeral synthesis of PGI_2 has been consistently observed under a variety of experimental protocols (3, 4, 6, 7) and appears to result from the self-inactivation of prostaglandin synthetase (26, 27).

Table IV. Agonist-induced PAF and PGI_2 Production by Primary Endothelial Cell Cultures

Agonist	PAF		PGI_2	
	cpm/10^6 cells	*(n)*	*ng/10^6 cells*	*(n)*
Thrombin	13,169±6,419	(6)	33.9±11.6	(4)
Histamine	4,194±2,211	(6)	16.8±13.2	(4)
Bradykinin	950±69	(3)	5±1.1	(4)
ATP	979±461	(4)	2.2±0.7	(7)

Confluent monolayers of endothelial cells derived from various umbilical veins were stimulated with an agonist for 10 min and the amount of PAF or PGI_2 generated was quantitated as described in Methods. The background in each experiment was subtracted before calculation of the mean of the stated number of experiments.

Endothelial cells synthesized both PAF and PGI_2 in response to exogenous ATP. The specificity demonstrated by endothelial cells for exogenous ATP, or deoxy-ATP, to induce the synthesis of PAF and PGI_2 suggests that a single type of receptor may mediate the activation of both synthetic pathways. The human endothelial cell ATP receptor does not display the accepted specificity of a P_1-purinergic receptor (28), nor does the human umbilical endothelial cell display the P_2-purinergic receptor responsible for the ATP-, ADP-, or β,γ-imido-ATP-dependent release of PGI_2 from cultured porcine or bovine aortic endothelial cells (7, 29). The synthesis of both PAF and PGI_2 by human endothelial cells most closely resembles the ATP- or deoxy-ATP-specific release of histamine from rat mast cells (30). Stimulation of ATP receptors by ATP may occur in response to two sources of extracellular ATP: secretion of the contents of platelet-dense granules releases nearly as much ATP as ADP; and thrombin stimulation of human endothelial cells causes the selective release of a majority of the ATP pool of the cell (22). The presence of extracellular ATP in vivo is also suggested by an endothelial cell ecto-ATP hydrolase activity (22). The rapid hydrolysis of extracellular ATP by this activity may serve to limit the stimulation of PAF and PGI_2 by secreted ATP.

We have employed agonists and antagonists specific for histamine or bradykinin receptor subtypes to show that the induction of both PAF and PGI_2 synthesis proceeds from the stimulation of a single receptor subtype. Specifically, this was a histamine H_1 or a bradykinin B_2 receptor. The inflammatory response induced by agents such as bradykinin and histamine may depend upon this concerted synthesis of PAF and PGI_2. The actions of histamine previously have been characterized as the direct inhibition of polymorphonuclear neutrophil (PMN) function (31), as well as the synthesis by endothelium of the polymorphonuclear leukocyte inhibitor, PGI_2 (4). PAF is the first activator of inflammatory cell types found to be synthesized in response to histamine and bradykinin. Thus, this endothelial cell-associated PAF may recruit inflammatory cell types to the site of inflammation and then activate them. PGI_2, which temporarily may antagonize the effect of PAF on blood-borne cells, may aid this PAF-mediated cellular recruitment through capillary bed vasodilation and increased blood flow. There is as yet no evidence that PAF can directly participate in this vasodilation. Although PAF will cause profound hypotension upon injection into a test animal (24, 32), and the same agonists that induce endothelial cell PAF synthesis also induce the synthesis of EDRF (23), PAF was shown to have no effect on the vascular smooth muscle tension of a major vessel. The

stimulation of PAF and PGI_2 synthesis by histamine and bradykinin shows that endothelial cells are an active participant in the process of acute inflammation, a role of endothelium originally postulated by Metchnikoff (33).

Endothelium normally presents a nonthrombotic surface to the lumen of the vessel, yet under some circumstances it does become thrombogenic. The demonstration that human endothelial cells synthesize and retain PAF in response to physiologic agonists may be a mechanism by which this alteration is effected. The association of PAF with endothelial cells was not altered by the presence of physiologic concentrations of albumin, which is capable of sequestering PAF (34). Although this PAF was not readily extractable, phospholipase digestion indicates that at least a portion of this PAF is present at the cell surface (Prescott, S. M., G. A. Zimmerman, and T. M. McIntyre, unpublished observations). Alteration of the surface of the endothelium and the enhanced binding and activation of neutrophils and platelets would have several sequelae: thrombus formation; damage of the endothelium by degradative enzymes and activated oxygen species; access of blood-borne elements, including lipoproteins, to the subendothelial space; and the release of platelet-derived growth factor, a potent smooth muscle and fibroblast mitogen. Several of these potential consequences have been hypothesized to be pathogenic events in the formation of an atherosclerotic plaque (35) and in other forms of vascular injury (36). The co-induction of PGI_2 synthesis might temporarily counteract these events, due to its short-lived synthesis, but also may potentiate these cell-mediated sequelae through vasodilation-induced cell recruitment. The production of PAF by endothelium may also function as a homeostatic process. Platelets have been shown to possess an endothelial cell growth factor (37), and it is known that in some manner platelets are required for the maintenance of vascular integrity (38, 39). In addition, PAF has recently been shown to induce angiogenesis in the chick chorio-allantoic membrane (40). It is apparent that through the induction of PAF synthesis and accumulation by endothelial cells, and the co-induction of PGI_2 synthesis, that the endothelium can be an active participant in inflammatory, homeostatic, and pathologic processes.

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