Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when stimulated with thrombin

(endothelial cell–platelet interaction/atherogenesis/thrombosis)

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ABSTRACT Cultured human endothelial cells produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) when stimulated with human thrombin. The response to thrombin is dose dependent, with a half-maximal effect at 0.17 unit/ml. The product is identified as PAF by the incorporation of radiolabeled precursors, its behavior in chromatographic systems, the recovery of biological activity, and the effect of treatment with phospholipase A2. Incorporation of [3H]acetate into PAF is maximal by 5 min and decreases thereafter. Endothelial cells produce 0.10–0.17 nmol of PAF per 10^6 cells in a 5-min exposure to thrombin, as judged by the amount of neutrophil-aggregating activity. The production of this potent agonist for platelet activation and neutrophil chemotaxis by endothelial cells suggests that it may play a role in the maintenance of vascular integrity and perhaps in pathological events such as thrombosis and atherogenesis.

The interactions of endothelium with cellular and humoral components of the blood are complex, but one important role of the endothelium is the presentation of a nonthrombogenic surface under most circumstances (1, 2). One mechanism by which endothelial cells accomplish this is the production of prostacyclin (3, 4).

Conversely, under some conditions the endothelium loses its nonthrombogenic properties, so that platelets and neutrophilic polymorphonuclear leukocytes (PMNs) adhere to it. This may have a homeostatic role since platelets are required for the maintenance of vascular integrity (5, 6). However, platelet and PMN adhesion and release of bioactive materials have been associated with pathological events, including thrombosis and atherogenesis (2, 7). The mechanism for the loss of the nonthrombogenic properties of endothelium is unknown, but presumably the loss could result from the production of a thrombogenic substance by endothelium.

Platelet-activating factor (PAF) is a potent stimulus for platelet aggregation and neutrophil aggregation and chemotaxis (8). The chemical identity of PAF has been shown to be 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine by Demopoulos and co-workers (9, 10), and its biosynthesis (11–13), degradation (14, 15), and effects on other cells and tissues (16–20) have been extensively investigated recently. We have examined cultured human endothelial cells for production of PAF since such a response could explain the increased interaction of endothelium with platelets and neutrophils under some physiological and pathological circumstances. We demonstrate here that endothelial cells produce PAF when stimulated with thrombin, an agent that is known to increase platelet adhesion to endothelium (21).

MATERIALS AND METHODS

Materials. 1-O-[1',2',3'H]alkyl-2-acetyl-sn-glycero-3-phosphocholine ([3H]PAF; 45 Ci/mmol; 1 Ci = 37 GBq) and the sodium salt of [3H]acetate acid (2.8 Ci/mmol) were purchased from New England Nuclear. Unlabeled PAF and other lipid standards were purchased from Avanti. Purified human thrombin (2920 units/mg of protein) and antithrombin III were generously provided by George Broze (Washington University, St. Louis, MO).

Culture of Endothelial Cells. We used a modification of the methods of Jaffe et al. (22). The cells were cultured in medium 199 containing 25 mM Hepes buffer, supplemented with L-glutamine (2.0 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 20% pooled human serum. The cultures, on 35-mm gelatin-coated plates, reached confluency in 5 days, at which time the plates were completely covered by uniform, contact-inhibited monolayers of flattened, closely apposed endothelial cells. Under these conditions there were 7.6 × 10^5 endothelial cells per plate (9.4 × 10^5 cells per cm² of surface area). Contamination by smooth muscle cells, fibroblasts, or other cells was estimated by microscopy to be less than 0.2% in all cultures used. The identity of endothelial cells grown in this fashion was verified by morphology and demonstration of factor VIII antigen by indirect immunofluorescence. Endothelial isolates from six veins were used for these experiments.

Assay of [3H]Acetate Incorporation into PAF. This assay was performed as described by Mueller et al. (13). The medium was removed from confluent monolayers of endothelial cells and replaced with 2 ml of Hanks' balanced salt solution (HBSS) that contained 50 μCi of [3H]acetate and the appropriate concentration of thrombin (or other agonist). The incubation was performed for various times, at 25°C, and stopped by scraping the cells from the plate and quickly transferring them and the supernatant buffer to extraction solvent (23) that also contained 50 μg of unlabeled PAF. The entire extracted sample was fractionated by thin-layer chromatography (TLC) on precoated plates of silica gel 60 (Merck, Darmstadt, F.R.G.) in solvent system I of Mueller et al. (13). After drying, the silica was scraped in increments into vials and the radioactivity was estimated by scintillation spectrometry.

Characterization of the [3H]Acetate-Containing Polar Lipid. The extracts from several plates in each of two experiments (different isolates) were chromatographed on TLC plates as above. The area containing authentic PAF was scraped into a centrifuge tube and the lipids were eluted with

Abbreviations: PAF, platelet-activating factor; [3H]PAF, 1-O-[1',2',3'H]alkyl-2-acetyl-sn-glycero-3-phosphocholine; PMN, neutrophilic polymorphonuclear leukocyte; Gro/Cho, sn-glycero-3-phosphocholine.
this extract was taken for analysis by high-performance liquid chromatography (HPLC) with a procedure based on that of Blank and Snyder (24) (Fig. 4).

Another portion was dissolved in 1.0 ml of diethyl ether and treated with phospholipase A₂ from Crotalus adamanteus as described (25). Control incubations received no enzyme. The extracts of these incubations were analyzed by TLC as above.

Metabolism of 1-[³H]Alkyl-sn-glycerol-3-phosphocholine (1-³H)Alkyl-GroPCho. 1-[³H]Alkyl-GroPCho was generated from [³H]PAF by saponification in 0.12 M NaOH in CHCl₃/MeOH (1:4, vol/vol) for 10 min at 37°C and was purified by TLC (above). Endothelial cells were labeled with 1.5 μCi of 1-[³H]Alkyl-GroPCho (dissolved in 0.5 ml of medium) for 4 hr at 37°C. The labeling medium was removed and replaced with regular culture medium containing either no additions or human thrombin (2 units/ml). After 5 min the reaction was terminated, and the cells were scraped from the plate and extracted in solvent that contained 10 nmol each of 1-alkyl-GroPCho and PAF. A portion of the lipid extract was taken for direct scintillation spectrometry and the remainder was applied to a TLC plate for separation and quantitation as described above. In these experiments, 1-alkyl-GroPCho and PAF were clearly separated (R₁ = 0.13 and 0.21, respectively) and in the labeling experiments an intermediate area did not contain radioactivity above background.

Recovery of Biological Activity. Six dishes of endothelial cells were stimulated for 5 min with thrombin at 2 units/ml and extracted as described above (without carrier PAF). The extract was applied to the HPLC system and 0.5-ml fractions of the effluent were collected. The fractions were dried under nitrogen, redissolved in 75 μl of 0.15 M NaCl that contained fatty acid-free bovine serum albumin at 1 mg/ml, and tested as described below. After the separation of the unknown sample, 5 nmol of unlabeled authentic PAF was applied to the HPLC and the resultant fractions were handled in the same way as the unknown except that each was redissolved in 0.5 ml.

To estimate the quantity of PAF from endothelial cells, portions from each fraction found to contain bioactivity were combined and dilutions of the resultant solution were assayed for PAF-accumulating activity. A portion of the combined product was treated with phospholipase A₂ (as above) and compared with another portion that had been treated with buffer for biological activity.

Granulocyte and Platelet Aggregation. The method of Craddock et al. (26) was used to examine purified products of stimulated endothelial cells for PMN-aggregating activity. Isolated human PMNs, 0.55 × 10⁷ cells/ml in HBSS with human albumin at 5 mg/ml, were treated with cytochalasin B (5 μg/ml) for 15 min at 37°C. Aggregation was performed by stirring (900 rpm) 0.45 ml of the PMN solution in a Payton (Buffalo) model 300 BD aggregometer at 37°C for 2 min and then adding 50 μl of the solution to be tested. Light transmission (%) was monitored and recorded on a strip chart continuously for 3–5 min. The maximal ΔΤ, excluding the dilution artifact, induced by each sample was measured. Under the conditions of these experiments, the maximal ΔΤ is proportional to the area under the aggregation curve. Human platelets were prepared and their aggregation (27) and serotonin release (28) were measured as described.

PMN Adherence to Endothelial Cells. Duplicate endothelial cell monolayers were incubated with thrombin (Sigma) at 0.5 units/ml for 2 min at 37°C. The supernatant solutions were removed and replaced with suspensions of isolated PMNs (5.5 × 10⁶/ml). Adherence of PMNs was measured by removing the supernatant, after 15 min, and determining the number of PMNs remaining in suspension (29). Microscopic studies have confirmed that the decrease in number of PMNs can be accounted for by those adherent to the monolayer.

RESULTS

Cultured Human Endothelial Cells Incorporate [³H]Acetate into a Polar Lipid When Stimulated with Thrombin. Endothelial cells were stimulated with thrombin for 15 min in buffer and radiolabel was recovered in several fractions after separation of polar lipids by TLC (Fig. 1). When incorporation by stimulated cells was compared to controls, there was a clear increase in the amount of radiolabel recovered in the fraction with the same R₁ as authentic PAF in the thrombin-treated cells. Cells from each isolate demonstrated this response. The time course of acetate incorporation into this lipid showed the effect to be maximal by 5 min (Fig. 2), with a subsequent slow loss of label. The response to thrombin was concentration dependent (Fig. 3) and occurred over the same range that activated platelets; the half-maximal response for both [³H]acetate incorporation by endothelial cells and [³H]serotonin release by platelets was approximately 0.17 units/ml. Thrombin that had been inactivated by exposure to phenylmethanesulfonyl fluoride or to antithrombin III did

Fig. 1. Endothelial cells stimulated with thrombin incorporate acetate into a polar lipid. Confluent monolayers of human endothelial cells were incubated for 15 min in buffer containing 50 μCi of [³H]acetate; in some dishes human thrombin was present in a final concentration of 2 units/ml (hatched bars, thrombin; open bars, control). A lipid extract was analyzed by TLC. The results shown represent the mean of duplicate incubations, which were within 4% of the mean, from cells obtained from a single umbilical vein. PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidyethanolamine; Origin, origin; NL, neutral lipids; SF, solvent front.

PMNs can be accounted for by those adherent to the monolayer.
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FIG. 3. Incorporation of [3H]acetate into a polar lipid is dependent on the concentration of thrombin. Endothelial cells were stimulated for 5 min with thrombin at various concentrations. The products were assayed as described in Fig. 1 and Materials and Methods. In experiments on the same day, using the same solutions of thrombin, the release of [3H]serotonin from washed, prelabeled human platelets was determined. In both cases the results are expressed as percent of maximal response: for endothelial cell lipid-acetate production (●) maximal response was incorporation of 3750 dpm; for platelets (○ and ●, duplicates) maximal response was release of greater than 80% of the radiolabeled serotonin.

not induce this response in endothelial cells (Table 1). The response has a requirement for divalent cations since stimulation performed in Ca2+- and Mg2+-free buffer resulted in only 24% as much [3H]acetate incorporation into the polar lipid as when these cations were present (Table 1). In experiments designed to determine whether the acetate-containing lipid was released into the supernatant buffer, we found that the lipid remained associated with the endothelial cells (Table 1). This was true even if fatty acid-free bovine serum albumin was included in the supernatant buffer during the incubation. Mechanical stimulation and incubation with unlabeled PAF (10 μM) both failed to stimulate the incorporation of acetate into this lipid (Table 1).

Identification of the [3H]Acetate-Containing Polar Lipid as PAF. The migration of a [3H]acetate-containing lipid with authentic PAF in the TLC system suggested that the compound was radiolabeled PAF. To test this conclusion, we eluted the [3H]-containing lipid from thin-layer chromatograms for further examination. When incubated with phospholipase A2, it lost greater than 90% of its radiolabel as compared to material incubated with buffer (two experiments, data not shown). These results confirmed that the [3H]acetate was located at the sn-2 position on the glycerol backbone.

The lipid containing [3H]acetate at sn-2 was analyzed by HPLC (24) as shown in Fig. 4. The endothelial cell-derived product eluted with authentic [3H]PAF and more than 85% of the added radioactivity was recovered in the peak fractions.

Endothelial Cells Metabolize L-[[3H]Alkyl-GroPCho. To further validate the identity of the acetate-containing compound as PAF, we examined endothelial cells for the ability to metabolize alkyl lipids. Endothelial cells accumulate 1-[3H]alkyl-GroPCho and have the appropriate enzyme activities to acylate it since 1-[3H]alkyl-2-acetyl-GroPCho was found in cells incubated with the lysolipid (Table 2). Additionally, they can convert either the 1-[3H]alkyl-GroPCho or the 1-[3H]alkyl-2-acetyl-GroPCho (or both) to [3H]PAF (Table 2). Small quantities of the latter were found even in monolayers that had not been exposed to thrombin, although some aspect of the incubation conditions may have stimulated them. The addition of thrombin resulted in an increase in both the absolute amount and percentage of radiolabel found in the PAF-containing fraction of the TLC (Table 2).

Biological Activity Typical of PAF Is Recovered After HPLC Separation of Extracts from Stimulated Endothelial Cells. The lipid extract from thrombin-stimulated endothelial cells was separated by HPLC and fractions of the effluent were tested for their ability to induce the aggregation of

Table 1. Endothelial cell production of [3H]acetate-labeled PAF under various conditions

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>% of maximal response</th>
<th>dpm of [3H]acetate in PAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin* (2 units/ml)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Released</td>
<td>5118</td>
<td>100</td>
</tr>
<tr>
<td>Cell-associated</td>
<td>438</td>
<td>9</td>
</tr>
<tr>
<td>Thrombin/AT-III</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin/PhMeSO2F</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>1 mM</td>
<td>1242</td>
<td>24</td>
</tr>
<tr>
<td>Thrombin, Ca2+-/Mg2+-free buffer</td>
<td>69</td>
<td>1</td>
</tr>
<tr>
<td>Mechanical*</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

*The supernatant buffer was removed and assayed separately from the monolayer of cells at the conclusion of the incubation. The same results were obtained with or without fatty acid-free bovine serum albumin (1 mg/ml) in the buffer.

1Thrombin was incubated for 30 min with an 8-fold molar excess of antithrombin III and heparin at 2.0 units/ml.

2Thrombin was incubated for 60 min with either 10 mM or 1 mM phenylmethylsulfonyl fluoride (PhMeSO2F). A portion of this reaction mixture was added, in a 1:1000 dilution, to achieve what would have been a final thrombin concentration of 2 units/ml.

3The [3H]acetate-containing buffer was added to the monolayer and the cells were scraped from the dish with a rubber policeman. The cells and buffer were allowed to incubate for 5 min and then were extracted.

FIG. 4. HPLC analysis of [3H]acetate-labeled polar lipid and authentic [3H]PAF. Samples of the [3H]acetate-labeled polar lipid were recovered from the TLC gel. A portion of the sample was injected onto a 4.6 × 300 mm Varian Micropak Si-5 column and eluted with a mobile phase (1 ml/min) that initially consisted of 96% hexane/2-propanol (1:1, vol/vol) and 4% (vol/vol) water. Between 5 and 20 min after injection the amount of water was increased to 8% in a linear gradient. Fractions were collected every 0.5 min from 20–32 minutes after injection and the radioactivity was estimated. A sample of authentic [3H]PAF was analyzed in the same manner. PtdCho and Lyso-PtdCho denote the retention times of authentic phosphatidylycholine and lysophosphatidylycholine, respectively.
PMNs. As shown in Fig. 5, endothelial cells produced such activity and it eluted with authentic PAF. In the experiment shown, the PAF from six plates of cells (approximately 4.6 × 10⁶ cells) was assayed. We estimate from this assay that the amount of PAF produced is in the range of 0.10 to 0.17 nmol per 10⁶ cells. Treatment of a portion of the combined fractions with phospholipase A2 resulted in greater than 90% loss of aggregating activity as compared to an equal portion treated with buffer (not shown). The compound recovered in these fractions also potently stimulated the aggregation of platelets (not shown).

**TREATMENT OF ENDOTHELIAL CELL MONOLAYERS WITH THROMBIN**

**RESULTS IN INCREASED GRANULOCYTE ADHERENCE.** To examine a potential physiological or pathological effect of PAF production by endothelial cells, we exposed them to thrombin at 0.5 unit/ml or buffer and measured the adherence of PMNs. Basal adherence (buffer stimulus) of PMNs to endothelial cells was 24% and the treatment with thrombin increased this to 70%. In another experiment, a similarly augmented adherence in response to thrombin persisted even though the monolayers were washed prior to the addition of the PMNs.

**DISCUSSION**

In these studies, we have demonstrated that cultured endothelial cells synthesize PAF in response to stimulation with thrombin. The stimulation of the PAF production by endothelium occurs at concentrations of thrombin that are known to elicit other responses (3, 30) by endothelial cells, as well as responses in other cells (Fig. 3). Active thrombin is required, as is the presence of divalent cations, and the PAF that is produced remains associated with the cells under the conditions of our experiments (Table 1).

The production of PAF previously has been described in blood cells (10, 13, 31) and macrophages (32), and PAF has been found in amniotic fluid (33). In addition to its effects on inflammatory cells, PAF has been shown to affect smooth muscle preparations (18) and pulmonary function (19) and to stimulate glucose production by perfused liver (20). Whether endothelial cell-derived PAF plays a role in these effects is uncertain. The finding that this potent stimulus for platelet and PMN activation is synthesized by endothelial cells may explain the increased adherence of these blood cells to endothelium under some circumstances. For example, the perfusion of isolated umbilical veins with a solution of thrombin followed by perfusion with platelet-rich plasma led to increased platelet adherence to endothelial cells when compared to controls (21). The addition of thrombin to cultures of endothelium results in contraction of the cells which, in turn, results in the presence of gaps in the monolayer (34). A corresponding response in vivo would cause disruption of the endothelial lining, and the production of PAF by endothelial cells may promote platelet adhesion as a means of maintaining vascular integrity under such conditions. However, augmented production of PAF, generated by stimuli that injure the endothelium, could result in thrombosis or amplify the response to endothelial injury. The latter possibility suggests that PAF production by endothelial cells may play a role in atherogenesis or syndromes of vascular injury mediated by platelets or PMNs.

While this manuscript was under review Camussi et al. reported the production of PAF by cultured endothelial cells (35). Their results differ from those reported here in that they found no response to thrombin unless it was added repeatedly, and they found PAF to be released into the supernatant buffer.

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