Platelet-Activating Factor Effects on Bovine Pulmonary Artery Endothelial Cells

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Endothelial cells (ECs) were isolated from bovine pulmonary artery and maintained in long-term culture. On reaching confluency, ECs formed a characteristic "cobblestone" monolayer. One hour after addition of 1 nM platelet-activating factor (PAF) to the growth medium, ECs underwent dramatic changes in shape from their normal polygonal morphology to more elongated spindle-shaped forms. More pronounced effects were evident in the presence of 0.1 nM phorbol-12-myristate-13-acetate (PMA), a potent activator of C kinase. It was found that at concentrations from 10^{-11} to 10^{-7} M, PAF stimulates the phosphoinositide turnover in EC. The half-maximal activation in the release of inositol phosphates was at 10^{-8} M. This finding suggested that an increase in intracellular Ca^{2+} concentration and activation of protein kinase C were involved in the mechanism of action of PAF on EC. The metabolic responses of EC were evaluated by measuring the activity of β-adrenergic receptor-coupled adenylate cyclase (AC) in a crude membrane fraction and by assay of prostacyclin and thromboxane released by cultured EC. AC from control membranes was activated by isoproterenol in a dose-dependent manner (EC_{50} = 30 nM) from 0.8-5.5 pmol cAMP/min/mg protein. If the membranes were isolated after preincubation of ECs with 1 nM PAF or 0.1 nM PMA, the AC activity was decreased by 70 and 90%, respectively; in both cases, affinity for isoproterenol was lowered threefold (EC_{50} = 100 nM). Our data suggest that PAF interaction with EC leads to an apparent β-adrenergic receptor desensitization that probably acts via a phosphorylation mechanism involving C kinase. Incubation of EC for 30 minutes with 0.1-1.0 nM PAF caused inhibition of both prostacyclin and thromboxane production (55 and 75%, respectively) indicating that PAF acts at a level common to both pathways of arachidonate metabolism. Similar results were obtained using PMA (0.1 nM) but not with phorbol-12,13-didecanoate, an inactive analogue of PMA. Taken together, these data indicate that C kinase is involved in PAF-induced alteration in receptor sensitivity at the plasma membrane level as well as in intracellular enzymes responsible for prostacyclin and thromboxane synthesis by EC. This down-regulation of metabolic activity of EC is accompanied by concomitant shape changes. (Circulation Research 1987;61:389-395)

Platelet-Activating factor (PAF; AGEPC, 1-0-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a very potent mediator that is involved in many inflammatory and allergic tissue reactions. PAF may exert its effects as a result of direct actions on neutrophils, macrophages, vascular endothelium, or vascular smooth muscle as well as via platelet activation.

The effect of PAF on the arterial wall has been studied recently using an in vivo thrombosis model. It was demonstrated that in guinea pig, a topical PAF superfusion beside the thrombus formation leads to massive cytoplasmic vacuolation of adjoining endothelial cells.

Many pathologic states in lungs may result either from a direct effect of PAF on the vessel wall or from the generation and release of leukotrienes, thromboxanes, or other mediators by leukocytes, platelets, and even endothelial cells. However, under in vitro conditions, the effect of PAF on endothelial cells has not previously been investigated. The aim of our study was to determine the effect of PAF on metabolic (functional) activities of cultured bovine pulmonary artery endothelial cells.

Materials and Methods

Chemicals

Cell culture media were purchased from Gibco (Grand Island, N.Y.). PAF was obtained dissolved in chloroform solution (Sigma #P9525, Sigma Chemical Company, St. Louis, Mo., 2 mg/ml, 99% pure). An aliquot of this solution (in a polypropylene tube) was evaporated under a stream of nitrogen, dissolved in DMSO at a concentration of 10^{-4} M, and stored at -30° C. 4β-phorbol-12β-myristate-13α-acetate (PMA), 4α-phorbol-12,13-didecanoate (PDD), and other chemicals were obtained from Sigma Chemical Company. PMA was dissolved in ethanol at 10^{-8} M and stored at -30° C. The concentrations of PAF and PMA were below their critical micelle concentrations. Labelled compounds were from Amersham (Great Britain).
Cell Culture

Endothelial cells (ECs) were isolated from bovine pulmonary artery and maintained in long-term culture without exposure to proteolytic enzymes. 10,11 Membrane Fraction

Confluent monolayers of ECs grown in T75 plastic culture flasks were washed three times with serum-free medium 199. Therefore, 5 ml drug-containing medium was added to each of 4 flasks in a group (control, 1 nM PAF, 0.1 nM PMA, or 0.1 nM PDD) and incubated for 1 hour at 37°C. Then, all flasks were placed on ice, washed twice with medium 199 and three times with a buffer containing (in mM) NaCl 150, ethylenediaminetetraacetate (EDTA) 1, and HEPES-NaOH 10 (pH 7.5 at 4°C), and scraped off using a rubber policeman. After centrifugation for 10 minutes at 1,200g, the cells were resuspended in hypotonic buffer (EDTA 1 mM, HEPES-NaPH 10 mM, pH 7.5), homogenized in a glass tissue grinder (20 strokes), and centrifuged for 15 minutes at 3,000g. The pellet was washed three times with hypotonic buffer, and the final pellet was resuspended in a volume of buffer sufficient to yield a protein concentration of 1 mg/ml.

The effect of PAF on phosphatidylinositol turnover was studied, as described recently. 10,11 Using a modification of the method of Berridge et al. 10 Briefly, ECs were seeded in 24-well culture dishes. On the second day after seeding, cells were incubated for 24-48 hours in the presence of myo-[2-3H]inositol (10 μCi/ml). Then, the medium containing [3H]inositol was removed, and confluent cell layers were washed five times with 1 ml medium 199. To each well, 1 ml medium containing LiCl was added, and cells were incubated for 30 minutes at 37°C. Then, this medium was aspirated, and 1 ml buffer containing (in mM) NaCl 140, KCl 2.8, CaCl2 1, MgCl2 2, and 10 HEPES-NaOH (pH 7.5) was added. After 5 minutes of incubation with PAF, 1 ml hot 1% (wt/vol) sodium dodecyl sulphate/30 mM EDTA was added to each well to terminate the reaction. The cell lysates were diluted to an ionic strength less than 60 mM and applied onto columns containing 0.50 ml Dowex 1 x 4 (Aldrich Chemicals, Milwaukee, Wis.). Columns were first washed with 14 ml H2O to wash out myo-[3H]inositol. Subsequently, inositol phosphates were eluted by stepwise addition of solutions containing increasing amounts of formic acid. 14

Adenylate cyclase was assayed as described previously. 15 The incubation medium contained 20 mM HEPES-NaOH (pH 7.5), 0.1 mM ATP, [γ-32P]-ATP (600–800 cpm/pmol), 2 mM MgSO4, 1 mM EGTA, 0.5 mM cyclic adenosine 3',5' monophosphate (cAMP), 0.5 mM 3- methyl-1-isobutylxanthine, 1 mM guanosine triphosphate (GTP), 0.2 mg/ml creatine kinase, 10 mM creatine phosphate, and 25 μg membrane protein in a total volume of 50 μl. Incubation was carried out at 37°C and stopped with 0.2 ml 0.5N HCl. After boiling for 6 minutes, samples were neutralized with 0.2 ml 1.5 M imidazole, applied onto alumina columns, and eluted with 5 ml 10 mM imidazole-HCl (pH 7.5) directly into scintillation vials. Radioactivity was counted using a liquid scintillation counter.

For the assay of prostacyclin and thromboxane as well as for morphology studies, cells were grown as confluent monolayers in 8-well culture dishes. On the day of the experiment, growth medium was exchanged for serum-free medium 199, and cells were incubated for 3 hours. Then, monolayers of cells were washed with fresh medium, and 1 ml drug-containing medium was added. At the end of the incubation, aliquots of medium were assayed for thromboxane B2 and prostaglandin 6-keto-F1α. Thromboxane B2 and 6-keto-PGF1α were assayed using Amersham RIA kits as described previously. 16 Protein was assayed using a standard Bio-Rad assay kit.

Results

To investigate the influence of PAF on bovine pulmonary artery endothelial cells (BPAECs), 10th to 20th passages of BPAECs cultured in Ryan Red medium were used. 14 On reaching confluency, BPAECs formed a characteristic "cobblestone" monolayer. Three hours prior to the beginning of the experiment, the growth medium was changed for serum-free medium 199. At the beginning of the experiment, the medium was aspirated, and fresh medium-containing vehicle (PAF 1 nM, PMA 0.1 nM, or PDD 0.1 nM) was added to each well. During the first hour of incubation at 37°C in a standard CO2 incubator, BPAECs in the presence of 1 nM PAF underwent dramatic shape changes from their normal polygonal morphology (Figure 1) to more elongated spindle-shaped forms (Figure 2) as did the addition of 0.1 nM PMA, a potent activator of protein kinase C (Figure 3). PMA and PAF together induced more pronounced effects; BPAECs changed their shape and nearly 50% of them detached. In the control experiment using 0.1 nM PDD, an inactive analogue of PMA, 17 no shape change was evident. These data are very similar to previously published observations on PMA-induced morphologic changes in cultured cells 14 and appear to be a prelude to increased migration and cell division. 14,19,20

It is well documented that PAF stimulates phosphoinositide turnover in platelets, thus leading to activation of protein kinase C and elevation in the intracellular free Ca2+ concentration. 20 We were able to show that the same mechanism of PAF signal transduction exists in BPAEC. Figure 4 shows that at concentrations of 10-10–10-7 M, PAF stimulates the formation of inositol phosphate, inositol bisphosphate, and inositol trisphosphate. The maximal stimulation was two–three times at 10-8 M PAF; EC50 was at 1 nM.

It seemed likely that the pronounced morphologic changes would be accompanied by alterations in metabolic responses of BPAEC. We chose β-adrenergic receptor-coupled adenylate cyclase as a test system for evaluation of PAF-induced changes in metabolic responses of BPAEC. The β-adrenergic adenylate cyclase system has recently been demonstrated in endothelial cells. 21,22 For our experiments, BPAECs were grown in T75 flasks to obtain enough
FIGURE 1. Control monolayer demonstrating cobblestone morphology (magnification 155 ×).

FIGURE 2. Monolayer after exposure to 10⁻⁴ M PAF for 72 hours; cells have become markedly elongated (magnification 155 ×).
FIGURE 3. Monolayer after exposure to $10^{-9}$ M PMA for 72 hours; cells are elongated, and monolayer is irregular and densely packed (magnification 155×).

Material for measuring isoproterenol-stimulated adenylate cyclase activity in crude membrane fractions. Figure 5 shows the isoproterenol dose-response curve for adenylate cyclase activity of a crude membrane fraction prepared from BPAECs grown on four T75 culture flasks. From the control curve, it is evident that isoproterenol activated BPAEC adenylate cyclase from 0.8–5.5 pmol cAMP/min/mg protein (almost sevenfold) with $EC_{50} = 30$ nM. If BPAECs were preincubated for 1 hour with 1 nM PAF prior to membrane isolation, adenylate cyclase activity was decreased by 70%, and $EC_{50}$ for isoproterenol activation became almost 100 nM. However, although the effectiveness of isoproterenol was decreased threefold, the degree of adenylate cyclase activation by isoproterenol remained unchanged.

Pretreatment of BPAECs with 0.1 nM PMA (Figure 6) yielded results that suggest participation of protein kinase C in decreasing the adenylate cyclase activity after PAF treatment. Figure 6 demonstrates that after PMA pretreatment, isoproterenol-stimulated adenylate cyclase activity was decreased by 90%. This inhibition of enzyme activity was accompanied by reduction of hormone potency ($EC_{50} = 100$ nM) as well as by preservation of the degree of stimulation of the

**Figure 4.** Dose-response curves for PAF-stimulated release of inositol phosphates by BPAEC.

**Figure 5.** Dose-response curves for isoproterenol-sensitive adenylate cyclase from membranes of BPAEC preincubated in the absence (○) or presence of 1 nM PAF (●). Data are mean ± SD of representative experiment performed in triplicate.
Concentration of Isoproterenol (H)

Figure 6. Dose-response curves for isoproterenol-sensitive adenylate cyclase from membranes of BPAEC preincubated in the presence of 0.1 nM PDD (○) or 0.1 nM PMA (●). Data are mean ± SD of representative experiment performed in triplicate.

remaining adenylate cyclase activity by isoproterenol. In control experiments, 0.1 nM PDD, the inactive analogue of PMA, was completely without effect.

BPAECs are capable of synthesizing and releasing both prostacyclin and thromboxane A₂ into the incubation medium. It is possible to detect their stable metabolites, 6-keto-PGF₁α and thromboxane B₂, using appropriate RIA kits. We studied the effect of PAF on this metabolic function of BPAEC.

BPAECs were grown in 8-well culture dishes. Two hours before the experiment, growth medium was exchanged for serum-free medium 199. At zero time, this was exchanged for fresh medium supplemented with various concentrations of PAF. After 30 minutes of incubation at 37°C, aliquots were withdrawn and assayed for thromboxane B₂ and 6-keto-PGF₁α. As can be seen from Figures 7 and 8, PAF at 0.1 and 1 nM significantly decreased the release (and probably the synthesis) of both prostacyclin and thromboxane by 55 and 75%, respectively. However, at 10 nM, this effect disappeared. Moreover, there was marked stimulation of the release of prostacyclin and thromboxane into the incubation medium. Unfortunately, it was not possible to increase PAF concentration further because of its highly cytotoxic effect on BPAEC.

Figure 7. Dose-dependent effect of PAF on 6-keto-PGF₁α production by BPAEC. Data are mean ± SD of representative experiment performed in duplicate.

Figure 8. Dose-dependent effect of PAF on thromboxane B₂ production by BPAEC. Data are mean ± SD of representative experiment performed in duplicate.

Only the inhibitory phase of the PAF effect on prostacyclin and thromboxane release involves protein kinase C action since PMA produced an inhibitory effect (Figure 9). Our results show that in the range of concentrations from 0.01–1 nM, PMA inhibits the release of thromboxane by 50%, while PDD remains ineffective (Figure 9). Exactly the same effect was observed on prostacyclin release (data not shown). We were not able to investigate higher concentrations of PMA because of its high cytotoxicity.

Discussion

Recently, it has been reported that endothelial cells from large vessels contain β-adrenergic receptors coupled to adenylate cyclase. The ability of phorbol esters, such as PMA, to induce desensitization to β-adrenergic agonists in the adenylate cyclase system has been reported for various cells, suggesting the involvement of protein kinase C in this process. In our study, PAF induced desensitization of β-adrenergic responses of BPAEC adenylate cyclase in the same manner (Figure 5). PMA also caused loss of adenylate cyclase sensitivity to isoproterenol, thus confirming the general mechanism of its action (Figure 6). Both PMA and PAF shifted EC₅₀ from 30 to 100 nM and decreased specific activity of adenylate cyclase in both the absence and the presence of isoproterenol (Figures 5 and 6). However, the degree of adenylate cyclase
stimulation by 10 μM isoproterenol was substantially unchanged, suggesting that coupling of receptor to adenylate cyclase through the N-protein complex did not change. It is more likely that reduction in the amount of β-adrenergic receptors might explain the observed effect. Our speculation is supported by recently published results of several investigators: Braquet et al40 reported that incubation of rat cerebellum homogenates with PAF dramatically inhibited the number of β2-adrenergic receptors. Limas and Limas23 demonstrated that incubation of cardiac myocytes with diacylglycerol induced desensitization of β2-adrenergic receptors reducing both their affinity and the total number detectable with [3H]-CGP-12177. Yamashita et al9 observed PMA- and diacylglycerol-induced desensitization to β-adrenergic agonist stimulation in the adenylate cyclase system of rat reticulocytes. This is consistent with our results on PAF-stimulation of release of inositol phosphates by BPAEC (Figure 4).

It should be noted, however, that vascular effects of PAF may not be solely the consequence of its action on catecholamine responsiveness of endothelium. Our results indicate that PAF significantly influences the prostacyclin and thromboxane release by BPAECs (Figures 7 and 8). At low concentrations, PAF inhibits the release of prostacyclin and thromboxane; however, at 10 nM, the increase in their release is evident. Such a biphasic effect of PAF may be explained as follows: the inhibitory action of PAF may result from protein kinase C activation since PMA produces the same inhibitory action (Figure 9). It has been reported recently that activation of protein kinase C is inhibitory for thromboxane generation in platelets because of inhibition of cytosolic free calcium.37 It is known that PAF induces a rise in cytosolic free calcium in platelets not only through stimulation of inositol phospholipid metabolism36 but also receptor-operated calcium channels.28 The same mechanisms appear to exist in BPAECs as well.29,30

At high PAF concentrations, ability to increase cytosolic free calcium by either mechanism may overcome the inhibitory action of protein kinase C and thus result in activation of prostacyclin and thromboxane production. However, the possibility that some unique regulatory pathways different from those present in platelets exist in endothelial cells cannot be excluded.

In addition to playing a role in signal transduction resulting in metabolic responses, C kinase activation may also play a role in the endothelial shape-change responses. It was recently demonstrated that treatment with PMA causes dramatic changes in the morphology of human aorta endothelial cells in a primary culture.31 By analogy with other stimulating agents, the alterations in endothelial morphology caused by PAF appear to signal activation of endothelial cells in terms of migration and division behavior as assessed by video time-lapse microscopy and computer analysis of cell behavior.31,32 Furthermore, signal transduction during activation responses of endothelial cells in general appears to involve activation of protein kinase C and Ca2+ mobilization34,35-36 and perhaps are synergistically effective in eliciting a full cellular response.36 Cell division is accompanied by increased intracellular Ca2+ levels at anaphase.37 Intracellular Ca2+ is elevated in response to PAF,13 and it is tempting to speculate that, like the metabolic responses, the shape and behavior changes seen here in response to PAF involve C kinase activation and may also be dependent on Ca2+ mobilization.

In conclusion, our results demonstrate that PAF induces significant alteration in BPAEC morphology and metabolic functions. The mechanism of its action involves protein kinase C since PAF stimulates phosphoinositide turnover; PMA, a well-established activator of protein kinase C, mimics the effects of PAF on the β-adrenergic receptor-adenylate cyclase complex as well as on prostacyclin and thromboxane release. These findings open new avenues for the elucidation of PAF action on endothelium and its role in vascular pathology.

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KEY WORDS • platelet-activating factor • endothelium • phorbol esters • beta-adrenergic receptors • protein kinase C • prostacyclin • thromboxane