Evidence for an intracellular action of platelet-activating factor in bovine cultured aortic endothelial cells

¹Alastair G. Stewart, Philip N. Dubbin, Trudi Harris & Gregory J. Dusting

Department of Physiology, University of Melbourne, Parkville, Victoria 3052, Australia

Bovine culture endothelial cells (BAECs) generate platelet-activating factor (Paf) following activation by bradykinin (Bk 0.1 nM), the ionophore, A23187 (3 μ M), and ATP (10 μ M), but Paf is not released from the cells. These stimuli also elicit generation of prostacyclin (PGI₂). The specific and competitive Paf receptor antagonists, WEB 2086 (0.1–1.0 μ M) and CV 6209 (0.01–0.1 μ M), inhibited Bk-, A23187- and, to a lesser extent, ATP-induced PGI₂ generation but had no effect on basal PGI₂ generation. These data suggest a role for intracellular Paf in signal transduction.

Introduction Platelet-activating factor (Paf) is regarded as a pro-inflammatory mediator (Braquet *et al.*, 1987) that requires release from the cell of origin for its action. Macrophages and polymorphonuclear leukocytes (PMNs) synthesize and release Paf in response to immune complexes, endotoxin and zymosan but these cells also retain a significant portion within the cell. In contrast, endothelial cells synthesize, but do not release Paf in response to bradykinin (Bk), the ionophore A23187, ATP, histamine and thrombin (McIntyre *et al.*, 1985). The function of intracellular Paf has not been identified.

We have observed an inhibitory effect of several Paf receptor antagonists on macrophage prostacyclin (PGI₂) generation in the absence of exogenous Paf (Stewart & Dusting, 1988). This observation, together with extensive data linking exogenous Paf to arachidonic acid mobilization (Braquet *et al.*, 1987), led us to investigate the role of intracellular Paf in eicosanoid generation by endothelial cells since in these cells all the Paf synthesized is retained intracellularly and the stimuli for Paf generation also cause PGI₂ generation. Antagonism of Paf was examined by use of the potent, specific and structurally distinct Paf receptor antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and CV 6209 (Terashita *et al.*, 1987).

Methods Bovine aortic endothelial cells were cultured in monolayers as previously described (Booyse

et al., 1975; Dusting *et al.*, 1988) and were grown to confluence in Linbro plastic culture dishes using RPMI 1640 (containing 20% v/v foetal calf serum, 15 mM HEPES, pH 7.4, streptomycin, 100 μ g ml⁻¹ and penicillin, 100 u ml⁻¹). The medium was exchanged immediately before experiments for one containing 0.25% w/v bovine serum albumin (BSA) in place of foetal calf serum.

Paf receptor antagonists were added 15 min before stimuli and the incubations continued for a further 60 min. The supernatants were harvested for radioimmunoassay (RIA) of the stable PGI₂ product 6-oxo-PGF_{1 α} (Salmon, 1978). In experiments in which Paf was assayed, incubations were carried out for 5 min since this period corresponded to the maximum stimulation of Paf which subsequently declined rapidly. The supernatants were assayed for Paf content without further extraction using rabbit washed platelets (Parente & Flower, 1985; Stewart & Dusting, 1988). Paf was extracted from the cell monolayer by addition of 1.0 ml ice-cold 80% ethanol. The extract was evaporated to dryness then reconstituted in Tyrode solution (plus 0.25% BSA, 1.8 mM Ca²⁺, 10 mM HEPES, pH 7.40) before bioassay.

RPMI 1640 and foetal calf serum were obtained from CSL (Australia) and Flow Laboratories, respectively. Sources of chemicals: ATP; A23187; bovine serum albumin (BSA), grade 5, essentially fatty acid free (Sigma Chemical Co.); hexadecyl platelet-activating factor (Paf, Bachem); 6-oxo-PGF_{1 α} (Upjohn); 6-oxo-PGF_{1 α} antiserum (Dr J. Salmon, Wellcome Research Laboratories); 6-keto [5,8,9,11,12,14,15 (n)-³H] prostaglandin F_{1 α} , 170 Ci mmol⁻¹ (Amersham); [hexadecyl-1',2'-³H(N)]-2-acetyl-sn-glyceryl-3-phosphorylcholine, 59.5 Ci mmol⁻¹ (New England Nuclear); HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid, BDH chemicals); CV 6209 (2-[N-acetyl-N-(2-methoxy-3-octadecyl-carbamoyloxy-propoxycarbonyl)amino-methyl]-1-ethylpyridinium chloride), (Takeda Chemical Industries Ltd.); WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)-triazolo(4,3-a),(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone), Boehringer Ingelheim, West Germany.

¹ Author for correspondence.

Results and Discussion Concentrations of Bk (0.1 nM), A23187 (3 μ M), and ATP (10 μ M) that were equi-active and submaximal for BAEC generation of 6-oxo-PGF_{1 α} were determined from the concentration-response curves for each of these stimuli. In separate experiments, Bk, A23187 and ATP also induced the synthesis, but not release, of Paf whereas unstimulated BAECs did not contain detectable Paf levels (Figure 1). Pre-incubation of BAECs with the Paf receptor antagonists, WEB 2086 (1 μ M) and CV 6209 (0.1 μ M) caused marked reductions in Bk-stimulated 6-oxo-PGF_{1 α} , smaller inhibition of A23187 and ATP, but no inhibition of basal 6-oxo-PGF_{1 α} generation. These concentrations of WEB 2086 and CV 6209 were maximally effective for inhibition of Bk-stimulated 6-oxo-PGF_{1 α} generation and produced 1.63 ± 0.16 and 2.59 ± 0.18 log unit ($n = 6$) rightward shifts of Paf-induced aggregation of rabbit washed platelets, respectively.

The specificity of the Paf receptor antagonism at these concentrations is supported by the failure of either compound to inhibit platelet aggregation induced by a range of stimuli including arachidonic acid, A23187, ADP and collagen (Casals-Stenzel *et al.*, 1987; Terashita *et al.*, 1987). Furthermore, CV 6209 failed to show any direct inhibitory effects on either cyclo-oxygenase or phospholipase A₂ (Terashita *et al.*, 1987).

The extent of the inhibition by the Paf receptor antagonists of PGI₂ generation was clearly dependent on the stimulus used (Figure 1). The lack of inhibition of basal PGI₂ generation suggests that neither Paf receptor antagonist inhibits directly the enzymes involved in PGI₂ synthesis and is consistent with the failure to detect Paf in unstimulated BAECs. The inhibition by the Paf antagonists of PGI₂ generation stimulated by Bk, A23187 and ATP does coincide with the generation of intracellular Paf. The differences in the extent of inhibition between A23187, ATP and Bk may be explained by the distinct mechanisms of BAEC activation: A23187 causes a large and persistent increase in intracellular Ca²⁺; ATP activates the Na⁺/H⁺ exchanger (Kitazono *et al.*, 1988); Bk activates phospholipase C (Clark *et al.*, 1986).

Exogenous Paf does not stimulate PGI₂ generation by BAECs (data not shown). However, endothelial cells have a high capacity to metabolize Paf (McIntyre *et al.*, 1985) which may preclude the

Figure 1 Basal, bradykinin (Bk 0.1 nM), A23187 (3 μ M) and ATP (10 μ M)-induced generation of 6-oxo-PGF_{1 α} (open columns) and Paf (filled columns) by BAECs (a) and inhibition of 6-oxo-PGF_{1 α} by the Paf receptor antagonists (b), WEB 2086 (1 μ M, open columns) and CV 6209 (0.1 μ M, filled columns). The data are presented as the means of 3–8 observations; s.e.mean shown by vertical bars. * $P < 0.05$, paired t test.

ability of exogenous Paf to mimic the proposed intracellular action of mobilizing arachidonic acid.

The present data suggest a role for Paf in the signal transduction mechanism for Bk-stimulated PGI₂ generation. The site of intracellular action of Paf has not been identified but recent data suggest that Bk activates phospholipase C in BAECs (Clark *et al.*, 1986) and a link between Paf receptors and phospholipase C activation is well established (Braquet *et al.*, 1987).

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