Platelet Activating Factor Inhibits Fluid Transport by Corneal Endothelium

Zhaorong Zhu,* Kunyan Kuang,* Fengying Kang,* Jun Li,* and Jorge Fischbarg*†

Purpose. Given reports of corneal edema after endothelial exposure to platelet activating factor (PAF), the authors have investigated whether PAF can affect the function of corneal endothelium in vitro.

Methods. The endothelial side of deepithelialized rabbit corneas was perfused with BSS+ and test agents: PAF, its inactive receptor ligand analog Lyso-PAF, and its antagonist BN52021. Stromal thickness was determined by specular microscopy. Translayer-specific electrical resistance ($\rho$) was measured in cultured bovine corneal endothelial cells grown on permeable substrates at 36.5°C.

Results. Control corneas perfused with BSS+ or with BSS+ containing Lyso-PAF swelled at a very slow rate (6.2 ± 0.1, and 7.9 ± 0.2 μm/hour, respectively). Corneas exposed to PAF swelled appreciably faster and at rates that were a saturable function of PAF (K_m, 2.1 μM); maximal rates of swelling were <20 μm/hour, indicating no appreciable damage to intercellular junctions. BN52021 prevented PAF-induced swelling (K, 1.1 μM). PAF led also to a decrease in $\rho$ (from 42.8 ± 1.4 to 24.5 ± 0.6 Ω cm^2 in 1 hour; 46.8 ± 1.5 to 38.3 ± 1.4 Ω cm^2 in control layers; and 43.0 ± 1.2 to 30.8 ± 1.6 Ω cm^2 in layers exposed to PAF + BN52021). Such $\rho$ changes are consistent with swelling of intercellular spaces.

Conclusions. Results suggest that PAF inhibits transendothelial fluid transport on binding to an endothelial cell receptor for it; continuous stimulation of a PAF-induced signaling cascade may lead to such inhibition. From these and other results, fluid transport might result from cascades activating sequentially basolateral and apical transporters or channels. Invest Ophthalmol Vis Sci. 1996;37:1899–1906.

Platelet activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine) is a potent mediator known to stimulate a wide span of biologic responses, ranging from aggregation and degranulation of platelets and neutrophils to cellular processes. On noxious stimuli, PAF can be synthesized rapidly by cells, and it plays a major role in inflammation, which includes increasing vascular permeability and stimulating leukocyte chemotaxis. PAF acts on target cells through a receptor that has been cloned in guinea pig lung and in human lung, placenta, and differentiated HL60 granulocytes; sequence analysis indicates that the PAF receptor belongs to the superfamily of G-protein-coupled receptors. On binding, PAF can stimulate activation of phospholipase C, with subsequent release of intracellular Ca++ and activation of protein kinase C (PKC). PAF-like activity has been detected in several ocular tissues subsequent to inflammation; iris, ciliary body, retina, vascular endothelium, and leukocytes could each contribute to the presence of this mediator. PAF also is found in inflamed rabbit cornea and alkali-burned cornea. Injected intracamerally into rabbits, PAF induces severe aqueous flare, pupillary constriction, change in intraocular pressure, and corneal edema. Therefore, we have investigated whether PAF exerts a direct effect on corneal endothelium by using rabbit corneal endothelial preparations and cultured bovine corneal endothelial cells.
**MATERIALS AND METHODS**

**Materials and Experimental Solutions**

PAF and lyso-PAF were obtained from Sigma (St. Louis, MO). PAF antagonist BN52021 (BN) and solvent for it were kindly supplied by Dr. P. Braquet (Institut Henri Beaufour, Paris, France). The test solutions were: BSS+ (Alcon, Forth Worth, TX; balanced salt solution enriched with bicarbonate, dextrose, and glutathione) or Dulbecco's modified Eagle's culture medium (DMEM; Gibco, Grand Island, NY). PAF and lyso-PAF in chloroform stock solutions were dried in a speed vacuum dryer and redissolved in test solutions containing 0.1% ethanol at different concentrations: 0.96 µM (0.5 µg/ml); 1.9 µM (1 µg/ml); 4.8 µM (2.5 µg/ml); and 9.6 µM (5 µg/ml). Lyso-PAF was dried and redissolved at 9.6 µM (4.6 µg/ml). BN was prepared shortly before the experiments at the following concentrations: 2.5 µM (1 µg/ml), 25 µM (10 µg/ml), and 250 µM (100 µg/ml). Final concentration of the solvent for it (dimethyl sulfoxide) was 0.1%.

**Measurement of Stromal Thickness of Deepithelialized Rabbit Cornea**

Animal procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits, each weighing 3 kg, were killed with sodium pentobarbital solution injected into the marginal ear vein. The eyes were enucleated immediately and dissected using the technique of Dikstein and Maurice. The epithelium was scraped off, and the corneal preparations were mounted in an experimental chamber held in a thermal jacket kept at 36.5°C. The endothelial side of the cornea was perfused with solutions at a rate of 1.4 ml/hour using a syringe pump (Razel Scientific Instruments, Stamford, CT), whereas the stromal surface was covered by silicone oil. The cornea was viewed with a specular microscope, and the stromal thickness was measured in conventional fashion by focusing in sequence on the stroma–oil and endothelium–fluid interfaces and using the microscope micrometer to gauge their separation; readings were taken every 15 minutes.
PAF Inhibits Corneal Endothelial Fluid Transport

Figure 3. As in Figures 1 and 2, but comparing the effect of platelet activating factor (PAF) with that of lyso-PAF. N = 4 in each group.

minutes for 3 hours. A correction was applied for index of refraction differences between the different media. Each experiment was performed with a different ocular preparation; four experiments were performed for each of the experimental groups shown in Figures 1 to 5.

Measurement of the Transendothelial Resistance

Cultured bovine corneal endothelial cells (CBCEC) were prepared as in Narula et al. Briefly, bovine calf eyes from a local slaughterhouse were washed with physiological saline containing 50 μg/ml garamycin. Corneas were excised under sterile conditions and were placed in a hemispheric plastic holder (endothelial layer up). The endothelial layer was covered with 0.5 ml 0.05% trypsin–0.53 mM EDTA-Na (Gibco) and was incubated at 37°C for 5 minutes. Subsequently, the cornea was scraped gently with a rubber policeman. The dislodged cells were aspirated with a pipette and added to 5 ml of DMEM containing 6% fetal bovine serum (FBS; Gibco), 2 ng/ml human recombinant basic fibroblast growth factor (FGF), and antibiotics (100 U/ml penicillin G, and 100 ng/ml streptomycin). The endothelial cell suspension was centrifuged at low speed (1000 rpm, Sorvall type A rotor, 5 to 8 minutes), and the supernatant was discarded. The pellet was resuspended in the same medium, and the cells were then apportioned into 75-cm² tissue culture flasks (Falcon; Becton Dickinson, Lincoln Park, NJ). The cells were fed with fresh DMEM–FBS–FGF–antibiotics (as above) every 2 to 3 days. After reaching confluence, CBCEC cells were harvested and seeded on permeable substrates (10 mm tissue culture inserts [TCI]; Nunc, Naperville, IL; membrane diameter 8 mm, transparent when wet) at a density of 5 × 10⁴ cells per insert. Monolayers reached confluence within 1 week, and experiments were performed 2 to 3 days after confluence. The electrical resistance of the monolayer was measured with a chamber and meter (Endohm chamber and EVOM meter; World Precision Instruments, Sarasota, FL). For each measurement, the electrical resistance of a blank TCI was subtracted from that of a cell-bearing TCI, and the difference was multiplied by the surface area of the insert (0.5 cm²). Deviations are SEM; we used Student's t-test for statistical analysis of the data. Multiparameter fits were performed with a Levenberg–Marquardt algorithm for nonlinear fits contained in the graphics program Origin (Microcal, Northampton, MA).

RESULTS

Effects of Platelet activating factor on the Stromal Thickness of Deepithelialized Rabbit Cornea

After setting up a rabbit corneal preparation, we monitored its thickness. Under control conditions, corneas swelled slowly and in a nearly linear fashion (Fig. 1), reflecting the slow deterioration of in vitro preparations. Control corneas perfused with 0.1% ethanol—
BSS+ swelled at a rate of 6.2 ± 0.1 μm/hour; there was no significant difference (Fig. 1) between that rate and the rate of swelling of corneas perfused with BSS+ alone (5.2 ± 0.1 μm/hour, P > 0.05). However, corneas perfused with 0.1% ethanol–BSS+ plus different concentrations of PAF (in μM: 0.96, 1.9, 4.8, 9.6) swelled much faster than the controls in a dose-dependent manner (Fig. 2). The rates of corneal swelling were 10.3 ± 0.1, 12 ± 0.2, 17.3 ± 0.2, and 18.7 ± 0.1 μm/hour, respectively (compared with the rates of swelling for control corneas; P < 0.05 for all four PAF-treated groups). As the inset in Figure 2 shows, the rate of swelling was a saturable function of [PAF]; in the standard hyperbolic fit shown, the computed Kᵣ was 2.1 μM (Fig. 2). In contrast, the corneas perfused with 0.1% ethanol–BSS+ plus 9.6 μM/l of the inactive PAF receptor ligand lyso-PAF (Fig. 3) swelled at a rate (7.9 ± 0.2 μm/hour), similar to that seen in control preparations (P > 0.05) and, hence, much slower than that in corneas exposed to the same concentration of PAF (P < 0.005).

In another group of experiments, corneas were
preincubated for 10 minutes with the PAF antagonist BN at different concentrations (2.5, 25, and 250 μM), and subsequently were perfused with a solution containing PAF 4.8 μM plus the antagonist. Corneas swelled at rates lower than those seen with PAF alone (Fig. 4); the swelling rates were 12.2 ± 0.3 (P < 0.05), 9.3 ± 0.2 (P < 0.01), and 7.4 ± 0.2 μm/hour (P < 0.01), respectively. As Figure 4 shows, this antagonist protected corneas from PAF-induced corneal swelling in a pronounced manner, reaching maximal protection with as little as 50 pM. For the inset in Figure 4, the control (baseline) swelling rate in the presence of solvent and absence of PAF or BN was taken as 7.2 μm/h. The experimental swelling rates in the presence of PAF and BN were assumed to conform to standard competitive inhibitory kinetics, i.e.,

\[
\text{rate} = \text{rate}_{\text{be}} + \text{rate}_{\text{max}} * \frac{[\text{PAF}]}{[K_{m}]} * \left(1 + \frac{[\text{BN}]}{K_{\text{BN}}} + [\text{PAF}]\right),
\]

where \(K_{\text{BN}}\) is the inhibition constant for BN, and \(K_{m}\) is the dissociation constant for PAF (1.3 μM) calculated in the inset of Figure 2. The \(V_{m}\), \(K_{m_{\text{max}}}\), and \(K_{\text{BN}}\) calculated from fitting the equation above \((\chi^2 = 0.046)\) were 15.3 ± 1 μm/hour, 2.6 μM, and 1.1 μM, respectively. Lastly, for further comparisons, all the swelling rates depicted as slopes in Figures 1 to 4 are summarized in the bar diagram of Figure 5.

**Effects of Platelet Activating Factor on the Transendothelial Resistance in Cultured Bovine Corneal Endothelial Cells**

Cultured bovine corneal endothelial cells were grown to confluence on TCI using DMEM. For the 2 hours before a given measurement, the DMEM used was serum free and contained 0.1% ethanol. The transendothelial-specific resistance \(\rho\) of a given TCI was measured continually during exposure to control and test solutions. As Figure 6 shows, the control DMEM solution, containing 0.1% ethanol, led to a 15% decrease in \(\rho\) during the initial 30 minutes, after which the \(\rho\) values stabilized. On the other hand, exposure to PAF led to larger decreases in \(\rho\); for the first 10 minutes, \(\rho\) in CBCEC exposed to 4.8 μM PAF decreased much faster than in a control CBCEC group \((P < 0.001)\). The antagonist BN gave the cells partial protection; in cells preincubated in the presence of 2.5 μM antagonist for 10 minutes and then exposed to that same concentration of antagonist plus 4.8 μM PAF, the monolayers behaved as did those in the control group for the first 10 minutes of exposure and then exhibited a decrease in \(\rho\) intermediate between those of the control group and those exposed to PAF. Compared to the initial \(\rho\) values, after 1 hour of exposure, \(\rho\) was 82% in control layers (from 46.8 ± 1.5 to 38.3 ± 1.4 Ω cm²), 72% in the BN + PAF-treated group (from 43 ± 1.2 to 30.8 ± 1.6 Ω cm²), and 57% in PAF-treated layers (from 42.8 ± 1.4 to 24.5 ± 0.6 Ω cm²). All such differences were statistically significant \((\rho_{\text{control}} > \rho_{\text{BN}}, P = 0.01; \rho_{\text{control}} > \rho_{\text{PAF}}, P = 0.002; \rho_{\text{BN}} > \rho_{\text{PAF}}, P = 0.04)\).

**DISCUSSION**

A direct effect of PAF on corneal endothelial function is reported here for the first time. We used 0.1% ethanol in BSS* as a solvent for PAF, but the data in Figure 1 suggest that the effect of the solvent, if any, was small. Corneas exposed to PAF swelled much faster than did control corneas (Fig. 2; Table 1), and the \(I_{50}\) of PAF was less than 1 μM. This dosage was similar to that needed to induce conjunctival edema through a subconjunctival injection and much lower than needed to cause corneal edema by an intracameral injection in vivo. Such a difference may stem from the dilution of intracameral PAF by the circulation of aqueous humor. In contrast, in our experiments, the endothelial side of the cornea was perfused with test solutions at a rate of 1.4 ml/hour; hence, PAF was in contact with the corneal endothelium at a constant concentration and for prolonged periods, which may account for the sensitivity to PAF observed.

The acetyl-ester linkage at carbon 2 of PAF is critical for activity. Lyso-PAF (1-0-alkyl-2-hydroxyl-sn-glycerol-3-phosphorycholine) is a metabolic precursor.
and inactive metabolite of PAF and possesses virtually none of PAF's biologic activity. In our study, the effect of lyso-PAF was not significantly different from that of the control medium with solvent (Fig. 3; P > 0.05, Table 1) but was very different from that of PAF (Fig. 3; P < 0.005, Table 1).

There is sizable evidence that the effects of PAF on target cells are initiated by a receptor-mediated process. In this connection, high-affinity PAF binding sites are present in rabbit iris and ciliary body preparations. The inhibitory effect of PAF reported here is saturable (Fig. 2, inset). From the data in Figures 2 and 4, the $K_i$ for PAF inhibition of fluid transport is saturable (Fig. 4; $P < 0.011$, Table 1), with a computed $K_0$ for a low-affinity PAF binding site in rabbit platelets; in addition, although PAF is generally active in the nM range, it is required in micromolar concentrations to act on prolactin-secreting pituitary rat cells. Moreover, BN, the most potent PAF receptor antagonist among the ginkgolides, protects rabbit iris tissue against PAF. 17 The inhibitory effect of PAF reported here is specific for both PAF inhibition and BN protection clearly suggests that these effects are exerted at the level of a receptor specific for PAF in corneal endothelium.

In principle, PAF could cause corneal edema by inhibition of the endothelial fluid transport function or disruption of endothelial intercellular junctions leading to swelling driven by the stromal imbibition pressure. However, the rates of swelling seen after exposure to PAF were moderate, indicating that disruption of intercellular junctions was unlikely. A calculation bears this point; as proposed years ago, the fluid flow across the intercellular junctions driven by the stromal swelling pressure can be calculated under some simple assumptions. We give the following assumed values and the corresponding symbols: junctional length ($j_l$), 0.24 μm; junctional width ($j_w$), 30 μm; length of intercellular cell perimeter ($P_r$), 1200 cm per cm$^2$ of tissue area; stromal swelling (imbibition) pressure ($ΔP$), 55 mm Hg; viscosity ($η$), 6.9 × 10⁻³ poise. The transjunctional flow would then be: $J_v = (j_w^3 \times \eta \times j_l)/(12 \times η \times j_l) = 35 μm/hour$. Such flow is not far from the maximum rate of swelling currently observed (approximately 20 μm/hour), which is also the order of the rates at which corneas swell after ouabain inhibition of the Na pump (30 to 40 μm/hour) and without change in endothelial permeability. Corneas with disrupted intercellular junctions, such as those obtained by exposure to Ca²⁺-free solutions, swell at much higher rates ($≈100 μm/hour$).

We also explored whether the transendothelial-specific electrical resistance might be affected by PAF. For this purpose, we used bovine corneal endothelial cells grown as a monolayer on permeable substrates. Such preparation constitutes a useful model for the endothelium because, like their counterparts in vivo, these cultured cells pump fluid from the basal to the apical compartment and display measurable electrical resistances and potential differences across the monolayer. As implied above, the hydraulic conductance of the endothelium is dictated largely by the width of its junctions. In contradistinction, the specific resistance of the endothelium depends largely on the width of its intercellular spaces. Some illustrative calculations follow. We assume that, in addition to the junctional values above, the corresponding values for the intercellular spaces are:

- space length ($s_l$) = 12 μm;
- average space width ($s_w$) = 120 μm;
- equivalent conductance ($Λ$) = 122 (Siemens × cm²)/Equiv.

<table>
<thead>
<tr>
<th>Table 1. Statistical Comparisons of the Different Rates of Swelling</th>
<th>Figure</th>
<th>P Value</th>
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<tr>
<td>BSS⁺ &lt; BSS⁺ + 9.6 μM PAF</td>
<td>2</td>
<td>0.003</td>
</tr>
<tr>
<td>BSS⁺ &lt; BSS⁺ + 4.8 μM PAF</td>
<td>2</td>
<td>0.001</td>
</tr>
<tr>
<td>BSS⁺ &lt; BSS⁺ + 1.9 μM PAF</td>
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<td>0.010</td>
</tr>
<tr>
<td>BSS⁺ &lt; BSS⁺ + 0.96 μM PAF</td>
<td>2</td>
<td>0.047</td>
</tr>
<tr>
<td>BSS⁺ = BSS⁺ + 9.6 μM lyso-PAF</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9.6 μM PAF &lt; 9.6 μM PAF</td>
<td>4</td>
<td>0.0004</td>
</tr>
<tr>
<td>4.8 μM PAF + 2.5 μM BN &lt; 4.8 μM PAF</td>
<td>5</td>
<td>0.011</td>
</tr>
<tr>
<td>4.8 μM PAF + 25 μM BN &lt; 4.8 μM PAF</td>
<td>5</td>
<td>0.009</td>
</tr>
<tr>
<td>4.8 μM PAF + 250 μM BN &lt; 4.8 μM PAF</td>
<td>5</td>
<td>0.003</td>
</tr>
</tbody>
</table>

All solutions contained 0.1% ethanol; compounds listed were added to BSS⁺. BSS⁺ = balanced salt solution enriched with bicarbonate, dextrose, and glutathione; PAF = platelet activating factor; BN = PAF antagonist BN52021.
electrolyte concentration \( (C) = 0.15 \times 10^{-3} \text{ mol/cm}^3 \).

The specific resistances for the junction \((R_j)\) and space \((R_s)\), and the total resistance \((R_t = R_j + R_s)\) would be:

\[
R_j = j/(\Lambda \times \delta \times p \times w) = 3.6 \, \Omega \times \text{cm}^2;
\]
\[
R_s = s/(\Lambda \times \delta \times p \times w) = 45.5 \, \Omega \times \text{cm}^2;
\]
\[
R_t = 49.1 \, \Omega \times \text{cm}^2.
\]

The initial specific resistance we determined (approximately 48 \( \Omega \times \text{cm}^2 \)) would be explained by such values. The maximum decline seen (down to some 25 \( \Omega \times \text{cm}^2 \)) subsequent to PAF exposure would be explained by an enlargement of the intercellular spaces width to 250 Å. Such a moderate effect contrasts with the drastic decrease in \( R_t \) (down to approximately 5 \( \Omega \times \text{cm}^2 \)) seen when gross damage (junctional opening?) is imposed on a cultured endothelial layer by Ca-free exposure.\(^{24}\) The enlargement postulated here for the intercellular spaces would be consistent with the inhibition of net fluid transport observed if the inhibitory process leads to uncompensated changes in cell volume and shape. For instance, if PAF leads to excessive fluid exiting the cell apically without compensatory volume-regulatory basolateral entry, the ensuing cell shrinkage might result in enlargement of intercellular spaces.

Our results suggest that PAF causes corneal edema largely by a direct effect on corneal endothelial function. Moreover, they suggest that the PAF effects are mediated by binding to and activation of a PAF receptor located in the endothelial cells; this would trigger a signaling chain of cellular reactions that would eventually interfere with the fluid transport mechanism. As an alternative, the PAF-initiated signaling chain might be a normal element in the fluid transport process, and its excessive stimulation would lead to the observed interference. The link between signaling and endothelial fluid transport proposed here is consistent with several earlier findings. Thus, as can be seen from the work of Walkenbach and colleagues,\(^{20,27}\) cultured bovine endothelium has specific adenosine receptors that stimulate adenyl cyclase. That work was confirmed and extended recently by Riley et al.,\(^{28}\) who described how endothelial adeny cyclase is stimulated by a G-protein coupled to an adenosine receptor and how the resultant increase in cyclic adenosine monophosphate increases net endothelial fluid transport. Such results appear consistent with an adenosine-triggered cascade leading to protein kinase A (PKA) activation. In addition, a muscarinic receptor has been located recently in corneal endothelium by Lind and Cavagnagh.\(^{59}\) This is suggestive given the role of acetylcholine in stimulating fluid transport by a signaling cascade in systems such as salt gland; moreover, in recent results from our laboratory,\(^{31,32}\) acetylcholine increased the rate of rabbit stromal deturgescence and the transendothelial electrical potential difference. It is worth noting that in the steady state, the cascade presumably triggered by adenosine (PKA?) stimulates fluid transport,\(^{25,26}\) whereas the one that we postulate would follow PAF exposure (PKC?) inhibits fluid transport. That difference might arise if each of the two cascades activates (or turns off) a different set of transporters or channels. In a hypothetical example, sustained PKA activation would lead to increased electrolyte (NaCl, KCl?) and fluid influx across the basolateral membrane, leading to the increased overall fluid transport consistent with earlier findings, whereas sustained PKC activation would lead to initially increased electrolyte (KCl?) and fluid efflux across the apical membrane, followed by the depletion of cell electrolytes and the inhibition of fluid transport we currently report.

From these and other considerations, if the cascades are active during fluid transport, optimal efficiency would best be achieved if they acted with temporal separation, as in a continuous cycle. In fact, temporal changes in protein phosphorylation have been proposed to underlie cell volume regulatory phenomena.\(^{30,34}\) The current observations, therefore, may be consistent with vectorial fluid transport driven by a succession of volume regulatory events triggered alternatively across the basolateral and apical membranes of a polarized endothelial cell.

**Key Words**
corneal hydration, electrical resistance, platelet activating factor (PAF)

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**References**
5. Chao W, Liu H, Hanahan DJ, Olson MS. Platelet-activating factor-stimulated protein tyrosine phosphoryl-


