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Platelet-activating factor induces collagenase expression in corneal epithelial cells

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ABSTRACT **Platelet-activating factor (PAF), a potent lipid mediator involved in inflammatory and immune responses, accumulates rapidly in response to injury in a variety of tissues, including the corneal epithelium. However, the precise role of this compound in the cascade of events following insult has not been defined. Here we examined the effect of PAF on gene expression in the epithelial cells of rabbit corneas in organ culture. We found that incubation with 100 nM methylcarbamoyl PAF, a nonhydrolyzable analog of PAF, produced rapid transient 2.8- and 3.5-fold increases in the expression of c-*fos* and c-*jun*, respectively, at 1 hr, followed by increased expression of the collagenase type I gene beginning at 3 hr and peaking at 14-fold by 8 hr. Addition of the protein-synthesis-inhibitor cycloheximide superinduced c-*fos* and c-*jun*, strongly potentiating the PAF effect, but inhibited the induction of collagenase type I expression, suggesting the existence of a transcriptional factor linking the two events. BN-50730, a selective antagonist of intracellular PAF-binding sites, blocked the expression of the immediate-early genes as well as the increase in collagenase type I mRNA. Our results suggest that one of the functions of PAF may be to enhance the breakdown of the extracellular matrix as a part of the remodeling process during corneal wound healing after injury. Pathologically, a PAF-induced overproduction of collagenase may be a factor in the development of corneal ulcers, as well as other pathophysiological conditions such as cartilage destruction in arthritis. If so, inhibitors of this signal-transduction pathway may be useful as tools for further investigation and, eventually, as therapeutic agents to treat such disorders.**

Platelet-activating factor (PAF; 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a biologically active ether phospholipid involved in inflammatory and immune responses (1, 2), is rapidly generated after tissue injury (3), triggering a number of signaling systems. In the cornea, PAF is undetectable under normal conditions but appears as soon as 30 min after alkali injury, suggesting that corneal cells synthesize this potent mediator (3, 4). In the rabbit cornea, 4% of the choline glycerolipids correspond to the membrane PAF precursor 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (5).

The function and pathological significance of PAF in injured tissues, however, have not been defined. Recently, a PAF receptor was cloned (6–8) that includes a putative G protein-binding domain that may couple to phospholipases as well as to other effector proteins. The use of PAF antagonists in binding studies has identified receptor sites on the cell surface as well as on intracellular membranes (9). The intracellular sites have been postulated to mediate gene expression (9). It has been demonstrated that PAF is a transcriptional activator of c-*fos* and c-*jun* in neuroblastoma cells (10), and a PAF-responsive region in the c-*fos* promoter has been identified (11). Moreover, upon generation, PAF is usually retained intracellularly rather than being released to the extracellular medium (1, 3, 12).

Here we show that, in the cornea, PAF induces the expression of c-*fos* and c-*jun*, followed by the accumulation of collagenase type I mRNA. Because collagen degradation is an important step in the process of corneal wound healing (13) and collagen type I is the most abundant collagen in the cornea (14), these results suggest a different mode of action for this lipid mediator as well as a possible function in the remodeling processes after corneal injury.

MATERIALS AND METHODS

Materials. An analog of PAF (1-*O*-hexadecyl-2-*N*-methylcarbamoyl-*sn*-glycerol-3-phosphocholine; methylcarbamoyl PAF; cPAF, Calbiochem), in a 1 μM stock solution in ethanol, was divided into aliquots and stored at −20°C. Before use, the solution was diluted 1:100 (vol/vol) in Dulbecco's phosphate-buffered saline. BN-50730 [tetrahedra-4,7,8,10-methyl-1-(chloro-2-phenyl)-6-(methoxy-4-phenyl-carbamoyl-9-pyrido-(4',3'-4,5)-theine(3,2-*f*)-triazolo-1,2,4-(4,3-*a*)-diazopine-1,4], provided by P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France), was dissolved in dimethyl sulfoxide and added to the medium. The final concentration of both dimethyl sulfoxide and ethanol was <0.01%, which we found not to affect the expression of the genes studied in the cornea (data not shown). Other chemicals used in this study were [α-^{32}P]dCTP and [γ-^{32}P]ATP (NEN), Hybond-N (Amersham), dextran T-500 (Pharmacia), Eagle's minimum essential medium (MEM), Hepes buffer, mycostatin, amikacin, and glutamine (all from Sigma).

Corneal Organ Culture. Rabbit eyes (Pel-Freez Biologicals, Rogers, AR) were maintained on ice until delivery to the laboratory. Previously described organ culture conditions (15) were modified as follows: corneas were dissected to include a scleral rim and were precultured for 2 hr (two corneas per 15 ml of medium) at 37°C, 5% CO_2/95% air, in MEM supplemented with 1% horse serum, dextran T-500 at 16 g/liter, 2 nM glutamine, and amikacin and mycostatin each at 4 mg/liter. cPAF was added to a final concentration of 100 nM, and incubations were continued for the times specified in each experiment. When PAF antagonists were used, the corneas were preincubated for 1 hr in the presence of 10 μM of these antagonists before cPAF addition.

RNA Extraction. Corneal epithelial cells were scraped with a sterilized blade (two corneas per sample) and homogenized on ice in 0.25 ml of a solution of 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.1 M 2-mercaptoethanol. RNA was extracted using the phenol/chloroform/guanidine isothiocyanate method of Chomczyski and Sacchi (16). Briefly, 200 μl of 2 M sodium acetate (pH 4) was added to 2 ml of tissue homogenate, followed by 2 ml of

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Abbreviations: PAF, platelet-activating factor; cPAF, methylcarbamyl PAF; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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phenol/chloroform/isoamyl alcohol, 3:2.55:0.05 (vol/vol/vol). The samples were chilled on ice for 20 min and centrifuged at 6600 × *g*, 4°C, for 20 min. The upper phase, which contained the RNA, was carefully transferred to a tube containing an equal volume of isopropyl alcohol, mixed, and stored at −20°C overnight. After centrifugation (6600 × *g*, 20 min), the pellet was washed with cold ethanol, air dried, and dissolved in diethylpyrocarbonate-treated water. An aliquot was taken to determine the quantity and purity of RNA by spectrophotometry; the amount of RNA per sample was 40–50 μg.

Northern Blot Hybridization. RNA (7–10 μg per lane) was loaded onto a 1.2% agarose/formaldehyde gel, electrophoretically separated (17), transferred onto Hybond nylon membranes, and immobilized by exposure to UV light for 5 min. Membranes were prehybridized at 42°C for 6 hr in a solution containing 50% (wt/vol) formamide, 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.4), 5× Denhardt's solution, 0.5% SDS, and salmon sperm DNA at 150 μg/ml.

Hybridization was done under the same conditions with labeled probes (2–4 × 10^6 cpm/ml) for 18 hr at 42°C. The membranes were washed in 2× SSPE for 15 min at room temperature, in 2× SSPE for 30 min at 55°C, and twice with 0.1× SSPE for 10 min at 57°C, then exposed to a storage phosphor screen, and scanned by using a Molecular Dynamics series 400 PhosphorImager analyzer. Counts in each band were assessed with Molecular Dynamics ImageQuant V. 3.11 software. Quantities were standardized relative to the signal for the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a constitutively expressed gene, as an internal control in each lane.

Preparation of Probes. The rat c-fos cDNA containing plasmid pSP65 c-*fos* 1A was from Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ). It was digested with *Eco*RI to give a cDNA fragment of 2.1 kb. The mouse c-jun cDNA-containing plasmid pGEM-jun was from Douglas Jones (Salk Institute, San Diego). It was digested with *Eco*RI and *Hin*dIII to give a fragment of 1.9 kb. The GAPDH cDNA-containing plasmid pHcGAP (American Type Culture Collection) was digested with *Pst* I and *Xba* I to give a 0.75-kb fragment. The cDNA fragments were labeled with [α-^{32}P]dCTP by random-primer extension (18, 19). GAPDH was used to determine the relative amount of RNA loaded in each lane by rehybridizing the blots that had previously been probed with c-*fos*, c-*jun*, or collagenase.

An oligonucleotide (5′-GCAGTAGCAGCAGCAGAGGCAAGCCGGGC-3′) complementary to the rabbit collagenase mRNA from position 64 to 92 (20) was labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP (21). A GenBank DNA data base search indicated that this oligonucleotide hybridizes only to rabbit collagenase type I gene at high stringency.

RESULTS AND DISCUSSION

PAF Is a Transcriptional Activator of c-*fos* and c-*jun* in the Epithelium of Rabbit Corneas Maintained in Organ Culture. The addition of 100 nM cPAF to corneal organ culture caused an increased expression of c-fos and c-jun mRNA in the epithelial cells, compared with unstimulated controls (Fig. 1*A*). After 1 hr of incubation with cPAF, the relative amounts of c-fos and c-jun mRNAs were 2.8 ± 0.2-fold and 3.5 ± 0.4-fold greater than controls, respectively (Fig. 1*C*). Much lower values were observed for c-fos mRNA after 2 hr of exposure to cPAF and for c-jun mRNA after 4 hr, suggesting the operation of an active signal-termination system by which the accumulated mRNA is rapidly degraded in corneal epithelial cells. In our system, the induction of c-*fos* was delayed, peaking at 1 hr, compared with increases in the expression of this gene observed after 30 min in other cells (22–24).

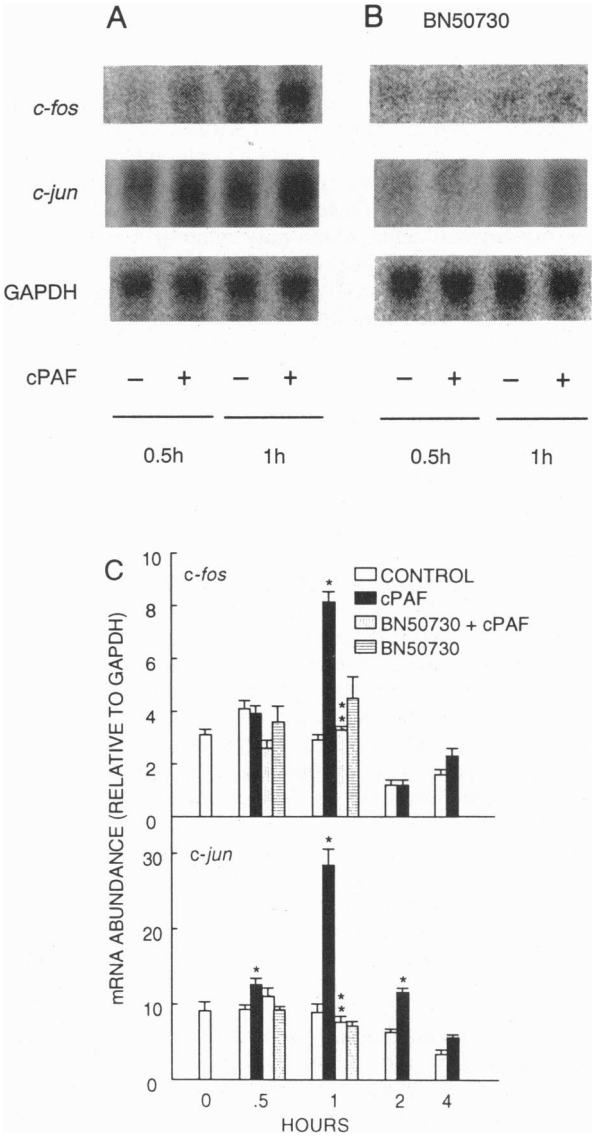

FIG. 1. cPAF induces the expression of c-*fos* and c-*jun* in rabbit corneal epithelium. (*A*) RNA blot autoradiograph of c-fos and c-jun mRNA from epithelial cells from organ-cultured rabbit corneas 30 min and 1 hr after cPAF treatment. RNA (7 μg) was loaded in each lane. The blot was initially probed with either a rat c-fos cDNA fragment or a rat c-jun cDNA fragment and reprobed after stripping with a human GAPDH cDNA fragment as an internal control for the amount of RNA recovered. The c-fos cDNA probe recognized a single mRNA species at 2.2 kb. The c-jun probe recognized an mRNA species at 2.8 kb. The GAPDH cDNA probe recognized an mRNA species at 1.6 kb. (*B*) Inhibition of c-fos and c-jun mRNA accumulation by the PAF antagonist BN-50730. (*C*) Effect of cPAF and the antagonist BN-50730 on time course of expression of c-*fos* and c-*jun*. The white bar represents the basal control level without added cPAF or BN-50730. Values, which were quantified by using a PhosphorImager analyzer, correspond to means ± SDS of three to five samples and are represented in arbitrary units of band intensity. *, Significant differences ($P < 0.05$, Student's *t* test) with respect to control (0 time); **, significant differences with respect to cPAF treatment alone at that time point.

The experimental design allowed for the study of PAF effects on the whole cornea maintained in organ culture. At the time of sampling, an intact layer of epithelial cells was obtained by rapid scraping (25, 26). Both the organ culture conditions and the scraping of the epithelial cells may have

contributed to the relatively high basal levels of immediate-early gene expression measured in controls. In human neuroblastoma cells *in vitro*, PAF stimulates a Fos/Jun/AP_1 transcriptional pathway, eliciting a rapid but transient effect on accumulated messages (10). PAF has subsequently been found to activate c-*fos* and other immediate-early genes in other transformed cells (22–24). Our use of organ culture conditions permitted these experiments to be performed in nontransformed cells, which may reflect the effects of PAF under more physiological conditions.

Although cPAF has only 20% of the biological potency of the naturally occurring lipid mediator (27), this nonhydrolyzable PAF analog was used in preference to PAF because there is high PAF acetylhydrolase activity in both cornea (28) and serum (29) that leads to the formation of the biologically inactive lyso-PAF.

BN-50730, an Intracellular PAF Antagonist, Inhibits the Lipid Mediator-Induced c-*fos* and c-*jun* Expression. BN-50730 (10 μM) incubated with corneal organ cultures for 1 hr before the addition of cPAF completely abolished the cPAF-induced stimulation of c-*fos* and c-*jun* expression (Fig. 1 *B* and *C*). BN-50730, a hetrazepine based on the triazolodiazepine framework (30), is a selective antagonist of intracellular PAF-binding sites (9) and is effective as an antagonist of stimulation-induced immediate-early gene expression in brain (31) and in transformed neural cells exposed to PAF in culture (32). A chemically different PAF antagonist, BN-52021, preferential for neural-cell plasma-membrane-binding sites as compared with intracellular membranes (9), was less effective in inhibiting the PAF-induced gene expression in the cornea (Table 1).

PAF Triggers Collagenase Expression. Epithelial cells from corneas cultured for several hours displayed a dramatic cPAF-induced accumulation of collagenase type I mRNA (Fig. 2). The increase in collagenase expression began at 3 hr, peaked at 8 hr, and declined to basal levels by 24 hr (Fig. 2*C*). This message was not detectable on RNA blots in the absence of added cPAF (Fig. 2*A*).

Our experimental paradigm—short-term corneal organ culture followed by the isolation of epithelial cells—lacks the complexities of multiple cellular elements involved in the extracellular matrix; microvascular constituents as well as inflammatory cells are absent. Thus, potential alternative sources for the increased collagenase mRNA expression are eliminated, and the model closely resembles the *in vivo* situation in the cornea immediately after injury.

PAF-Induced Expression of Collagenase Type I mRNA Is Inhibited by Both BN-50730 and Cycloheximide. To test the hypothesis that PAF-induced immediate-early gene expression may lead to the subsequent activation of AP-1-containing target genes and that the collagenase gene may be one of them, two further studies were done in this corneal organ-culture system: (*i*) an investigation of the effect of a PAF-receptor antagonist and (*ii*) an examination of the effect of a protein-synthesis inhibitor. If a PAF-initiated gene cascade does lead to collagenase expression, the intracellular PAF-receptor antagonist BN-50730 should block collagenase transcriptional activation. BN-50730 abolished the PAF-induced increase in the expression of the immediate-early genes, and, similarly, we found that 60-min preincubation with BN-50730 also completely eliminated the PAF-induced accumulation of collagenase mRNA (Fig. 2*B* and *Insert* in *C*).

Inhibition of protein synthesis with cycloheximide (35 μM) strongly potentiated cPAF-triggered c-*fos* and c-*jun* expression but suppressed the cPAF-induced accumulation of collagenase mRNA (Fig. 3). Superinduction of immediate-early genes by protein-synthesis inhibitors such as cycloheximide is well known, although not completely understood (33). The superinduction effect has been explained as inhibition of the synthesis of a protein necessary for mRNA degradation or of a labile protein that represses transcription, thus delaying transcriptional shut-off (34). The elimination of cPAF-induced collagenase expression by cycloheximide indicates

Table 1. Selective effect of the intracellular PAF-receptor antagonist BN-50730 on c-*fos* and c-*jun* expression

	mRNA abundance (relative to GAPDH), mean ± SD	
	c-fos	c-jun
Control	2.9 ± 0.2	8.9 ± 1.1
PAF (100 nM)	8.1 ± 0.4*	28.3 ± 2.1*
+ 1 μM BN-50730	3.3 ± 0.1†	17.1 ± 2.8†
+ 10 μM BN-50730	3.2 ± 0.1†	7.1 ± 0.6†
+ 1 μM BN-52021	7.4 ± 0.4	21.4 ± 4.2
+ 10 μM BN-52021	7.1	21.8

Corneas in organ culture were preincubated for 1 hr in the presence of 1 μM or 10 μM BN-52021, an extracellular PAF-receptor antagonist (9), or BN-50730, an intracellular PAF antagonist (9, 32), and then incubated for 1 hr in the presence of 100 nM cPAF. Values are the means ± SDS of three to five samples, except the last row which represents the average of two samples.
*Significant differences with respect to controls.
†Significant differences with respect to cPAF treatment alone.

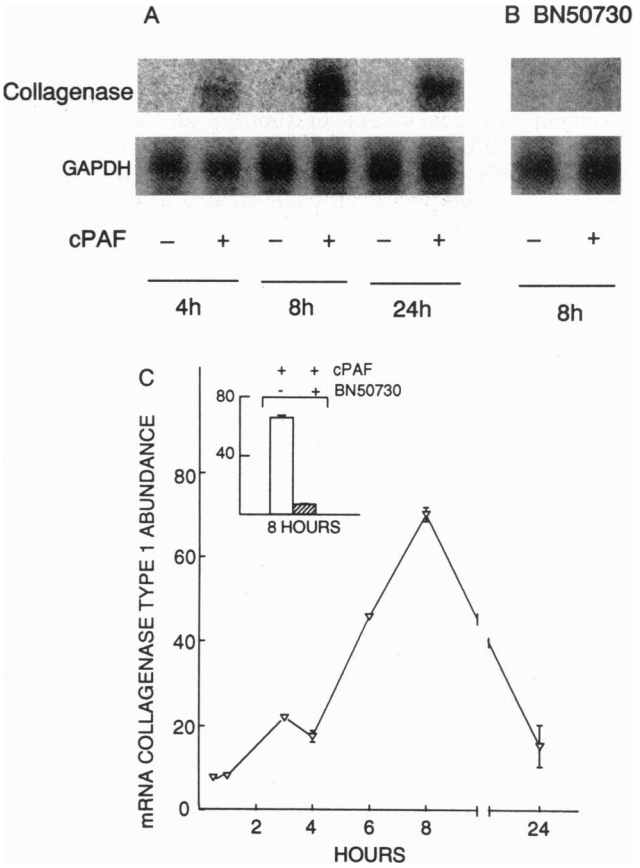

FIG. 2. cPAF causes induction of collagenase mRNA in rabbit corneal epithelium. (*A*) RNA blot autoradiograph of collagenase type I mRNA from epithelium obtained from organ-cultured rabbit corneas after incubation for 4, 8, and 24 hr with 100 nM cPAF. The collagenase oligonucleotide probe was hybridized to an mRNA species of 2.1 kb. (*B*) Inhibitory effect of 10 μM BN-50730 on cPAF-induced collagenase expression. (*C*) Time course of collagenase mRNA accumulation in the presence of cPAF measured by PhosphorImager analysis. (*Insert*) Effect of PAF antagonist BN-50730 (10 μM) after 8-hr incubation with cPAF. All values correspond to the means ± SDS of four experiments and were normalized to the values obtained for GAPDH.

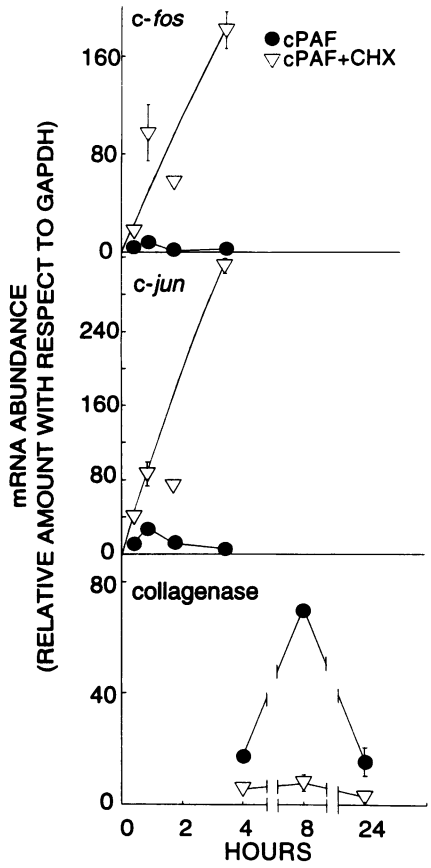

FIG. 3. Cycloheximide superinduces c-*fos* and c-*jun* and inhibits cPAF-induced collagenase expression in rabbit corneal epithelium. Rabbit corneas in organ culture were preincubated in the presence of 35 μM cycloheximide (CHX) for 1 hr and then incubated with 100 nM cPAF. The c-fos, c-jun, and collagenase type I mRNA levels were quantified as described.

the requirement for synthesis of a transcriptional factor after the early events. Evidence for the involvement of immediate-early genes in collagenase expression has been demonstrated in human fibroblasts, where tumor necrosis factor α stimulates collagenase gene transcription via an AP-1 site responsive to c-*jun* (35). Also, antisense DNA for c-fos mRNA blocks the induction of collagenase by phorbol esters (36).

The translation product of collagenase mRNA is an inactive proenzyme which, upon being secreted into the extracellular space, is converted to an active enzyme by proteolytic cleavage (37). The enzyme is one of several secreted metalloproteinases that are actively involved in the remodeling of the cornea during wound healing (13). The expression and release of these metalloproteinases are tightly regulated to prevent damage to the extracellular matrix, where most collagen is found. Transcription of collagenase genes is activated by hormones, growth factors, cytokines, and tumor promoters (38–40), and is inhibited by steroids, transforming growth factor β, and retinoic acid (41–43). The PAF-induced activation of collagenase expression reported here may represent another mechanism by which PAF contributes to corneal development, as well as other physiological processes in which active remodeling of the extracellular matrix occurs. For example, PAF can play a physiological role in ovoimplantation and pregnancy (44), and increased levels of collagenase mRNA have been reported in embryos at the time of implantation in the uterine wall (45, 46). Therefore, PAF may activate collagenase expression to facilitate tissue remodeling.

Because PAF is a mediator generated in the cornea in response to injury and inflammation (3, 4) and is accumulated in the cornea up to 24 hr after injury (the longest time point we tested) (3), it may also play a pathophysiological role as a trigger for the expression of collagenase. *In vivo* corneal inflammation induced the migration of polymorphonuclear leukocytes, also a rich source of PAF formation, to the tissue. Sustained activation of collagenase expression may lead to abnormal wound healing and abnormalities in collagen deposition, scar formation, and distortion of the refractive properties of the cornea. Collagenase is activated during corneal alkali burns and often leads to ulcers 1–2 weeks after the injury (13). PAF may also be involved in pathological processes outside the eye. In several arthropathies, including rheumatoid arthritis, extensive cartilage breakdown occurs, and expression of collagenase at abnormally high levels seems to be a major factor in the destruction of the tissue (47–49). PAF antagonists that block collagenase expression may be useful in the precise identification of these pathological mechanisms, as well as in the eventual development of potential therapeutic strategies to control collagen degradation in these diseases.

In summary, we have demonstrated that PAF transiently activates c-*fos* and c-*jun* expression and subsequently leads to the expression of collagenase type I mRNA in the epithelial cells of organ-cultured corneas. Corneal epithelial cells not exposed to PAF did not express collagenase type I mRNA. The effect of the lipid mediator suggests the triggering of a gene cascade because an antagonist of the intracellular PAF receptor inhibited the expression of both c-*fos* and c-*jun*, as well as that of collagenase type I. Additionally, the time course of expression supports this idea, in that the transient increases in c-*jun* and c-*fos* peaked at 1 hr of incubation and collagenase starts to increase after 3 hr with maximum accumulation at 8 hr. Further evidence for a gene cascade initiated by PAF-mediated transcriptional activation of c-*jun* and c-*fos* followed by the synthesis of a transcription factor(s) is based on the observation that the protein-synthesis inhibitor cycloheximide superinduced the expression of the immediate-early genes but inhibited the PAF-induced collagenase type I mRNA accumulation.

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