The elastin peptides-mediated induction of pro-collagenase-1 production by human fibroblasts involves activation of MEK/ERK pathway via PKA- and PI_3K-dependent signaling

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Abstract Elastin peptides, such as κ-elastin (kE), bind to the elastin receptor at the cell surface of human dermal fibroblasts and stimulate collagenase-1 expression at the gene and protein levels. Using specific inhibitors and phosphospecific antibodies, we show here that the binding of elastin peptides to their receptor activates the extracellular signal-regulated kinase (ERK) pathway; this activation is essential for the induction of pro-collagenase-1 production. Moreover, protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI_3K) signaling were found to participate in ERK activation. Concomitantly, we demonstrate that stimulation by elastin peptides leads to enhanced DNA binding of activator protein-1 (AP-1). Our data indicate that the up-regulation of collagenase-1 following treatment of fibroblasts with elastin peptides results from a cross-talk between PKA, PI_3K and the ERK signaling pathways and that this regulation is accompanied by activation of AP-1 transcription factors. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Fibroblast; κ-Elastin; Collagenase-1; Mitogen-activated protein kinase/extracellular signal-regulated kinase; Activator protein-1; Protein kinase A; Phosphatidylinositol 3-kinase

1. Introduction

Elastin, a cross-linked polymer of tropoelastin, is found in the extracellular space of several vertebrates tissues such as lung, major arteries and skin [1]. Degradation of insoluble elastin by elastases leads to the production of elastin peptides [2] which exhibit activities influencing cell chemotaxis [3], cell proliferation [4,5] and gene expression [6]. These effects are mediated through binding of elastin peptides to their cognate receptor.

The elastin receptor complex is composed of three distinct subunits: the elastin-binding protein (EBP), a protective protein and a neuraminidase [7]. The 67-kDa EBP is an enzymatically inactive, spliced variant of β-galactosidase and possesses an elastin-binding and a galactolectin site. Occupancy of the galactolectin site considerably reduces the affinity of the receptor for elastin leading to the shedding of EBP from the cell surface [8].

In a previous investigation on human skin fibroblasts, we have shown that, following binding to EBP, κ-elastin (kE), a chemical hydrolysate of insoluble elastin, or the elastin synthetic peptide VGVAPG, up-regulated collagenase-1 (matrix metalloproteinase-1, MMP-1) and stromelysin-1 at the gene and protein levels [6].

The promoter of MMP-1 contains several regulatory elements; the activator protein-1 (AP-1) site located at −72 bp was reported to play an important function in the control of MMP-1 production [9–11]. We therefore hypothesized that the stimulation of pro-MMP-1 production observed when human skin fibroblasts were cultured in the presence of kE could involve AP-1 signaling and studied the upstream involvement of mitogen-activated protein kinases (MAP kinases), as those enzymes occur upstream of AP-1 signaling by controlling its activation [12]. As, in the case of MMP-1 production control, the importance of the MAP/extracellular signal-regulated kinase (MEK)/ERK signaling cascade had been reported several times [13–15], its possible involvement in kE-induced pro-MMP-1 production by human skin fibroblasts was investigated.

Our data demonstrate that activation of the MEK/ERK signaling pathway is crucial for kE signaling leading to MMP-1 production. Additionally, we establish that the activation of this MAP kinase cascade involves synergistic protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI_3K) signaling and that fibroblasts stimulation by elastin peptides leads to activation of AP-1 transcription factors.

2. Materials and methods

2.1. Reagents

kE was prepared as described previously [6]. Lactose, alkaline phosphatase-conjugated anti-sheep antibody, nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate alkaline-phosphatase substrate and isobutylmethylxanthine were purchased from Sigma (Saint Quentin Fallavier, France).

The sheep anti-human MMP-1 polyclonal antibody, protein kinase

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Abbreviations: AP-1, activator protein-1; CREB, cAMP response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; EBP, elastin-binding protein; ERK, extracellular signal-regulated kinase; kE, κ-elastin; MAP kinase, mitogen-activated protein kinase; MEK, MAP/ERK kinase; MMP-1, matrix metalloproteinase-1 or collagenase-1; NF-κB, nuclear factor κB; pERK, phosphorylated ERK; PI_3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C

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C (PKC) inhibitor GF109203X, PKA inhibitor H-89 and forskolin were obtained from Calbiochem (France Biochem, Meudon, France). The rabbit anti-human double-phosphorylated ERK1/2 (pERK1/2), horseradish peroxidase-conjugated anti-sheep and anti-rabbit polyclonal antibodies, PI_3K inhibitor LY294002 and MEK1/2 inhibitor U0126 were purchased from New England Biolabs (Saint-Quentin-en-Yvelines, France). The enhanced chemiluminescence substrate solution and [γ-^{32}P]ATP were from Amersham Biosciences (Saclay, France). Reagents for cell culture were from Life Technologies (Cergy Pontoise, France). All reagents used for electrophoretic mobility shift assays were obtained from Promega (Charbonnières, France). All others reagents were from Sigma.

2.2. Cell culture

Human skin fibroblast strains were established from explants of human adult skin biopsies obtained from informed healthy volunteers (age: 21–49 years). Cells were grown as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 200 U/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine in the presence of 5% CO_2. Cells at subcultures 2–10 were used. For experiments, fibroblasts were grown to confluence in 10% serum-containing medium. Before stimulation, confluent cells were incubated for 18 h in DMEM containing 0.5% fetal calf serum, washed twice with phosphate-buffered saline (PBS) and incubated in serum-free medium with or without kE, in the presence or absence of cell signaling effectors. Inhibitors and lactose were added 1 h prior to kE stimulation.

2.3. Western blot analysis

The protocol used for pro-MMP-1 detection has been described [6]. Briefly, fibroblasts were treated with kE for 24 h and the culture media were harvested and centrifuged (500×*g*, 10 min, 4°C) to remove cellular debris. The supernatants were then concentrated using Microcon YM10 (Millipore, Molsheim, France) and the protein content resolved by SDS–PAGE under reducing conditions. The membranes were saturated with 5% (w/v) non-fat dry milk, 0.1% (v/v) Tween-20 in TBS (Tris 50 mM, NaCl 150 mM, pH 7.5), treated with sheep polyclonal anti-human MMP-1, and then incubated with alkaline phosphatase-conjugated anti-sheep antibody. Immunocomplexes were visualized using nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate alkaline phosphatase substrate. The bands were further quantified by densitometry using Phosphor Analyst software (Bio-Rad, Marne-la-Vallée, France).

To study ERK1/2 activation, cells were treated with kE and incubations were pursued for 5–60 min. Stimulation was stopped by adding ice-cold PBS containing 50 μM Na_3VO_4. Cells were then sonicated in lysis buffer (PBS pH 7.4, 0.5% Triton X-100, 80 mM β-glycerophosphate, 50 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, and the protease inhibitor cocktail from Sigma). Proteins were resolved on SDS–PAGE under reducing conditions. Protein transfer and blocking of non-specific binding sites were performed as described above. Active forms of ERK were visualized using a rabbit anti-human pERK1/2 primary antibody and a horseradish peroxidase-coupled anti-rabbit secondary antibody.

2.4. Electrophoretic mobility shift assay

Fibroblasts were incubated with kE for 2–8 h. After removal of the culture medium, cell layers were scraped, centrifuged, and nuclear extracts were prepared as described by Staal and co-workers [16].

Binding reactions were established in 20 μl of binding buffer as previously described [17], using 2 μg of nuclear extract protein per assay. Nuclear extracts were incubated for 20 min at room temperature with [γ-^{32}P]ATP end-labelled double-strand AP-1 consensus oligonucleotides, using T4 polynucleotide kinase. The AP-1 oligonucleotide used for the electrophoretic mobility assay was 5′-CGC TTG ATG AGT CAG CCG GAA-3′.

For competition assays, the nuclear extracts were incubated for 10 min at room temperature with an unlabelled probe before adding AP-1-labelled probe: AP-1; nuclear factor κB (NF-κB), 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; cAMP response-element-binding protein (CREB), 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′. The unlabelled/labelled probe concentration ratio was 100:1. Samples were electrophoresed through an 8% polyacrylamide gel at a constant voltage of 180 V and gels were dried before autoradiography.

2.5. Radioimmunoassay of cAMP

Cells were incubated for 5 min at 37°C in DMEM containing 1 mM isobutylmethylxanthine and activators (forskolin or kE). Reactions were stopped by addition of 1 M final concentration of $HClO_4$ followed by immersion into an ice bath. The cells were homogenized and their cAMP content quantified by a radioimmunological method as described by Cailla and co-workers [18], except that bound and free ligand were separated by precipitation of bound ligand with a mixture of γ-globulin (2.5 mg/ml) in citrate buffer (pH 6.2) and polyethyleneglycol 6000 (20% w/v).

2.6. Statistical analysis

Experiments were performed in triplicate. Results are expressed as mean ± S.E.M. Differences between control means and treated groups were assessed using Student's *t*-test.

3. Results

Consistent with our previous investigation [6], human skin fibroblasts in culture produced a low level of pro-MMP-1 following 24 h of culture (Fig. 1, lane 1). Depending on the donor, one or two immunoreactive species having apparent molecular weight of 57 kDa and 53 kDa were detected corresponding to the glycosylated and non-glycosylated pro-MMP-1 isoforms respectively. Culturing human skin fibroblasts in presence of 50 μg/ml kE leads to a six-fold increase in pro-MMP-1 production (Fig. 1, lane 2). When cells were treated for 24 h in the presence of both 50 μg/ml kE and 10 μM U0126, pro-MMP-1 production returned to basal level (Fig. 1, lane 3), while U0126 alone had no effect (Fig. 1, lane 4). It suggested that kE-mediated MMP-1 over-expression was triggered by the MEK/ERK signaling pathway.

To further investigate these MAP kinase cascade events, we analyzed ERK phosphorylation by Western blot after kE stimulation (Fig. 2). When cells were treated without kE, two faint bands corresponding to the 44 and 42 kDa double-phosphorylated forms of ERK-1 and ERK-2 were observed (Fig. 2, lane 1). Supplementation of cell culture medium with kE enhanced the intensity of those bands, up to an apparent maximum after 30 min of stimulation (Fig. 2, lane 4). In keeping with the relationship between AP-1 transcription factors, ERK pathway and transcriptional control of collagenase-1 gene, we thus investigated AP-1 activation following treatment of cells with kE. The electrophoretic mobility shift assay experiments presented in Fig. 3 show that a transcription factor belonging to the AP-1 family is activated following kE treatment in a time-dependent manner. The specificity of DNA binding was assessed by competition using unlabelled AP-1, NF-κB and CREB probes (Fig. 3, lanes A, N, CR respectively). The addition of a 100-fold excess of the non-radioactive AP-1 probe totally removed the shifted AP-1 signal demonstrating the specificity of the binding; on the contrary, unlabelled CREB and NF-κB sequences competed partly and totally respectively with the radioactive AP-1 probe.

Upstream regulation of the MEK/ERK cascade typically involves tyrosine kinases, PKCs and/or cAMP-regulated pathways [19–21]. Tyrosine kinases could be excluded since herbimycin A did not interfere with kE-induced expression of MMP-1 by fibroblasts [6].

As PKC involvement had been reported in cell chemotaxis to elastin peptides [22], we investigated the possibility that the MEK/ERK cascade could be activated by such a kinase.

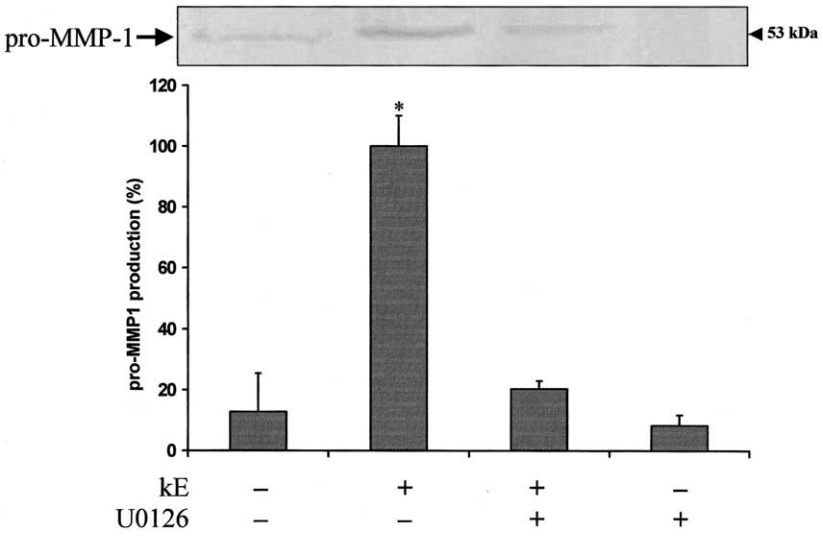


Fig. 1. Inhibition of the MEK/ERK signaling pathway blocks the effect of kE on pro-MMP-1 production. Upper panel: Western blot detection of pro-MMP-1. Lower panel: Densitometric analysis of Western blots. Stimulation of cells was achieved using 50 μg/ml kE. The MEK-specific inhibitor U0126 was used at 10 μM. $*P < 0.05$.

GF129203X, a highly specific inhibitor of cPKC, was ineffective at modulating enzyme production (data not shown).

When fibroblasts were treated with kE (50 μg/ml) or forskolin (10^{-4} M) as a positive control for 5 min, the intracellular cAMP level was increased six- and 19-fold respectively (Fig. 4A). In parallel, a 24 h chronic forskolin stimulation (10^{-5} M) of cells significantly stimulated the production of pro-MMP-1 by human skin fibroblasts (Fig. 4B, lane 3). Altogether, these findings pointed out that a cAMP-dependent pathway was probably involved in kE signaling.

PKA has been shown to activate Raf which, in turn, may trigger the MEK/ERK cascade [21]. In order to check the involvement of this cAMP-regulated kinase, we used H-89, a highly specific PKA inhibitor. Inhibition of PKA (Fig. 5, lane 3) reduced the kE-induced pro-MMP-1 production by about 50% (Fig. 5, lane 2), while H-89 alone had no effect (Fig. 5, lane 4). This result clearly indicated that a PKA was involved in kE-triggered signaling. However, as PKA inhibition led to partial inhibition of the kE effect on MMP-1 expression, other signaling pathways leading to MEK/ERK activation were suspected to be involved.

Several investigators have recently pointed out that matrix components could activate PI_3K further inducing MEK/ERK activation [23,24]. We thus tested the influence of PI_3K inhibition on pro-MMP-1 production (Fig. 6, lane 3). Inhibition of PI_3K by LY294002 in cells treated with kE resulted in an important reduction (about 60%) of the kE-induced pro-MMP-1 production, whereas LY294002 alone had no effect suggesting that PI_3K was involved in the signaling events leading to increased pro-MMP-1 production.

In order to establish a possible cross-talk between the PKA and PI_3K pathways, we analyzed whether their simultaneous inhibitions exhibited additional effects on ERK phosphoryla-

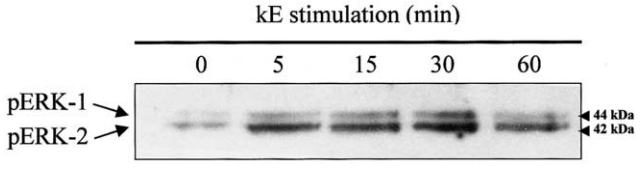


Fig. 2. Effect of kE treatment duration on ERK phosphorylation. Anti-pERK Western blot analysis. Fibroblasts were stimulated using kE (50 μg/ml) for the indicated durations. Cells were then harvested, cellular extracts prepared and analyzed as described in Section 2.

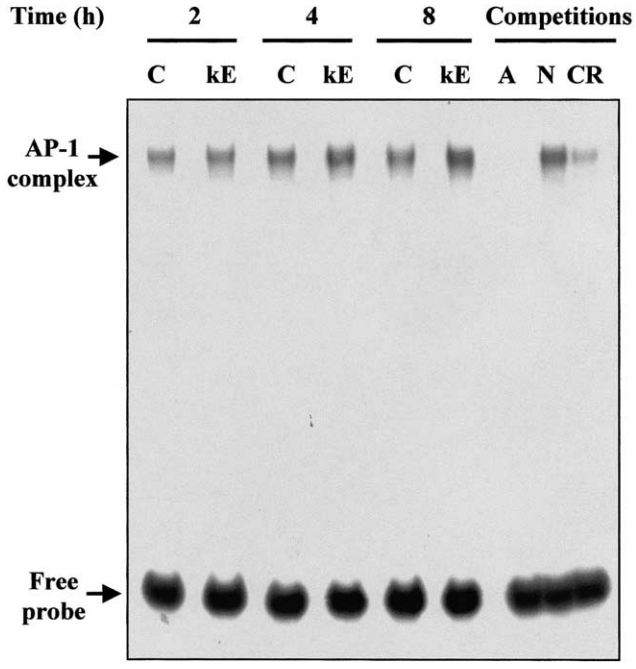


Fig. 3. Time course of AP-1 activation following treatment of cells with kE. Electrophoretic mobility shift assay experiments were performed using a [γ-^{32}P]-labelled AP-1 probe on nuclear extracts from cells incubated for 2, 4 or 8 h in the absence (C) or presence (kE) of 50 μg/ml kE. Competition assays were realized with nuclear extracts from cells treated with kE for 8 h. The radiolabelled AP-1 probe was added after the nuclear proteins had been incubated for 10 min in the presence of an excess of the following cold consensus sequences: AP-1 (A), NF-κB (N) and CREB (CR).

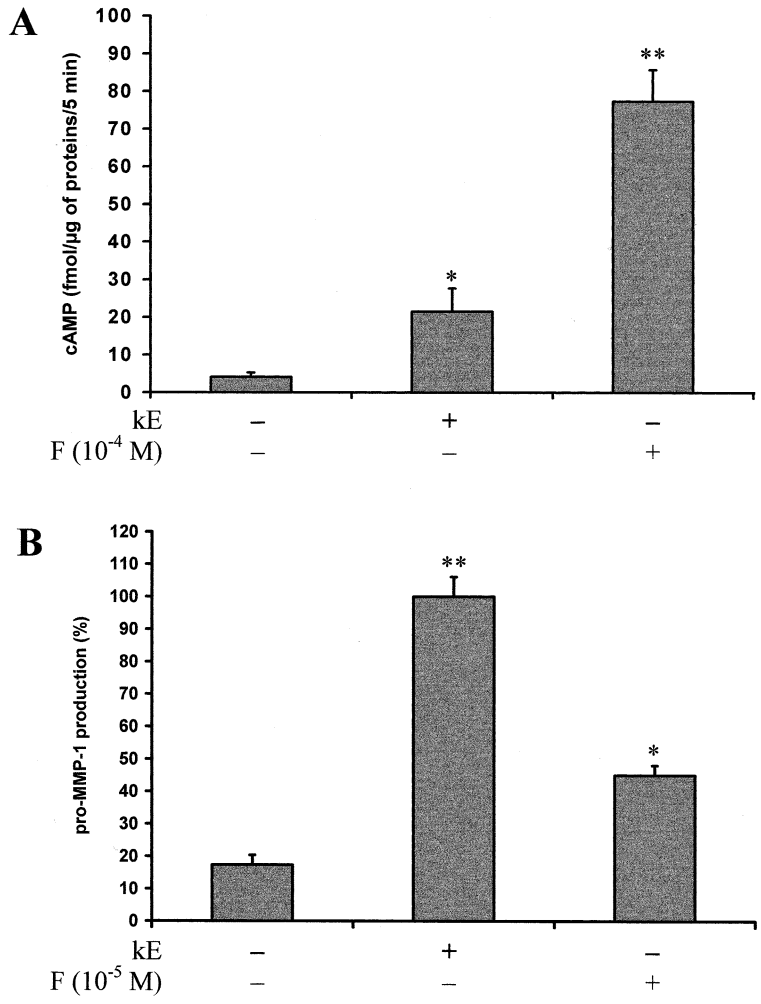


Fig. 4. Effect of kE treatment on the production of cAMP by cultured human skin fibroblasts and involvement of cAMP-dependent signaling pathways in the stimulation of pro-MMP-1 production after kE treatment. A: kE treatment increases the intracellular level of cAMP. Radioimmunoassay of cAMP. F, forskolin used at 10^{-4} M represents the positive control (acute stimulation). Cells were stimulated with kE (50 µg/ml) for 5 min. *$P < 0.05$; **$P < 0.01$. B: Forskolin (10^{-5} M) reproduces the effect of kE on the production of pro-MMP-1. Densitometric analysis of anti-MMP-1 Western blots. Cells were grown in the presence of 50 µg/ml kE for 24 h. F, forskolin was used at 10^{-5} M (chronic stimulation). *$P < 0.05$; **$P < 0.01$.

tion. Individual PKA and PI_3K inhibitions significantly reduced the level of pERK (Fig. 7, lane 3 and 4 respectively). Interestingly, when both kinases were simultaneously inhibited (Fig. 7, lane 5), ERK phosphorylation was further reduced (Fig. 7, lane 1) suggesting that PKA and PI_3K pathways were both involved in activation of MEK/ERK cascade. As we previously described (Fig. 2), intense phosphorylation of ERK-1 and ERK-2 was noticeable following treatment of cells with kE for 30 min (Fig. 7, lane 2). ERK phosphorylation decreased when cells were pretreated with 1 mM lactose (Fig. 7, lane 6), demonstrating that kE effects on ERK activation was mediated by EBP.

4. Discussion

MMP expression is regulated by several stimulatory factors such as phorbol esters, integrin-derived signals, extracellular matrix macromolecules or fragments, and changes in cell shape that influence a variety of signaling pathways [11]. A majority of those stimuli lead to activation of c-fos/jun proto-oncogenes, which heterodimerize and interact with AP-1 sites in MMP gene promoter as found in collagenase-1 [11]. We here demonstrated that stimulation of fibroblasts by elastin peptides also triggered AP-1 activation. The specificity of DNA binding was demonstrated by the use of a cold AP-1 consensus sequence and confirmed by the fact that unlabelled CREB consensus sequences, which bind AP-1 transcription factors with low affinity [25], only partially competed with the radioactive AP-1 probe, while NF-κB probe had no effect.

In most instances, the MEK/ERK MAP kinase pathway is the main cascade leading to collagenase-1 expression [13–15], which often involves AP-1 activation [10,11]. Using U0126, a MEK1/2 inhibitor, at a concentration known to not influence p38 and c-Jun N-terminal MAP kinases [26,27], we were able to demonstrate the specific implication of that kinase in MMP-1 expression. On the basis of inhibition of kE-mediated MMP-1 over-expression following supplementation of fibroblast culture media with RO-31-8220, we initially proposed cPKC as a key mediator of protease induction [6]. However, GF109203X, a more specific cPKC inhibitor, proved to be ineffective in modulating collagenase-1 expression suggesting that such kinase was not involved in elastin-induced effect.

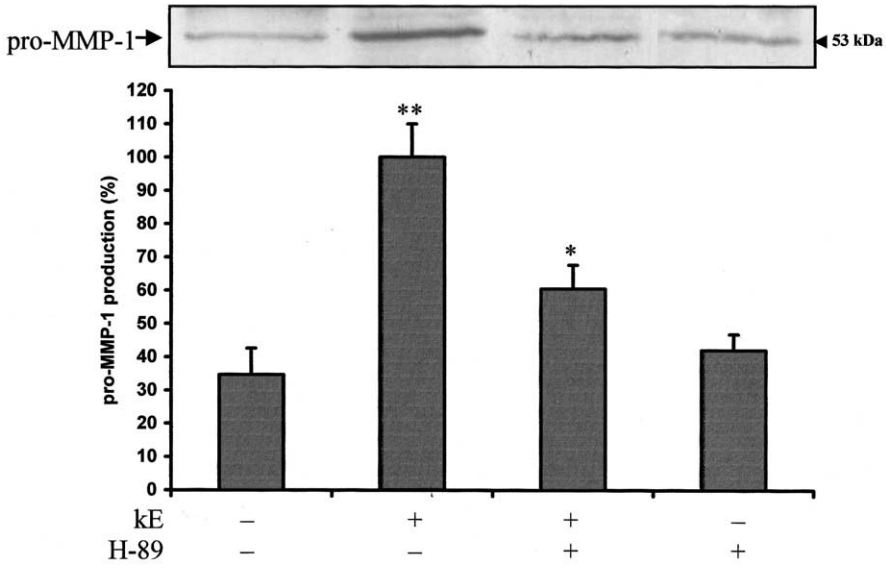


Fig. 5. Effect of PKA inhibition on the production of pro-MMP-1 induced by kE treatment. Upper panel: Anti-MMP-1 Western blot. Lower panel: Densitometric analysis. Stimulation of cells was achieved using 50 μg/ml kE. The PKA-specific inhibitor H-89 was used at 5 μM. $^{*}P < 0.05$; $^{**}P < 0.01$.

A rise of cAMP, which has been observed in monocytes treated with elastin peptides [29], is known to induce ERK phosphorylation [22,28]. In our cell system, a significant enhancement of cAMP level was observed as early as 5 min following cell stimulation; furthermore, forskolin, an activator of adenylylcyclase catalytic subunit, was found to induce MMP-1 production indicating cAMP was a mediator in the signaling cascade induced by elastin peptides. Since inhibition of PKA did not totally block ERK1/2 phosphorylation, we suspected that another pathway could be involved. Our results show that PI_3K is involved in this signaling as LY294002 inhibited kE-induced ERK phosphorylation. In conclusion, we show that PI_3K and PKA signaling contribute to the activation of the MEK/ERK cascade induced by treatment of fibroblasts with elastin peptides.

A number of potential mechanisms by which PI_3K can lead to ERK activation in cells have been described; atypical PKCs, notably PKCζ, are the most likely candidates. Indeed, the newly synthesized phosphatidylinositol 3,4,5-trisphosphate recruits phosphoinositide-dependent kinase-1 and PKCζ to the plasma membrane where a combination of lipid binding and phosphorylation by phosphoinositide-dependent kinase-1

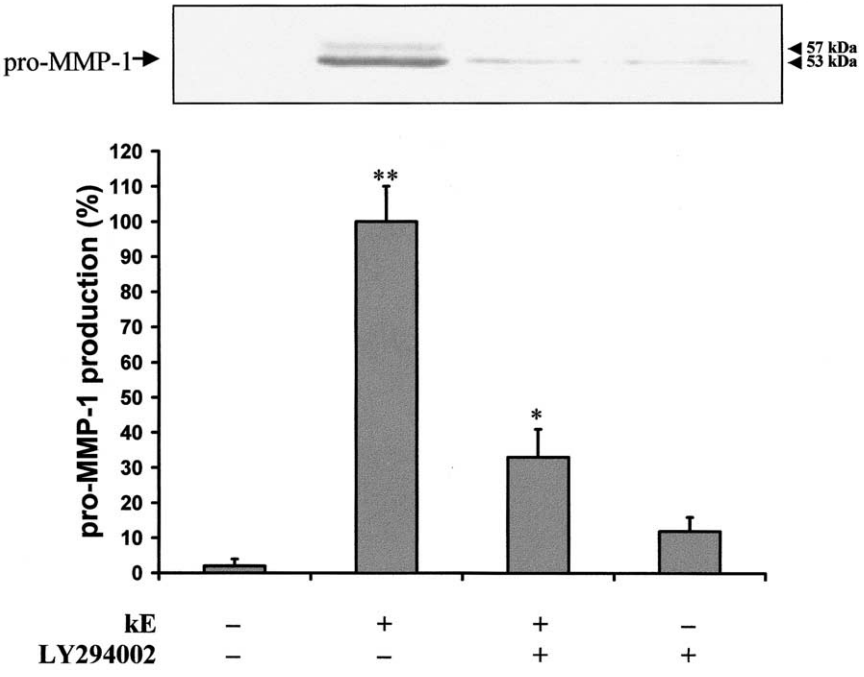


Fig. 6. Effect of PI_3K inhibition on the production of pro-MMP-1 induced by kE treatment. Upper panel: Anti-MMP-1 Western blot. Lower panel: Densitometric analysis. Stimulation of cells was achieved using 50 μg kE/ml. The PI_3K-specific inhibitor LY294002 was used at 25 μM. $^{*}P < 0.05$; $^{**}P < 0.01$.

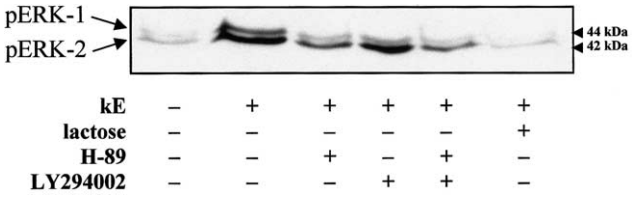


Fig. 7. Influence of simultaneous inhibition of PI_3K and PKA pathways on the phosphorylation of ERK following stimulation of cells with kE. Anti-pERK western blots. kE, lactose, H-89 and LY294002 were used at 50 μg/ml, 1 mM, 5 μM and 25 μM respectively. Cells were stimulated in the presence of kE for 30 min.

activates PKCζ [30]. PKCζ was also shown to phosphorylate both Raf-1 and MEK directly [31,32]. Another atypical PKC, PKCδ, is also known for its ability to activate the MEK/ERK pathway via c-Raf [33]. PI_3K was found associated with this aPKC in human hematopoietic cells as well as in platelets [34] and it seems that PI_3K could activate PKCδ by an unknown regulatory mechanism. Finally, PI_3K could activate the MEK/ERK pathway via its intrinsic Ser/Thr kinase activity (PI_3K-protein kinase). Indeed, Bondeva and co-workers [35] have shown that a PI_3K mutant, that had lost its lipid kinase activity but retained its protein kinase activity, could activate ERK. Nevertheless, the mechanism leading to ERK phosphorylation remains unknown.

To our knowledge, a cross-talk between PI_3K, PKA and ERK pathways leading to collagenase-1 expression has not been previously described. Our results demonstrate that interaction of fibroblasts with elastin peptides leads to activation of transcription factors belonging to the AP-1 family, and most importantly to PI_3K activation. Although our study focused exclusively on signaling events leading to pro-MMP-1 production, we feel that the occurrence of PI_3K activity downstream of EBP is particularly important. Indeed, besides their contribution to the regulation of several genes expression, these lipid kinases could be connected to many physiological events ([36] and references cited therein) that could be triggered by elastin peptides, such as cell proliferation [4], peptide-induced oxidative burst in neutrophils [29] and cell motility [3]. The involvement of a PI_3K in elastin peptide-triggered processes has now to be considered and extends the range of elastin peptides activities on cells.

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